Original Article

Biosynthesis Nanoparticles And Molecular Study To Detect Bacterial Isolated From Patients With Diabetic Foot Ulcers In Babylon Province

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Abstract

Objective:- Diabetic foot ulcers, the aim of the study for determine some types of microorganisms from diabetic foot ulcers that causes inflammation and long-term complications and activity study of ZnO NPs biosynthesis by Aspergillus niger against isolated bacteria, collected from infected patients at general Al -Hashimiya hospital in Babylon Governorate.

Methods:-collected 100 swabs, cultured it, bacteria was diagnostic by morphological and microscopic, standard biochemical tests and Vitec,the isolates identified by molecular diagnosis by extraction of DNA,using specific universal primer 16SrRNA (1239bp) and polymerase chain reaction (PCR), study biosynthesis,characterization of zinc Oxide Nanoparticles to inhibited bacterial growth.

Results:-The results were (26 aerobic bacterial isolates positive) present such as Gram positive Staph aureus , as regards the aerobic Gram negative bacilli, Klepsiella pneumoniae, Proteus mirabilis, Escherichia coli, The zinc oxide nanoparticles created by Aspergillus niger were spherical in shape with size 13.5nm that used as antibacterial.

Discussion:-Through determination of MIC were concentrations of ZnO NPs (31.25, 62.5µg /ml, 125µg/mL, 250µg/ml, 500µg /ml), (31.25, 62.5µg /ml) not prevent growth and not inhibition while 125µg/mL determine as MIC, two best concentrations are 250µg/ml, 500µg /ml both give inhibition zones.

conclusion:- Gram negative bacteria was predominantly for diabetic foot ulcers, Aspergillus niger was shown to be a stabilizer capable of producing spherical ZnO nanoparticles, due to is very suitable candidate to carry out antimicrobial operation, as a result to its small size.

Keywords: Diabetic foot ulcer, pathogenic bacteria, biochemical tests, DNA extraction and PCR, A. niger, ZnO NPs.

INTRODUCTION

One of the most prevalent endocrine diseases, diabetes mellitus it is characterized by decreased glucose uptake and caused by absolute or relative insulin deficiency, resulting in hyperglycemia [1],[2]. All forms of diabetes can cause major issues in a variety of body organs [3]. Diabetic foot ulcers are one of the most common long-term complications diabetes [4]. Diabetic foot infection (DFI) is the most common cause of diabetes patients, and reduction of quality of life in people with diabetes [5]. Nearly 80% of all lower limb amputations are a result of diabetic foot ulcer [6]. Diabetic foot ulcers (DFUs) are a complicated combination of risk factors that include peripheral neuropathy, peripheral vascular disease, trauma, and reduced infection resistance, and they continue to be a significant cause of lower extremity amputation worldwide [7]. Microbiological studies have indicated the most frequently identified isolates being aerobes including *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae* and others, infected diabetic foot ulcers bacteria remain the major problem [8]. For the treatment of DFUs are study nanomaterial as antibacterial for the treatment of DFUs and skin regeneration in the treatment of diabetic wounds [9]. ZnO NPs have antibacterial activity by rupturing cell membranes, most likely by producing reactive oxygen species like superoxide and hydroxyl radicals, the ZnO NPs may be responsible for the bacterial cell membrane damage and extrusion of the cytoplasmic contents, which leads to the death of the microbe.[10].

MATERIALS AND METHODS

Identification of Isolation Pathogenic bacteria

The first objective for using a variety of morphological traits for pathogenic bacterial detection and identification (colony color), microscopy using (gram stain), and for the purpose of making accurate diagnoses utilizing a panel of biochemical: oxidase, catalase, indole, citrate utilization and other tests and serological tests: coagulase test, test

techniques must be very specific for correctly identifying the bacterial genus and species used Vitiec-2 system (bioremix), and used molecular study for detection isolate used universal *16SrRNA* [11].

Biosynthesis of Zinc Oxide Nanoparticles

Isolation of Fungal and Identification

Isolate of *A. niger* through place grains of inventory rice for a certain period on potato dextrose agar (PDA) media.[12]. The diagnosis of isolate is depend diagnosis on colony color, a cultural features and morphology, that according standard microscopically identification by use stain Lacto-phenol Cotton Blue (LPCB), the obtained results for the fungal isolate showed that belonged to *A. niger*.

Preparation of ZnO NPs.

The prepared of ZnO NPs and obtained white powder by using the method of the Jacob.[13].

Structural Characteristics of ZnO NPs.

The synthesized nanoparticles of zinc oxide nanoparticles is using different spectroscopic and charactering technique, the techniques are UV-vis spectroscopy, X-ray power Diffractometric (XRD), Scanning Electron Microscopy (SEM) and Fourier transformed infrared spectroscopy (FTIR) [14].

Molecular diagnostic techniques of pathogenic bacteria

Genomic DNA extracted from pathogenic clinical isolates by using a commercial purification and a the kit extraction of Genomic DNA. (Mini Kit Genomic DNA from (Blood-Cultured Cell) Favorgen / Korea), the extraction was done following the manufacture's protocols recommended for bacteria and DNA was stored at -20ºC.

Polymerase Chain Reaction (PCR).

Using specific primer gene *16S rRNA* of bacteria that isolated was amplificated with universal primer PCR domain forward primer F- (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer R- (5'-GGTTACCTTGTTACGACTT-3'), using specific universal primer *16SrRNA* (1239 bp)[15].The reaction of Polymerase Chain (PCR) with condition initial denaturation95C at 3min., denaturation 95C at 30sec., annealing 60C at 30sec., extension 72C at 1min.,final extension 72Cat 5min [16],[17].

Determination of Minimum Inhibitory

For determination of MIC were concentrations of ZnO NPs (31.255µg /mL, 62.5µg /mL, 125µg/mL, 250µg/mL, 500µg /mL) incubated all these tubes at 37°C for 24 hr, with dark conditions, MIC is a lowest ZnO nanoparticles concentration that inhibited completely the bacterial growth and can detected and determined the concentration of tube first one 125µg/mL with to be the Minimum inhibitory concentration [18].

THE RESULTS

Identification of pathogenic bacteria

Depending on morphological and biochemical test results show that Gram negative bacteria reveals a high rate (18) isolates (69.23%) which includes *Klebsiella pneumoniae* that show a high percentage (7) (26.92 %), then *Proteus mirabilis* 6 (23.08 %), followed *Escherichia coli* 5 (19.23%). Whereas Gram-positive bacteria recorded Staph aureus 8 (30.77 %). While were results the highest number of infections were caused by *Staph aureus* infection rate (30.77%) was the most isolated bacteria in this study among all microorganisms and record lowest infection rate *Escherichia coli* was (19.23 %) The staphylococcus bacteria were dominant among the bacteria isolated from the diabetes foot ulcers, referring to Figure (1).

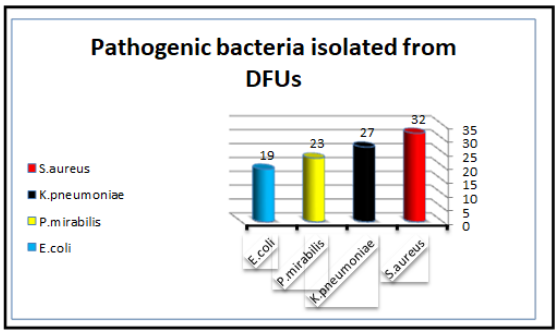

Figure (1): show of bacterial isolates and their percentages

Biosynthesis of ZnO NPs Using Fungus *Aspergillus niger*

ZnO NPs were obtained in the form of white powder as showed in Figure (2), *A. niger* was shown to be a stabilizer capable of producing spherical ZnO nanoparticles, after drying and weighing take, then, its properties were studied using UV , XRD, SEM, FTIR. The results of the current study agree with many studies [19].

STRUCTURAL CHARACTERISTICS OF ZNO NPS.

X-Ray Diffractometer (XRD)

The diffraction peaks of biosynthesized ZnO NPs unchanged, the diffraction peaks are crisp and narrow as showed in Figure (2) indicating product crystalline is naturally, calculated using Scherer equation derived from the XRD line broadening data, this demonstrates a minor lowering in crystalline, indicating the creation of smaller particles in size at 13.5nm.

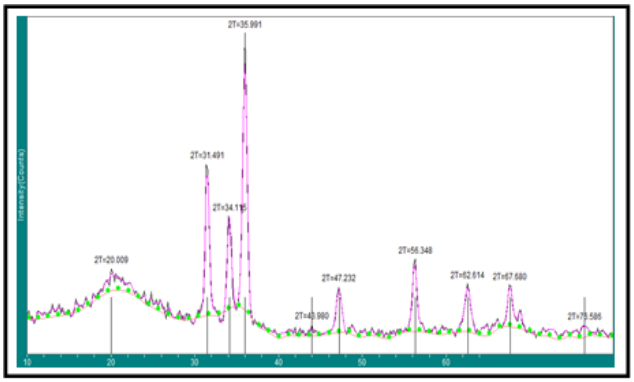

Figure (2): XRD graph of biosynthesis ZnO NPs from *A. niger*

Scanning Electron Microscope (SEM)

The SEM performed examination to determine the morphology of the ZnO NPs, SEM picture of ZnO NPs generated from fungal extract, the zinc oxide nanoparticles were measured and shaped using a scanning electron microscope, these photos indicated that the ZnO NPs created were spherical in shape as showed in Figure (3)according to [20]. The diffrer in shape of biosynthesized ZnO NPs, the discrepancy could be due to the type of microbes used for metal reduction.

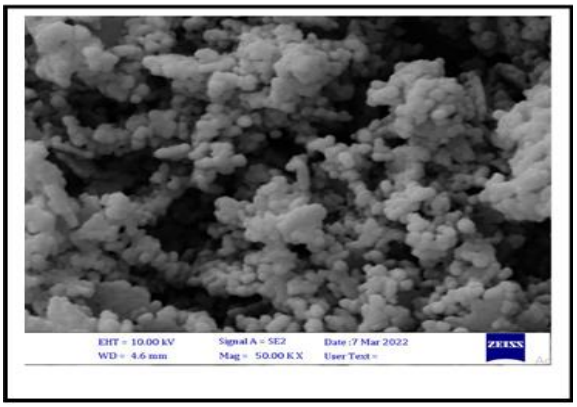

Figure (3): SEM image of biosynthesis ZnO NPs from *A. niger*

FTIR Spectroscopy

Showed the FTIR for ZnO NPs biosynthesized, using fungal extract, generally due to interatomic vibrations, ZnO nanoparticles absorbance bands in the range below 1000 cm1.

GENOTYPING IDENTIFICATION OF BACTERIA

Genomic DNA Extraction

In this research, molecular identification was used to identify the 26 isolates (*Proteus mirabilis, Escherichia coli, Staph aureus Klebsiella pneumoniae*). genomic DNA was successfully extracted from all bacterial isolates in this study by using Genomic DNA purification Kit a commercial (Blood-Cultured Cell) Favorgen / Korea), And according to company's instructions, the extraction results were good, and the quantification DNA , purification ,concentration was complete direct by spectrophotometer (Nano-drop) at (260-280) nm, the DNA concentration is obtained from all the isolates values range among (58 to142) ng/l, the extracted DNA was verified and examined by horizontal the gel electrophoresis in 1 percent agarose for 30 minutes at 75 voltages, and the purification was assessed as being between (1.8 to 2). as as depicted in Figure (4).

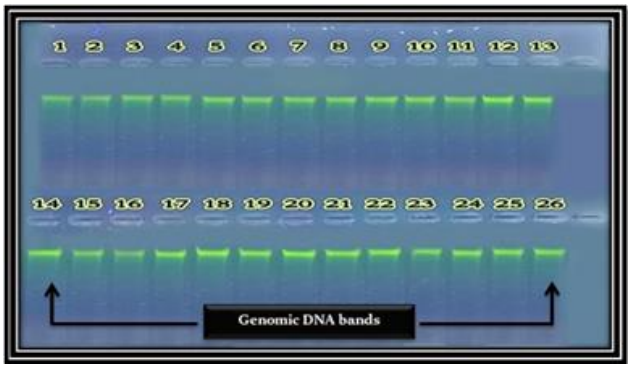

Figure (4): Presented the integrity of DNA which extracted from Pathogenic bacteria isolated from DFUs., *S. aureus* (1 — 8) *; E. coli* (9 —13); *K. pneumoniae* (14 —20) *; P. mirabilis* (21 —26). Agarose 1.3%, Volt 100, Time 45 minutes.

Molecular Identification of isolated bacteria by Detection *universal 16SrRNA*

The PCR technique to detect housekeeping genes for all isolates was also performed in the current study and began with the DNA extraction as a first step. A primer is specific for the responsible gene for determining the bacterial genus (*universal 16SrRNA*) identified all the isolates correctly and was used to perform a single step PCR. The results is exhibited that 8 were obtained from *S. aureus* and 7 for *K. pneumoniae* and 6 to *Proteus mirabilis* and 5 *E.coli* using housekeeping gene *universal 16SrRNA* with an amplified size of (1239bp). Electrophoresis over an agarose gel separated the bands, which were then stained with ethidium bromide and imaged under UV light, this show in Figure (5).

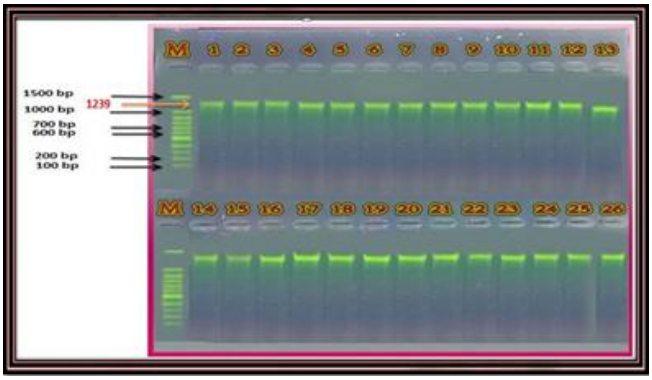

Figure (5):- Presented Amplification of *16SrRNA* to molecular conform DNA of pathogenic bacterial which isolated from DFUs., M:100 bp DNA *S. aureus* (1 — 8) *; E. coli* (9 —13); *K. pneumoniae* (14 —20) *; P. mirabilis* (21 —26). Agarose 1.3%, Volt 100, Time 45minutes.

Antibacterial Activities of ZnO Nanoparticles

ZnO NPs with pure structure hexagonal of polycrystalline ZnO NPs shown higher antimicrobial because the improved hexagonal bioactive surface , ZnO nanoparticles were reported to have antibacterial action against *E. coli*, and the impact increased with increasing ZnO particle concentration, the antimicrobial action of *A. niger* ZnO nanoparticle led in increased antibacterial impact of ZnO NPs, with significant antimicrobial property of green generated ZnO NPs realized *against E. coli* at 500 g/ml concentration, *S. aureus* appears to have a response to ZnO NPs as antibacterial activity in the current investigation when compared to the other three microorganisms optimum antibacterial. However, ZnO NP at doses of 250, 500 g/ml exhibits somewhat better *S. aureus* action, that results from of the synergistic antimicrobial pathways of ZnO NPs'. At a concentration of 500g/ml, *A. niger* extract had very strong against *S. aureus* activity. ZnO NPs have against *S. aureus* action, found a comparable increase in against *S. aureus* activity with higher ZnO concentrations. The antimicrobial activity of ZnO NP against *K. pneumoniae* is discernible at concentrations of 125g/ml, 250g/ml, and 500g/ml when compared to other microorganisms, the antibacterial activity of bio-fabricated ZnO nanoparticles *with A. niger against K. pneumoniae*, which showed the antibacterial action of ZnO nanoparticles showed lowering significant against *Proteus mirabilis* effect with 250µg/ml and 500µg/ml showing antimicrobial activity for others bacteria .

DISCUSSION

The results of current study agree with what was mentioned in previous studies, as the isolated bacterial species which was similar to isolate the bacteria in this study, as indicated by [21]. *S. aureus, K. pneumoniae, P. mirabilis, and E. coli* as the isolated bacterial species diabetic foot ulcers, the results of our current study agree with what was obtained by [22]. This supported the data of the previous diagnostic methods explained above, this confirmed the accuracy of the used tests and methods used for the identification of this bacterial species.They also nanoparticle formation shows that the biosynthesis process is not enzymatic, the biosynthesis of the metal NPs is do not dependent on NAD and NADH enzymes,but due to amino acidis, the zinc oxide NPs were measured shape using a scanning electron microscope,

indicated that the zinc oxide nanoparticles created were spherical in shape, according to studies [23]. This result is consistent with the other studies but this study were less size than others studies,The natural components from microbial extracts is responsible for the broad peak [24]. The results agree with the projected crystallite size from the SEM and XRD patterns, and it is obvious that the biosynthesis approach reduces the average diameter and size of the ZnO nanoparticles, antimicrobial pathways of ZnO nanoparticle and *A. niger* biologically active compounds which it ligated to the ZnO surface of nanoparticles, which are the ZnO NP are hexagonal plates, may have easily penetrated the membrane barrier mediating bacterial cell membrane damage and cell leakage resulting in cell death, according to [22]. In this regard, In this case also ZnO NP has enhanced antimicrobial efficacy than ZnO NP again suggesting the antibacterial effect of ZnO nanoparticle having increased surface bioactivity, these results are consistent with the results of MIC, also reveals that the antibacterial activities increased as the concentration of zinc oxide nanoparticles increased [25].

CONCLUSION

Most of Gram-negative bacteria in diabetic foot ulcer infection, but one of Gram positive were dominant *S. aureus*, the bacterial species isolated in this study were characterized by their high resistance to antibiotics reflects the unstudied use of antibiotics in the treatment of patients, the possibility of preparing Nano-products from bacteria and fungi and using them to inhibit a treatment diabetic foot ulcers, efficiency of ZnO to inhibit the bacteria causing contamination of diabetic foot ulcers isolated in this study.

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