The Synergistic Effect between Antibiotics and Biosynthesized ZnO Nanoparticles

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Abstract:
The effect of ZnO nanoparticles biosynthesized by probiotics bacteria, Lactobacillus gasseri, Leuconostoc mesenteroides ssp. dextranicum and Lactococcus lactis ssp. lactis combined with different antibiotics were investigated against MDR skin infection causative bacteria. The diameter of inhibition zones (mm) around the different antibiotic discs including (10) from different classes of antibiotics which include Imipenem, Cefazidime, Cefotaxime, Carbencillin, Aztreonam, Amikacin, Ciprofloxacin, Norfloxacin, Tetracycline and Oxytetracycline, with and without ZnO nanoparticles against isolates were measured. The bactericidal actions of some antibiotics have been elevated in the presence of (subMIC) biosynthesized ZnONPs against some skin infection causing bacteria. The results of the study showed the synergistic effect of ZnO nanoparticles with antibiotics used in study on the bacterial isolates of Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumanii and Staphylococcus aureus.

Keywords: ZnO nanoparticles, probiotics bacteria, Lactobacillus, Leuconostoc, Lactococcus, antibiotics, synergistic effect.

Note: The research is based on a PhD dissertation.

INTRODUCTION

Bionanotechnology is an important tool for the development of eco-friendly methodology for the synthesis of nanomaterials using biological sources. Nano sized particles possess several properties included larger surface to volume ratio and higher surface energy (Dhillon et al., 2012). Biological synthesis of nanoparticles using plant or plant extracts, microorganisms and enzymes have been suggested as eco-friendly, clean and nontoxic compared with chemical and physical methods (Vishaet
Cathrine et al. (2010) reported a green protocol to biosynthesis of silver nanoparticles using probiotic bacteria *L. acidophilus* and recorded its antibacterial activity. Researchers showed that exposure of lactic acid bacteria present in the whey of butter milk to mixtures of gold and silver ions could be synthesis nanoparticles of alloys of gold and silver (Sastry et al., 2003). Probiotic is defined as, a viable microorganisms preparation consumed by human beings and animals to induce beneficial effects influencing their gastrointestinal tract flora and modifying their immune states (Yanbo and Zirong, 2006).

A skin and skin structure infection is a bacterial infection of skin and associated tissues. The term ‘acute bacterial skin and skin structure infections’ (ABSSSIs), a group of common types of infection, including cellulitis, abscesses, and wound infections (Edelsberg et al., 2009), while superficial infections where the risk of anaerobic or Gram-negative pathogen involvement is higher, should be considered complicated infections (Shah and Shah, 2011). Gram-positive bacteria such as *S. aureus* and *S. pyogenes* are the dominant bacteriathatearlyisolated in the infectious process, whereas Gram-negative bacteria are found in chronic wounds (Cardona and Wilson, 2015). Eradication of the causative pathogenic bacteria requires effective antibiotic therapy, although clinical practice guidelines recommend a range of antibiotic therapies for each type of skin infection (Bassetti et al., 2014). The extreme level of resistance globally to many antibiotic drugs in the prevalent causative pathogens, and the presence of risk factors of treatment failure. There is one of suggesting that inappropriate antibiotic treatment is given to approximately 20–25% of patients, potentially prolonging their stay in hospital and increasing the risk of mortality and morbidity (Pulido-Cejudo et al., 2017). Combining antimicrobials was a potential strategy to reduce the growth of pathogenic microorganisms by expanding the spectrum of antimicrobial activity (Windiasti, 2016). The physiochemical properties of nanoparticles can provide antibacterial modes of action (Kumar et al., 2018). In the field of nanomedicine, the combination of antibiotics with nanoparticles has emerged as a novel route to increase the efficacy of both antibiotics and nanoparticles. Chemical composition of nanosurface enables, protection from enzymes, active targeting of antibiotics surface-functionalized at the target site and prolonged binding. The higher concentration of antibiotic within the cell eliminates the requirement of higher dosage, thereby reducing the side
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وتحت شعار (البحث العلمي بوايتنا للبناء والتقدم)

MATERIALS AND METHODS

Microorganisms:
Pathogenic bacteria: In this study (13) isolate of Pseudomonas aeruginosa, (11) isolate of Klebsiella pneumonia and (10) isolates for each of Acinetobacter baumannii and Staphylococcus aureus which isolated from burns and wounds. These isolates were identified throughout cultural, microscopical, biochemical test according to the criteria established by Forbes (2007) and Vitek2 system.

Probiotics bacteria:
Three isolates of probiotics bacteria included Lactobacillus gasseri, Leuconostoc mesenteroides ssp.dextranicum and Lactococcus lactis ssp.lactis, these isolates were identified throughout cultural, microscopical and biochemical test according to Goldman and Green (2015) and Vitek2 system.

Antibiotic Susceptibility Test:
Kirby – Bauer method was followed as described by WHO (2003) to carry the antibiotic susceptibility test for (10) different antibiotics. Pathogenic bacterial suspension was prepared by picking 4-5 colonies of each bacterial isolate from original culture and they were suspended in test tube containing 4 ml of normal saline, then turbidity was adjusted to obtain approximately 1.5 x 10^8 CFU/ml (0.5 MacFarland standard). By a sterile cotton swab a portion of bacterial suspension was transferred carefully and spread on Mueller – Hinton agar and left to dry at room temperature. The antibiotics discs were placed on the agar with a sterile forceps pressed firmly to ensure contact with the agar. The plates were inverted and incubated at 37°C for 24 h. Inhibition zones that developed around the discs were measured by millimeter (mm) using a metric ruler, according to clinical laboratory standards institute, the isolates were interpreted as susceptible or resistant to a particular antibiotic according to CLSI (2011).
Biosynthesis and Characterization of ZnO Nanoparticles

A pure culture of L. gasseri (9×10⁸ CFU/ml) was inoculated at 2% into the flask containing sterile De Man Rogosa and Sharpe (MRS) broth and incubated at 37 °C for 24h, same procedure for L. mesenteroides ssp.dextranicum, and L. lactis ssp. lactis isolates but incubated at 30°C for 24 h. After incubation pH of the culture broth was adjusted to 6 using 1 M NaOH. Analytical reagent grade Zinc Chloride (ZnCl₂) was taken into use for preparing a solution of 0.25(M) strength at room temperature, then added to the flask containing culture solution and heated on a water bath up to 80°C for 5-10 min (Prasad & Jha, 2009). A white precipitate appears at the bottom of flask indicates the transformation process and the flask was removed from the water bath, incubated at 37 °C for 12 h. (Salman et al., 2018). Then filtered and DW was added to ZnONanoparticles and centrifugation was done at 3000 rpm for 10 min and this step was repeated more than three times. Finally, the white pellet was washed using D.W then dried at 40 °C using a hot air oven for 8h. (Kadhim et al., 2018). ZnO nanoparticle was obtained as powdered form and characterized by Fourier Transform Infrared Spectroscopy (FTIR), Atomic Force Microscopy (AFM), X-ray diffraction (XRD) technique, scanning electron microscopic (SEM) and Energy-dispersive X-ray analysis (EDX) spectra (Kadhim et al., 2018; Salman et al., 2018)( data not shown).

Antibacterial activity of synthesized ZnO nanoparticle:

The antibacterial activity of the synthesized ZnO nanoparticles by Probiotics bacteria L. gasseri, L. mesenteroides ssp.dextranicum and L. lactis ssp. lactis was measured via determine the minimum inhibitory concentration (MIC) using micro dilution technique in the culture broth media. Further dilutions were prepared to concentrations ranging from (100-0.04) mg/ml. Briefly, 125 µl of sterile Muller Hinton broth was placed into the first column of the 96-well microplate and 125 µl of sterile Muller Hinton broth in the remaining wells. Subsequently, 125 µl of ZnO nanoparticles solution in PBS (100 mg/ml) was added to the first column of the microplate and mixed with the medium; this results in a ZnO nanoparticles concentration of 50 mg/ml; serially, 125 µl were transferred to the subsequent wells, discarding 125 µl of the mixture in the last column, the final volume for each well was 125 µl. All the wells were inoculated with 2.5 µl of an overnight culture (1.5× 10⁸ CFU/ml) of skin infection causative bacteria. Microplates were covered and incubated at 37 °C for 24 h. After that the MIC was determined (Salman et al., 2018).
Combined effect between antibiotics and biosynthesized ZnO nanoparticles:

To determine combined effects of antibiotics (Tetracycline, Amikacin, Carbencillin, Aztreonam, Cefotaxime, Ceftazidime, Ciprofloxacin, Imipenem, Norfloxacin, Oxytetracycline) and ZnO nanoparticles biosynthesized by choiced probiotics bacteria as described by Chauhan et al., (2014) with some modification, each standard paper disc was further impregnated with subMIC of the biosynthesized ZnO nanoparticles. A single colony of MDR isolate was suspended in 4ml of D.W then turbidity was adjusted to 0.5 McFarland, and by a sterile cotton swab a portion of bacterial suspension was spread on Mueller-Hinton agar plates, then the discs which immersed in sub-MIC of ZnO nanoparticles were placed. After incubation at 37°C for 24 h, the zones of inhibition were measured. The increase in the fold area of antibiotics alone and antibiotics plus ZnO nanoparticle can be calculated by the equation

\[
\text{Fold increase (\%)} = \frac{(b-a)}{a} \times 100
\]

Where “a” and “b” refer to the zones of inhibition for antibiotic alone and antibiotic with ZnO nanoparticles respectively (Murugan and Paulpandian 2013).

RESULTS AND DISCUSSION

Antibacterial activity of synthesized ZnO nanoparticle:

The antibacterial action of biosynthesized ZnO nanoparticles by probiotics bacteria L.gasseri, L.mesenteroidesssp.dextranicum and L.lactis ssp. Lactis was evaluated against bacterial isolates from skin infections. The MIC value for the synthesized nanoparticles varied depends on bacterial isolates. Results showed that the MIC of ZnO nanoparticles synthesized by L.gasseri was found to be 12.5mg/ml against A.baumannii and S.aureus isolates, the MIC against K.pneumoniae isolates was between (12.5 – 25) mg/ml, while against P.aeruginosa was 50mg/ml. The best activity of ZnO nanoparticles synthesized by L.mesenteroidesssp.dextranicum was found to be 25 mg/ml against A.baumannii and S.aureus, the MIC against K.pneumoniae was between (25-50) mg/ml, while in P.aeruginosa was 50mg/ml. The ZnO nanoparticle synthesized by L. lactisspp.lactis isolate was effective against A.baumannii and S.aureus with MIC recorded at 25mg/ml, but on P.aeruginosa (50 -100mg/ml).

Antibiotic susceptibility test:

The results revealed that all the bacterial isolates obtained from this study showed variable resistance to the tested ten antibiotics used. P.aeruginosa
isolates showed varied levels of resistances to antibiotics. All isolates of P. aeruginosa were resistant to Carbencillin, while sensitivity to other antibiotics at different proportions varied. Also results revealed that the 12 isolates of P. aeruginosa from 13 were resistant to tetracyclin, where the 11 isolates of P. aeruginosa from 13 isolates were resistant to Aztreonam. On the other hand, gram positive bacteria S. aureus isolates were reported varying resistance levels to Aztreonam and Carbencillin while resistance to other antibiotics at different proportions varied. Also results revealed that the 9 isolates of S. aureus from 10 were resistant to Amikacin. The results showed that all the isolates of A. baumannii were resistant to Cefotaxime and tetracycline, while 6 isolates of A. baumannii from 10 were sensitive to Amikacin and sensitivity to other antibiotics at different proportions varied. All isolates of K. pneumoniae were resistant to Aztreonam also results revealed that the 10 isolates of K. pneumoniae from 11 were resistant to Cefotaxime and Carbencillin while sensitivity to other antibiotics at different proportions varied.

It’s well known that nanomaterial has the ability to bind to electron donor groups such as Amides, carboxylates, Indoles, Hydroxyles and thiols and this in turn results in inactivation of cellular enzyme and DNA. Which was known to cause increased permeability and cell death through the formation of little pores in bacterial cell wall. (Ahmad and Sardar, 2013). The zinc oxide nanoparticles induces the production of ROS in bacteria and this can affect DNA as well as cellular machinery of bacteria (Beyth et al., 2015).

Combined effect between antibiotics and biosynthesized Zno nanoparticles:

The effect of biosynthesized ZnO nanoparticles by probiotics bacteria, L. gasseri, L. mesenteroidesssp. dextranicum and L. lactissssp. lactis combined with different antibiotics was investigated against eight of MDR skin infection causative bacteria using disk diffusion method. The diameter of inhibition zones (mm) around the different antibiotic discs including ten antibiotic from different classes of antibiotic which include Imipenem, Cefazidime, Cefotaxime, Carbencillin, Aztreonam, Amikacin, Ciprofloxacin, Norfloxacin, Tetracycline and Oxytetracycline, with and without ZnO- NPs against isolates were measured. The increase in the fold area of different pathogenic bacteria for antibiotics and antibiotics plus ZnO nanoparticle solution was calculated by fold increase (%) as shown in Tables (1), (2) and (3). The highest antibacterial activity of antibiotics and biosynthesized ZnO nanoparticles by
L. gasseri blends were observed with Ciprofloxacin and showed high fold increase (110) in zone of inhibition of A. baumanii (A6) (Table 1). Similarly, the antibacterial activity of some antibiotics Amikacin, Ceftazidime and Aztreonam also has been increased in the presence of ZnO nanoparticles by L. gasseri (SubMIC) against MDR isolates at different proportions varied. The molecules of antibiotic contain many active groups such as amide and hydroxyl groups, which may react easily with ZnO nanoparticles. The combined effect of ZnO NPs synthesized by L. gasseri and antibiotics was promising against K. pneumonia (K6) appeared sensitive to Aztreonam, Ciprofloxacin, Ceftazidime, Norfloxacine and Imipenem that combined with ZnO nanoparticles compared with antibiotics alone (Table 2). The more antibacterial activities of antibiotics combined with biosynthesized ZnO NPs by L. mesenteroidesssp. dextranicum was increased against S. aureus (S6) isolate for Oxytetracycline, Cefotaxime, Ciprofloxacin, Amikacin, Ceftazidime and Carbencillin with fold increase (162.5, 100, 75, 66.67, 90.91 and 120) respectively, while K. pneumonia (K6) appeared sensitive to Aztreonam, Ciprofloxacin, Ceftazidime, Norfloxacine and Imipenem that combined with ZnO nanoparticles compared with antibiotics alone (Table 2). The more antibacterial activities of antibiotics combined with biosynthesized ZnO nanoparticles by L. lacticissps. lactis and L. mesenteroidesssp. dextranicum was increased against S. aureus (A9) isolate for Oxytetracycline, Cefotaxime, Ciprofloxacin, Amikacin, Ceftazidime and Carbencillin with fold increase (162.5, 100, 75, 66.67, 90.91 and 120) respectively, while K. pneumonia (K6) appeared sensitive to Aztreonam, Ciprofloxacin, Ceftazidime, Norfloxacine and Imipenem that combined with ZnO nanoparticles compared with antibiotics alone (Table 2). The more antibacterial activities of antibiotics combined with biosynthesized ZnO nanoparticles by L. lactissps. lactis exhibited against S. aureus for Cefotaxime, Ciprofloxacin, Amikacin, Ceftazidime and Oxytetracycline, while A. baumanii appeared less affected compared with antibiotics alone (Table 3). Combining antimicrobial agent was a potential strategy to reduce the growth of pathogens by expanding the spectrum of antimicrobial activity. ZnO NPs have synergistic antimicrobial effect against Campylobacter jejuni. (Windiasti, 2016). The antimicrobial activity of antibiotics and in combination with ZnOnanoparticles against E. coli and Bacillus subtilis has been demonstrated by Chandrika et al. (2012). Ciprofloxacin-conjugated ZnO nanoparticles (Zn-CIPs) were prepared by Patra et al., (2014) and recorded their activity against MDR E. coli, S. aureus, and Klebsiella sp. and showed that the mode of action of the Zn-CIP complex was attributed to the Zn nanoparticles’ mediated damage the membrane of cells through ROS
الجانية والعشرون لقصما الحاشبات والعلوم
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(البحح العلمي ركيسة التهمية المصتدامة)
8-9 آيار 2022
(البحث العلمي بوابتنا للبناء والتقدم)

Generation, followed by the entry of ciprofloxacin into the cell, causing bacterial growth inhibition.

Table (1): Combined effect of biosynthesized ZnO2 by Lactobacillus gasseri and antibiotics against MDR bacterial skin infections

<table>
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<th>Inhibition zone (mm)</th>
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*AZ: Azithromycin, CTX: Cefotaxim, CIP: Ciprofloxacin, PIV: Pivmoxacin, CAZ: Cefazidime, MI: Minipen, OT: Oxytetracycline, NOR: Norfloxacin; 1-7: Diameter zones of inhibition for antibiotic-diameter zones of inhibition for antibiotic with ZnO2-NPs, %fold increase.
Table (2): Combined effect of biosynthesized ZnO2 by Leuconostoc mesenteroides ssp. dextrancum and antibiotics against MDR bacterial skin infections

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<th>Inhibition zone (mm)</th>
<th>OT</th>
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<th>CTX</th>
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- **OT**: Amikacin, **ATM**: Aztreonam, **CTX**: Cefotaxime, **CIP**: Ciprofloxacin, **PV**: Ceftriaxone, **CAZ**: Carbenicillin, **MI**: Imipenem, **NOR**: Oxytetracycline
- **ZnO2**: Zinc oxide
- **MDR**: Multi-drug resistant
- **S. aureus**: Staphylococcus aureus
- **S. epidermidis**: Staphylococcus epidermidis
- **E. coli**: Escherichia coli
- **P. aeruginosa**: Pseudomonas aeruginosa
- **B. subtilis**: Bacillus subtilis

**Notes**: The inhibition zones were measured in millimeters (mm) and were determined under the influence of biosynthesized ZnO2 by Leuconostoc mesenteroides ssp. dextrancum and selected antibiotics against MDR bacterial skin infections. The results show the effectiveness of the combination in inhibiting the growth of the tested bacteria.
Table (3): Combined effect of biosynthesized ZnO2 by Lactococcus lactis ssp. lactis and antibiotics against MDR bacterial skin infections

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**Notes:**
- **Py:** Pyrrolidonyl beta-naphthylamide.
- **ClO2:** Clostridium perfringens.
- **CAZ:** Ceftazidime.
- **AK:** Aztreonam.
- **OT:** Ofloxacin.
- **CIP:** Ciprofloxacin.
- **Piperacillin:** PIP.
- **Cephalosporins:** PIP, CEP, TETRAcycline.
- **Benzylpenicillin:** BENC.
- **Ampicillin:** AMPC.
- **Aminoglycosides:** TOBR.
- **Tetracycline:** TETR.
- **Linezolid:** LIN.

**Bioactivity of ZnO2:**
- **A1:** 12 10 8 8 8 8 8 8
- **A2:** 12 10 8 8 8 8 8 8
- **A3:** 12 10 8 8 8 8 8 8
- **A4:** 12 10 8 8 8 8 8 8
- **A5:** 12 10 8 8 8 8 8 8
- **A6:** 12 10 8 8 8 8 8 8
- **A7:** 12 10 8 8 8 8 8 8
- **A8:** 12 10 8 8 8 8 8 8

**Combined Effect:**
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<th>CAZ</th>
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CONCLUSION
The biosynthesized ZnO nanoparticles by probiotics bacteria combined with different antibiotics had synergistic effect against MDR skin infection causative bacteria.

References:
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8-9 أيار 2022

وتحت شعار (البحث العلمي بوابتنا للبناء والتقدم)

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التأثير التآزر بين المضادات الحيوية والزنك النانوي الصنف حيويا

عرفاء علي كاظن 1، جيهان عبد الناصر سلمان 1، عدومة جمعة حيدر 2
1 قسم علوم الحياة / كلية العلوم / الجامعة المستنصرية / بغداد، العراق
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مستخلص البحث:

كشف عن تأثير جسيمات الزنك النانوي المصنعة حيوياً بوساطة بكتريا المعززات الحيوية Lactobacillus gasseri و Leuconostoc mesenteroides ssp.dextranicum Lactococcus lactis ssp. lactis بانخفاضها للمضادات الحيوية المختلفة تجاه البكتريا المسببة لاصابات الجلد ذات المقاومة المتعددة للمضادات الحيوية باستخدام طريقة الانتشار بالأقراص. تم قياس اقطار مناطق التثبيط حول عشرة أنواع من المضادات الحيوية Imipenem و Ceftazidime و Cefotaxime و Carbencillin و Aztreonam و Amikacin و Ciprofloxacin و Norfloxacin و Tetracycline و Oxytetracycline و وجود و عدم وجود الزنك النانوي تجاه العزلات في الدراسة. لوحظ الفعل القاتل لبعض المضادات الحيوية وجود التركيز تحت المثبط الادني لالزنك النانوي المصنعة حيوياً و تبين تناثر الدراسة وجود تأثير تآزر بين الزنك النانوي والمضادات الحيوية تجاه العزلات البكتيرية Pseudomonas aeruginosa و Klebsiella pneumoniae و Acinetobacter baumanii و Staphylococcus aureus.

الكلمات المفتاحية: جسيمات الزنك النانوي، بكتريا المعززات الحيوية، Lactobacillus، Leuconostoc، Lactococcus، Lactobacillus

ملاحظة: البحث مستند إلى طروحات دكتوراه