Evaluation of the Biological Activity of Nickel Oxide Nanoparticles as Antibacterial and Anticancer Agents

Maha Fakhry Altaee*, Laith A. Yaqaqob1, Zaid K. Kamona2
1Department of Biotechnology, College of Science, University of Baghdad, Iraq
2Department of Student Accommodation Affairs, University of Baghdad, Iraq

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Abstract
In the present study, nickel oxide nanoparticles (NiO NPs) were evaluated as an antibacterial and anticancer agent. The nanoparticles of nickel oxide were synthesized using aloe vera leaves extract and characterized with AFM (showing an average diameter of 45.11 nm), XRD and FE-SEM analyses. Three different concentrations (125, 250 and 500 µg/ml) were prepared from the synthesized NiO NPs and investigated for their potential antibacterial activity against both Enterococcus faecalis (Gram-positive bacteria) and Acinobacter baumannii (Gram-negative bacteria). While cytotoxicity and apoptotic activity were measured on both MCF-7 and AMJ13 cancer cell lines by MTT and caspase-9 luminescence assays. The results showed that NiO NPs inhibit bacterial growth, as indicated by large inhibition zones against both tested bacteria, with all studied concentrations. Moreover, the results of cytotoxicity and caspase-9 activity assays were in concordance with those of antibacterial activity, showing high cytotoxicity and apoptotic effects against both of the studied cancer cell lines and with all the tested concentrations of NiO NPs. Both the antibacterial and anticancer activities of NiO NPs were dose-dependent.

Keywords: NiO nanoparticles, antibacterial activity, cytotoxicity, caspase-9, apoptosis.

*Tae et al.
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Introduction

Nanotechnology has attracted a great research interest in the last decades. In several areas of research, different methods have been used for the development of nanoscale materials, such as physical or chemical methods [1]. Within this context, nanotechnology involves the studying of extremely small structures, having a size of 0.1 to 100 nm, which confers them with unique properties as compared to the particles of microscopic scales [2].

Nanotechnology and nanoscience, often interchangeable, are defined by various means. Nanotechnology involves the design, synthesis, characterization, and application of materials which are characterized by the nanometer range of dimension [3]. While, nanoscience, which extend from studying individual atoms or molecules to submicrons, is the manufacturing and application of biological, chemical and physical systems at nanoscales [4].

Explaining of the dimensional evolution of different physical characteristics and some features which were previously unnoticed is an expanding field of novel knowledge creation. Within the empirical discipline of different nanotechnology, basic and applied scientific disciplines have been haled promptly [5]. Technological advances in a variety of applications are expected to be enhanced by nanotechnology, which shows an enormous promise for providing many breakthrough discoveries in the near future [6]. More improved and desirable products can be produced by using nano-systems than conventional materials. Enormous impacts in areas such as communication, electronics, cosmetics, medical device, architecture, medicine, agriculture, textile, metallurgy, food, space, defense, security, and many more are owed to advances in nanotechnology [7]. Recently, a large number of metal oxides were investigated for their biological activity, especially in biomedical fields. Among those, nickel oxide (NiO) was shown to be cost effective, photo-stable, abundantly available, and nontoxic to humans in short-term exposure [8]. Evaluation of the synthesized NiO nanoparticles by in vitro studies demonstrated that they may be mainly used for medicinal applications as antibacterial agents against many Gram-negative and Gram-positive bacteria. In addition, the cytotoxic potentiality of NiO nanoparticles against cancerous cells was reported [9].

The aim of the present study is to evaluate the biological activity of green synthesized nanoparticles (namely NiO NPS) as cost effective, available, and nontoxic antibacterial and anticancer agents.

Materials and methods

Synthesis of nickel oxide nanoparticles by aloe vera leaf extract

Preparation of aloe vera extract: The leaves of aloe vera were separated from the plant, thoroughly washed, and cut into small pieces. Then 25g of the leaves was mixed with 100ml distilled water and boiled for 2hours at 90°C. Next, Whatmann filter paper was used to obtain the extract and the filtrate was stored to be used in the synthesis of nanoparticles [8].

Synthesis and characterization of nickel oxide nanoparticles: All the chemicals and reagents used for the preparation of NiO nanoparticles were obtained from Sigma, Ltd, UAS, and the preparation of NiO NP was performed according to the procedure described by Ezharasria et al. [9], with minor modification. 10g of nickel (II) chloride hexahydrate (NiCl2) was dissolved in 250ml deionized distilled water and dispersed by an ultrasonication bath for 30 minutes. Then 10ml of the plant extract was added and dispersed by the ultrasonication bath for 60 minutes. Finally, 10M sodium hydroxide solution was gradually dropped into the reaction mixture until a solid black NiO NPs was formed. Following the formation of nanoparticles, Whatmann filter paper was used to separate the particles that were washed with water repeatedly to remove any remaining by-products. After that, different concentrations of the synthesized NiO NPs (125,250 and 500 µg/ml) were prepared.

NP Characterization Techniques: The average crystalline size was measured by atomic force microscopy (AFM) to illustrate the 2D and 3D topologies of the synthesized NiO NPs, as shown in Figure- 1: A. The crystal structure was measured by X-ray diffraction patterns, as shown in Figure- 1:
B. Further characterization was achieved by using Field emission scanning electron microscopy (FE-SEM) as shown in Figure- 1: C). The preparation of NiO NPs was performed in the Biotechnology Department, Nanotechnology Laboratory / College of Science / University of Baghdad / Iraq, while the characterization was conducted in the Chemistry Department / College of Science / University of Baghdad / Iraq.

**Evaluation of antibacterial activity**

**Preparation of bacterial isolates:** The bacterial isolates (Enterococcus faecalis and Acinobacter baumannii) were obtained from the Biotechnology Department Laboratories/ College of Science / University of Baghdad. All the confirmation tests of the present study were performed in the laboratory prior to the experiments. Then the bacteria were activated by growing in nutrient broth and incubated at 37 °C for 24 hours to be used for the determination of antibacterial activity experiments.

**Antibacterial activity:** The potential antibacterial activity of NiO NPs was investigated against Enterococcus faecalis (Gram-positive bacteria) and Acinobacter baumannii (Gram-negative bacteria). The antibacterial activity was estimated using agar well diffusion technique. For this purpose, aliquots of 25 ml of the Müller Hinton agar sterilized medium were distributed into sterilized petri dishes and allowed to solidify at room temperature. The overnight growth of activated tested bacteria was transported and spread over the agar medium using a sterile cotton swab. This was followed by making wells in each plate to examine the antibacterial activity of the synthesized NiO NPs. After that, different concentrations of NiO NPs (125, 250 and 500 µg/ml) were added to the wells. The plates were incubated for 24 hours at 37 °C and the inhibition zones around the wells were measured after incubation [10].

**Evaluation of anticancer activity**

**Cell culture:** The MCF-7 and AMJ13 cancer cell lines (provided by the Iraqi Center for Cancer and Medical Genetic Research / Almustansyria University/ Iraq) were seeded in RPMI 1640 medium supplemented with 10,000 IU penicillin, 10% fetal bovine serum, and 100 µg/ml streptomycin as antibiotics in 96-well culture plates. The culturing conditions included humidified atmosphere of 5% CO2, where the plates were incubated at 37 °C in a CO2 incubator [11].

**MTT Cytotoxicity Assay:** All the procedure of solutions preparation and the experimental tests followed the kit manufacturer's instructions (MTT Kit /Intron Biotech, Korea). 1 x 10⁵ cells/ ml were cultured in 96-well plates and the volume was completed to 200µl with RPMI 1640 medium for each well. The plates were covered with a sterile parafilm, gently stirred, and incubated for 24 hours at 37 °C with 5% CO2. After that, the medium was removed and 200µl of NiO NPs (125, 250 and 500 µg/ml) was added to the wells. In addition to other wells which contained positive control (doxorubicin 50 mg/ml) and negative control (DMSO) in each experimental repeat Three replicates were performed for each control and concentration treatment. The plates were re-incubated for 48 hours at 37 °C with 5% CO2. After treatment with NiO NPs, 10µl of MTT solution was added to each well and the plates were re-incubated for four hours at 37 °C, 5% CO2. Thereafter, 100µl of DMSO solution was added to each well after the removal of the medium and incubated for five minutes. The cell viability was estimated by measuring the optical density at a wavelength of 575 nm of absorbance and calculated according to the following formula:

\[
\text{Cell Viability} \% = \frac{\text{Optical density of sample} \times 100}{\text{Optical density of control}}
\]

**Caspase-9 luminescent assay:** This test was utilized to explain the mechanisms of cell death that was potentially induced by the synthesized NiO NPs since caspase 9 is a member of the caspases family which is involved in apoptosis pathways. The preparation of all solutions as well as the test procedure were performed according to the kit manufacturer’s instructions (Caspase-Glo® 9/ Promega, USA). Cells were seeded, at a concentration of 1 x 10⁴ cells/ well in 96-well plates, at 100 µl of complete medium as a final volume per each well. The plates were incubated for 24 hours at 37 °C with 5% CO2. The medium was removed after incubation and NiO NPs were added to the wells in either of the three concentrations used. Cells were treated with doxorubicin 50 mg/ml which was used as positive a control, while DMSO-treated cells were considered as negative controls. Caspase-Glo® 9 reagent at aliquots of 100µL was added to each well and the plate was thoroughly mixed at 300-500 rpm for 0.5-2 minutes using a plate shaker then incubated at room temperature for 30 min. Ultimately, the luminescence was measured using ELISA reader at 405 nm.
**Statistical analysis:** One-way analysis of variance (ANOVA) was used in this study. The significance of the differences and the correlations among the results were evaluated using SPSS version 23. p values ≤ 0.05 were adopted to denote statistically significant differences. Data were expressed as mean ± standard deviation.

**Results and Discussion**

Because of their wide application in various areas such as drug delivery, catalysis of reactions, and production of sensors, the fields of green nanoscience and technology have recently developed in a large scale[12, 13]. Studies were concentrated to look for alternative drugs with certain antimicrobial and anticancer activities with less side effects and costs as well as higher availability[14].

Nanomolcule analysis is an important challenge in today's nanoscale metrology. By measuring the height of the nanoparticles, the size of the nanoparticles can be easily determined using the AFM (Avg. Diameter: 45.11 nm). Images produced by AFM have several advantages for the distinction of nanomolcules compared to those produced using SEM/TEM [12]. The image from the AFM represents three-dimensional data, thus the height of the nanoparticles can be quantitatively measured. The results of the AFM analysis of the present study are shown in Figure- (1, A).
The results of the X-ray diffraction (XRD) of the synthesized NiO nanoparticles are shown in Figure 1-B. Face-centered cubic (FCC) crystalline structure of nickel oxide can be recognized from the peaks appearing at 2θ = 37.3º, 43.3º, and 63.18º which are indexed as 101, 210, and 240, respectively. Typical NiO patterns for all the resulted diffraction peaks were compared with those of the conventional Shimadzu XRD-7000 card. The sample showed a single phase according to the XRD pattern without any impurities in the diffraction peaks.
The morphological features of the prepared NiO nanostructures were investigated using SEM, as shown in Figure-1- C. The results suggest spherical shapes ranging from 10 to 40 nm in diameter. Besides, because of the heat produced in the annealing process, some of particles were slightly agglomerated. However, the small-sized particles were very reactive because of their sharp edges, which have a high volume - to - surface ratio (higher portions of external atoms) with lower energy than that of the bulk materials.

Results of the antibacterial activity of NiO NPs showed a high antibacterial activity for all tested concentrations (125, 250, and 500 µg/ml) against both tested bacteria (Enterococcus faecalis and Acinobacter baumannii). The antibacterial activity was increased to 16mm and 19mm at higher concentration (500 µg/ml), respectively, as shown Figure-2.

One of the suggested mechanisms for the increased antibacterial activity of NiO NPs was the interaction between the positive charge of bacterial surface, especially in Gram positive bacteria which has strong positive charge on its surface, and the negative charge on the surfaces of nanoparticles [9]. Also, the active metabolic compounds in aloe vera may play a role in increasing the antibacterial activity of the synthesized NiO nanoparticles. However, the antibacterial activity of NiO NPs was previously examined against different types of Gram +ve and Gram -ve bacteria and the results showed mainly large zones of inhibition for some bacterial species [13].
Moreover, the results of anticancer activity were in concordance with those of antibacterial activity. Figure-3 shows the cytotoxicity effects of NiO NPs against two cancer cell lines measured by MTT assay. Three different concentrations of NiO NPs (125, 250 and 500 µg/ml) were tested and a significant \( P \leq 0.0001 \) cytotoxicity effect was observed for all studied concentrations against both tested types of cancer cells in comparison with positive (DMSO) and negative (Serum free media) controls. Cell viability was decreased up to 32% for MCF-7 and 40% for AMJ13 at the highest concentration (500 µg/ml) in comparison with DMSO and serum free media, which reached to 20% and 98%, respectively.

**Figure 3**-In vitro Cytotoxicity of NiO NPs on MCF-7 and AMJ13 cell lines with three different concentrations.

MTT assay measures the number of viable cells to show the number of dead cells after any treatment [15], while caspase 9 analysis explains the mechanism of cell death. Our results of Caspase-9 analysis confirmed that NiO NPs induced apoptosis at all of the studied concentrations. This result was consistence with the findings of our cytotoxicity assay on the two studied cell lines. The results in Figure-4 exhibit that cancer cells treated with NiO nanoparticles reflected a dose-dependence induction of Caspase-9 activity after 24 hours and an increased activity at all treatment concentrations, at a p value of <0.0001. The significantly higher apoptotic activity reached to 78000 and 70000 for MCF-7 and AMJ13, respectively, in comparison with that of the controls which recorded 14000 for negative control and 89000 for positive control at 250 µg/ml.

**Figure 4**-Caspase-9 activity of NiO NPs on MCF-7 and AMJ13 cancer cell lines.
Evidence from previous in vitro animal models and epidemiological studies indicates that NiO NPs inhibit growth and induce apoptosis in a variety of cancer types. These characteristics of NiO nanoparticles may be related to antioxidant, anti-mutagenic, and other biological activities [9, 13].

One part of Caspase-specific proteases family is Caspase-9 that is activated during apoptosis [16, 17]. Caspase-9 activity, that acts as a key promoter factor in mammalian cells apoptotic intrinsic pathway, is measured by Caspase-Glo 9 assay which is a homogeneous luminescence assay [18]. The results obtained by our study indicate that cells treated with NiO NPs undergo apoptosis due to disrupting the cell membrane and cytochrome C release, because both are strongly related to the activation of Caspase-9 [19]. Caspase 9 is the downstream trigger activator of other scorpion Caspases (e.g. 3 and/or 7), leading to apoptosis [20]. Cysteine protease is a major driver of the ability for intracellular cleavage of primary substrates to induce cell death. Caspase family members are classified as initiators (caspase-2, 8, 9 and 10) or effectors (caspase-3, -6 and -7), depending upon their location in the dead signal cascade [21]. Activation of Caspase 9 and 3 is also involved in the mitochondria-dependent intrinsic pathway. The antiapoptotic and pro-apoptotic proteins interact and determine cell fate in mitochondria, in which also the intrinsic pathway is primarily regulated [22]. Protease activators and Caspases, which are pro-apoptotic molecules, in the presence of ATP, are released from the mitochondria during apoptosis [23, 24].

Overall, it can be concluded that the synthesized NiO NPs have significant antibacterial and anticancer effects on the tested bacterial strains and cancer cell lines. The synthesized NiO NPs hold the potential to be a promising antibacterial and anticancer agent.

Reference