Antimicrobial and antioxidant properties of biosynthesized of NiO nanoparticles using Raphanus sativus (R. sativus) extract

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Keywords: calcination, characterization, biological activity, scavenging potential, biosynthesis, Raphanus sativus

Abstract
The aim of this study was to explore the antibacterial and antioxidant efficacy of nickel oxide nanoparticles (NiO NPs). The Raphanus sativus (R. sativus) extract mediated NiO NPs were calcined at 100, 300, 600 and 900 °C in a muffle furnace for 3 h. The increased intensity of diffraction bands in the X-ray diffraction (XRD) spectrum suggest that the degree of crystallinity increases with increasing calcination temperature. The desired elements was depicted in the energy dispersive X-rays (EDX) spectrum confirm the purity of the NiO Sample. The variation in surface morphology and increase in the particles size from 12.78 to 51.42nm was determined from the transmission electron microscope (TEM) micrographs. The shift toward higher wavelength was observed in the diffuse reflectance spectroscopy analysis. The biological potential of the calcined NiO NPs was examined during the antibacterial and antioxidant experiments. The antibacterial effect of NiO NPs was studied using the agar well diffusion process, and the ABTS free radical scavenging potential of NiO NPs was also assessed. The activity of NiO NPs calcined at 100 °C is greater than that of those calcined at higher temperatures.

1. Introduction

Drug-resistant bacteria have emerged as a serious health concern around the world, owing to the widespread use of antibiotics for the care of animals and humans, which has resulted in the evolution of multiple resistant genes in the ecosystem [1, 2]. Drug resistance has intensified to the point that it is now lethal and impossible to handle. Most significantly, developed countries face significant challenges in treating those infections [3, 4]. Free radicals with various chemical origins are very unstable, and extracting electrons from other molecules to achieve equilibrium led to the degradation of the target molecule. Inside the human body, this highly reactive organism is continuously evolving, and it has the capacity to damage short-lived molecules and cellular components like protein, lipids, and DNA [5]. The ABTS− is a stable free radical that form stable diamagnetic molecules when it accepts an electron or hydrogen. By scavenging free radicals, the harmful effects of free radicals such as cancer, heart disease, and neurodegenerative disorders may be minimized [6]. As a result, there is a pressing need to devise some practical means of dealing with the world’s serious problems. Nanomaterials have gotten a lot of recognition as a result of researchers’ fascination with their unique physicochemical properties. The era of the commercial revolution resulted in the manufacturing of hundreds of nanomaterials due to their diverse
properties that allowed them to be used for a variety of applications [7]. Nanoparticles are distinguished from other particles of similar composition by their high surface area to volume ratio.

The NiO is a p-type semiconductor has gained more priority than other NPs owing to their magnetic, electrical, catalytic properties and electron exchange ability [8]. It has good thermal stability, effective hole transport properties, electron-blocking characteristics. The conversion of the anti-paramagnetic to ferromagnetic at room temperature is significant property of NiO NPs [9]. NiO NPs are best photocatalyst for decaying different organic pollutants from polluted water due to their some characteristics like cheap, photo stability, non-toxic and easily available. It has cytotoxic activity on different cells, for example, human breast cancer and human airway epithelial cells [10]. The demand for the simple and safe synthetic method for the synthesis of nanomaterials increases with passage of time. Thus, the plant mediated synthesis has fascinated because it’s economical, simple, non-toxic, and environment friendly nature [11]. With the passing of time, the need for an easy and secure synthetic process for the synthesis of nanomaterials grows. As a result, plant-mediated synthesis has piqued interest due to its cost-effective, quick, non-toxic, and environmentally friendly nature [12]. The NiO NPs fabricated using an aqueous extract of Sageretia thea and were tested for in-vitro pharmacognostic, antioxidant, and cytotoxic properties [13]. Similarly, NiO NPs were synthesized by using Agathosma betulina plant extract and Aspalathus Linearis natural extract as a reducing and oxidizing agent [14, 15].

This study, the facile synthesis of NiO NPs was carried out using R. sativus extract as reducing and capping agent and was calcined at 100 °C, 300 °C, 600 °C and 900 °C. The physicochemical characteristics were investigated through XRD, TEM, DRS, EDX and FTIR spectroscopy. All the calcined NiO NPs were tested against the G PB and GNB species using the Agar well diffusion method. The ABTS free radicals were scavenged through NiO NPs and the effect calcination of activity of NiO NPs was examined.

2. Material and methods

2.1. Material

All chemicals that utilized in this research work are of analytical grade with no further purification. Nickel acetate tetra-hydrate, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic) diammonium salt (ABTS), potassium persulphate, ethanediol and sodium hydroxide were bought from Sigma Aldrich and used without further purifications. Deionized water was used to prepare the working solutions. The glasswares were thoroughly washed with deionized water and rinsed with acetone before being oven dried at 100 °C. R. sativus plant roots were collected from Chakar district Jhelum valley to extract root extract for the formation of NiO NPs.

2.2. Extraction of R. sativus extract

The collected roots of R. sativus were taken, cleaned and washing with deionized water and were cut down into small pieces. The R. sativus extract was extracted with deionized water by sterile grinder. The obtained crude extract was filtered to remove suspended impurities and added into beaker and stored at 4 °C.

2.3. Synthesis of NiO NPs

The 40 mM stock solution aqueous solution of $\text{Ni(CH}_3\text{COO)}_2\cdot\text{4H}_2\text{O}$ was prepared by dissolving 9.95 g in 1000 ml. To synthesized NiO NPs, 80 ml of the stock solution was mixed with 20 ml of concentrated root extract and stirred for 10 min. The pH of the reaction mixture was then adjusted to 10 by adding 0.1 M NaOH solution, and heating and stirring was continued for another 30 min. After cooling for 12 h at room temperature, the reaction mixture was washed three times with deionized water, then ethanol. The solid product was oven dried at 100 °C followed by the calcination in a muffle furnace at 300 °C, 600 °C and 900 °C and pulverize into fine powder.

2.4. Characterization

Using Panalytical X-pert Pro at 2-theta (20°-80°), the difference in crystallographic parameters with calcination temperature was examined. The diameter at half maxima was used to measure the crystallite sizes using the Debye-Scherrer method. A Field electron and Ion Company (FEI) Tecnai G2 F20 S-Twin microscope with a 200 kV accelerating voltage was used for TEM research, while an EDX model INCA 200 was used to investigate elemental composition (UK). The transmittance edge was measured using a DRS spectrum in the 400-1000 nm range, while the band distance was calculated using Tauc’s map. To identify the functional groups present in the samples, an FTIR analysis was performed in the region of 4000-400 cm-1 using a Nicolet 6700 (USA) apparatus.

2.5. Antibacterial assay

The antibacterial efficacy of NiO NPs was tested using the agar well diffusion system against GNB (P. aeruginosa, E. coli) and GBP (S. aureus and B. subtilis) strains. First, agar nutrient plates were made by mixing agar with water
and leaving them in a laminar flow for a while to solidify. Then, wells were bored with a polystyrene tip and bacterial cultures were scattered over them. By ultrasonic dispersing, solutions or suspensions of NiO NPs were prepared in ethanediol (50 mg in 5 ml). Wells of these plates filled by specific volume in a microliter (μl) of the prepared suspension and kept in an incubator, 37 °C temperature was adjusted. After 24 h, the antimicrobial activity of NiO NPs against a bacterial strain was measured in millimeters (mm) [16].

2.6. Antioxidant assay
The antioxidant potential of the calcined NiO NPs was determined using a slightly changed version of the ABTS radical scavenging assay [5]. The decolorization that occurs when ABTS⁺⁺ is reduced to ABTS is used in this assay. 2.5 mM potassium persulphate and 7 mM ABTS were combined and left in the dark for 16 h to make ABTS⁺⁺ free radicals for the ABTS stock solution. Using a UV-double beam spectrophotometer, the absorbance of this solution was measured at 734 nm (Ao) (Shimadzu UV 1800). To make the NiO NPs solution, each calcined sample was dissolved in ethanediol at a concentration of 5 mgmL⁻¹. The research sample (Ai) was measured at 734 nm after diluting 1 mM of ABTS⁺⁺ solution with NiO NPs in a concentration range (10-400 gmL⁻¹) and diluting 1 mM of ABTS⁺⁺ solution with NiO NPs in a concentration range (10-400 gmL⁻¹). Equation 1 was used to measure the percent radical scavenging action, where Ao is the absorbance of the regulation and Ai is the absorbance of the test. The study used the same amounts of ascorbic acid as a reference.

\[
\%\text{RSA} = \left[ \frac{A_o - A_i}{A_o} \right] \times 100
\]

3. Results and discussion
The NiO NPs were prepared by an environmental friendly method using R. sativus extract and were calcined at 100 °C, 300 °C, 600 °C and 900 °C. XRD, TEM, DRS, EDX and FTIR techniques were used to study the optical properties, crystal structure and the surface functional moieties of the prepared NiO NPs. Phytochemical rules are believed to be reducing and capsuling agents, despite the fact that the exact composition of the green synthetic pathway is unknown. Metallic oxide nanoparticles are formed through a series of steps that include ion reduction, nucleation, clustering, growth, and oxidation [17]. The composition of the reducing agent, its concentration, the concentration of precursor salts, the temperature, and the pH all affect each of these phases. The most important phytochemicals found in R. sativus facilitate the formation nanoparticles [18]. Metal ions coated with phytochemicals are reduced by the hydroxyl groups of phytochemicals. In contrast to hydroxyl groups in other flavonoid and phytochemical types, the hydroxyl group in a flavonoid’s catechol moiety has a low dissociation force, according to previous DFT studies [19]. As metal ions are coated with phytochemicals and dried in the air, they become metal oxides [20]. In the presence of compounds containing hydroxyl groups, the chemical reaction for the synthesis of NiO NPs is shown below in figure 1.

\[
\text{NiO} + \text{Autooxidation} \xrightarrow{\text{Temperature}} \text{Ni(0)} + \text{e}^- + \text{H}^+
\]
3.1. EDX analysis
The EDX analysis was used to determine the elemental composition and percentage purity of NiO NPs. The EDX spectrum represented in figure 2, shows sharp peaks at 0.8, 7.9 and 8.5 keV assigned to Ni whereas the signal at the 0.6 keV attributed to O along with the band at the 0.4 keV ascribed to C in the analyzed sample. The corresponding weight percentages for Ni, O and C are 74.2, 24.4 and 1.4 % respectively. The peak for C in the spectrum was due to the carbon tape used for stacking of the sample [21].

3.2. XRD analysis
The XRD diffractograms of the synthesized NiO NPs were shown in figure 3, possess the characteristic peaks of diffraction. The XRD spectrum of as-prepared NiO NPs dried at 100 °C is noisy and lacks a diffraction peak, implying that the NiO NPs are amorphous. Figure 3(b) displays diffraction peaks at 2 points along the corresponding Miller indices of 37.27 (111), which correspond to reference card number 01-073-1519, confirming the cubic geometrical form of NiO NPs. The calculated crystallite size is 9.8 nm with 1.22 % imperfections in the crystal. The XRD pattern of NiO NPs calcined at 600 °C given in figure 3(c) possess three diffraction peaks at 37.48, 43.49 and 62.94 with corresponding hkl values (111), (200) and (220) respectively. The peaks, as well as the hkl values, matched those recorded in JCPDS card 01-073-1519, indicating that NiO NPs with cubic geometry were synthesised. The crystallite size calculated using Debye-Scherrer equation is 22.37 nm.
with 0.401% imperfection. In the XRD spectrum of NiO NPs calcined at 900 °C, diffraction peaks at 2θ position related to hkl values of 37.39 (111), 43.45 (200), and 62.94 are apparent, as seen in figure 3(d) (220). These peaks are all similar to those described in reference card 01-073-1519, suggesting that NiO NPs have a cubic geometry. The crystallite size calculated 100 nm with 0.091% crystal lattice. With the calcination temperature, the amplitude of diffraction bands increases, implying that the degree of crystallinity increases. The increased amplitude and shrunken broadening of diffraction bands due to the rise in crystallite size with calcination temperature whereas the gradual decrease in the crystal lattice with increasing calcination temperature suggests that the crystal obtained at high temperature are more stable.

3.3. TEM analysis
The TEM was used to investigate the influence of calcination temperature on the surface morphology of NiO NPs, and micrographs of all the calcined NiO NPs are shown in figures 4(a)–(d). As-synthesized particles have simple borders in the TEM micrograph (seen in figure 4(a)), but these boundaries vanish after the s NiO NPs are calcined at 300 °C, as seen in figure 4(b). This is due to the heat treatment cracking and transforming both the crystalline and amorphous content of the NiO NPs into clusters, which then break down into particles of comparatively larger size as the calcination temperature is raised to 600 °C, as seen in figure 4(c). The particles that form at 600 °C are interconnected. The noticeable distinctions between the particles vanish as the calcination temperature is increased to 900 °C (as seen in figure 4(d)), the visible boundaries between the particles vanish resulting in the formation of larger particles that are difficult to discern from one another. The
particle sizes estimated from TEM micrographs using ImageJ software for NiO NPs calcined at 100, 300, 600, and 900 °C are 12.78, 21.37, 34.89, and 51.42 nm, respectively.

3.4. DRS analysis
The wavelength of the transmittance edge in the DRS spectra (figures 5(a)–(d)) for NiO NPs calcined at different temperatures is calculated using a previous process \[22\]. The transmittance edges show a large decrease in transmittance due to light adsorption, which causes excitation of electrons from the valence band to the conduction band. The band gap energies for the NiO NPs analogue were calculated using the Tauc plot, and they were observed to decrease as the calcined temperature was increased. The NiO NPs has highest band gap of 3.12 eV whereas the NiO NPs calcined at 900 °C has a lowest ban gap of 2.86 eV as compared the as-synthesized NiO NPs calcined at lower temperatures. The reduction in band gap energy is due to an increase in electron energy, crystallite size, phase structure, and surface roughness, as well as an increase in crystallite size, phase structure, and surface roughness. The band gap energy is a size-dependent property, so as the calcined temperature increases, so does the size, resulting in a visible band gap decrease. An rise in electron energy, as well as an increase in crystallite size, phase structure, and surface roughness, has caused this decrease \[23\].

3.5. FTIR analysis
The FTIR spectra (figures 6(a)–(d)) of NiO NPs, a broad absorbance band in the region of 3541-3282 cm\(^{-1}\) attributed to the stretching vibration hydroxyl group \[24\]. The band at 1648.42 cm\(^{-1}\) is due to the bending vibration of O–H vibration \[25\]. With raising the calcination temperature of the sample, the amplitude of the above-mentioned bands decreased, implying that the water content evaporates at high temperatures. The peaks at 2926.20 and 2856.63 cm\(^{-1}\) along with the other absorbance peak in the range 2060 to 1236 cm\(^{-1}\) in the FTIR spectra of NiO NPs are assigned to C–H (aliphatic) and C–H (aromatic), C=O, C=C, N=C, N=C and NO\(_3\) moieties, which are seem to decompose at high temperature \[25\]. The presence of aliphatic C-H and aromatic C-H moieties are might be due to the use organic source (R sativus extract) as reducing and capping agent. With increasing calcination temperature, the amplitude of these peaks decreased, implying that these moieties decompose at higher temperatures. Heavy vibration of terminal metal hydroxide (Ni-OH) in the lattice structure caused the band at 1076.33 cm\(^{-1}\). The peak 618.43 cm\(^{-1}\) is due to stretching vibration of Ni-O while the bands at 545.28 and 468.12 cm\(^{-1}\) are the bending and the wagging vibration of Ni-O \[26, 27\]. The intensity of bands present in the FTIR spectra decrease with increasing calcination temperature is due to the restructuring and rearrangement of the crystal lattice.
The figure 7 shows experimental images of antibacterial activity of NiO NPs calcined at different temperatures against selected bacterial species. The antibacterial activity of NiO NPs against bacterial strains was measured in mm around each well, while there no zone of inhibition was observed for the control sample, as shown in table 1. The antibacterial...
effectiveness of NiO NPs calcined at 100 °C was higher against all bacteria studied, but it declined as the calcination temperature increased. The drop in NiO NP activity as the calcination temperature increases may be due to an increase in particle size. The surface area of nanoparticles decreased as particle size increased, resulting in a large reduction in reactive surface sites, reducing the ability NiO NPs to inhibit bacterial growth [11].

The mechanism of antibacterial action of metal oxide NPs can be explained in a variety of ways. In aqueous suspensions of NiO NPs, reactive species such as nickel cation, superoxide radical anions, and hydroxyl radical form. The Ni^{2+} ions released from nickel oxides, interfere with the thiol group of essential bacterial enzymes, causing inactivation and cell death, or radicals found in aqueous NiO NPs interact with the negatively charged bacterial cell surface, disrupting vital life processes such as respiration and cell replication, causing inactivation and cell death. The activity of NiO NPs against GNB was observed to be higher than that against GPB, which may be attributed to the differences in cell wall structure between the two pathogens. GPB has a dense and solid cell wall that is made up of multiple layers of peptidoglycan, teichoic acid, and lipoteichoic acid. Teichoic acid and lipoteichoic acid are chelating agents that chelate Ni^{2+} from NiO and transport it within the cell. The cell wall of GNP is composed of an outer membrane and several peptidoglycan layers. The major components of the outer membrane are lipoprotein, lipopolysaccharide, and phospholipids, while the peptidoglycan intact with lipoproteins in fluids, including periplasm allow the transport of NiO [28]. The presence of phosphate ions gives GPB a partial positive charge, while phospholipid and lipopolysaccharide give GNB a heavy surface negative charge. As a result, the negatively charged bacterial membrane is readily interfered with by the electrostatic interaction between the Ni^{2+} ions in the solution, upsetting the biochemical environment. Since the cell wall of GNB is thinner than the cell wall of GPB, Ni^{2+} has a greater capacity to penetrate within GNB than GPB. Furthermore, the oxygen released on NiO NPs’ surfaces reacts with water/moisture to create hydrogen peroxide, which penetrates pathogens and destroys them [29, 30].

3.7. Antioxidant activity
At various concentrations of NiO NPs calcined at different temperatures, table 2 shows the dose-dependent antioxidant efficacy of all NiO NPs calculated against ABTS^{+} radical cations, as well as the percentage radical scavenging operation. The results show that as the sample concentration grows, the amount of calcined NiO NPs that scavenge increases, which may be due to the increased amount of NiO NPs in the solution stabilising the ABTS reactive species [31]. IC_{50} values are the concentrations of NiO NPs needed to scavenge 50% of ABTS^{+} radical cations under the assay conditions. Table 2 shows the measured IC_{50} (gmL^{-1}) values for all calcined NiO NPs. With an IC_{50} of 258 gmL^{-1}, NiO calcined at 100 °C had the highest antioxidant potential of all the samples. If the calcination temperature rises from 100 to 900 °C, the antioxidant function declines. Under the assay conditions, the IC_{50} values are the concentrations of NiO NPs used to scavenge 50% of ABTS^{+} radical cations. The IC_{50} (gmL^{-1}) values for all synthesised samples are described in table 2. As compared to other NiO samples calcined at higher temperatures, NiO NPs calcined at 100 °C had the best antioxidant potential with an IC_{50} of 258 gmL^{-1}. This is because NiO NPs calcined at 100 °C have a greater active surface, which scavenges the ABTS^{+} radical cations. Particle size increases as the calcination temperature rises, according to XRD and SEM analysis. This implies that as the calcination temperature rises, the number of NiO NPs in the ethanediol solution decreases. As the calcination temperature increased, the activity decreased due to a drop in the volume of NiO NPs in solution, which is responsible for the stabilization of ABTS^{+} radicals [32].

Considering the antibacterial potency of the biosynthesized NiO NPs, the activity was considerably higher than previously recorded. This increased activity could be attributed to certain organized organic contents present in R. sativus extract, while the decreased activity of NiO NPs could be due to the deterioration of organic contents after heat treatment at high temperature, among other factors. The proposed mechanism metal (II) i.e. Ni^{2+} and Zn^{2+} based nanostructure like NiO and ZnO are given figure 1, whereas the possible mechanism for the nanomaterial where the metal cation has a +3 oxidation like Cr_{2}O_{3} and Eu_{2}O_{3} are given in figure 8. It is expected that the R. sativus natural extract will prove to be an effective reducing and oxidizing agent based on the
findings of this report. The findings of this study indicate that nano-sized materials synthesized with *R. sativus* natural extract would have higher activity than those previously mentioned [33–36].

4. Conclusion

The NiO NPs was successfully synthesized by a green method using *R. sativus* extract as a capping and reducing agent and were calcined at 100 °C, 300 °C, 600 °C, 900 °C. The XRD analysis confirms the synthesis of highly crystalline NiO NPs and the crystallinity increase with increasing calcination temperature accompanied by crystallite size. The reduction in band gap attributed to increase in particle size and surface roughness. The surface functional groups were studied and suggest the formation of pure NiO NPs. Both antibacterial and antioxidant activity was found higher for NiO NPs calcined at 100 °C as compared to those calcined at higher temperature. These properties are gradually decreasing with increasing calcination, due to the increase in particle size.

Acknowledgments

The authors are grateful to the Deanship of Scientific Research for funding this article by Taif University Researchers Supporting Project number (TURSP-2020/28), Taif University, Taif, Saudi Arabia.
Data availability statement

The data generated and/or analysed during the current study are not publicly available for legal/ethical reasons but are available from the corresponding author on reasonable request.

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