Green synthesis of NiO nanoparticles using *Aegle marmelos* leaf extract for the evaluation of in-vitro cytotoxicity, antibacterial and photocatalytic properties

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ABSTRACT

In the present study, we report the green synthesis of NiO nanoparticles using *Aegle marmelos* as a fuel and this method is ecofriendly and cost effective. The plant *Aegle marmelos* is used in the field of pharmaceuticals to cure diseases like chronic diarrhea, peptic ulcers and dysentery in India for nearly 5 centuries. The as-prepared nanoparticles were confirmed as pure face centered cubic phase and single crystalline in nature by XRD. The formation of agglomerated spherical nanoparticles was shown by HR-SEM and HR-TEM images. The particle size calculated from HR-SEM was in the range 8–10 nm and it matches with the average crystallite size calculated from the XRD pattern. NiO shows intense emission peaks at 363 and 412 nm in its PL spectra. The band gap of NiO is confirmed by DRS studies and the formation of pure Ni–O is confirmed by FT-IR spectra. The as-prepared NiO nanoparticles show super paramagnetic behavior, when magnetization studies are carried out. It is then evaluated for cytotoxic activity towards A549 cell culture, antibacterial activity and photocatalytic degradation (PCD) of 4-chlorophenol (4-CP), which is known as the endocrine disrupting chemical (EDC). From the results, it is found that the cell viability of A549 cells was effectively reduced and it showed better antibacterial activity towards gram positive bacterial strains. It is also proved to be an efficient and stable photocatalyst towards the degradation of 4-CP.

1. Introduction

The increasing environmental pollution has attracted the world wide researchers to work on the development of efficient photocatalysts based on semiconductors for the treatment of contaminated water resources by various organic pollutants that are released from many industries [1]. They impart toxic damage to the environment, human body and aquatic life [2]. Various methods were employed for water treatment like reverse osmosis, active sludge, coagulation and electrochemical oxidation, but most of the organic dyes persist in water even after treatment, because of their stability against temperature, light and chemicals. In recent research, photocatalytic degradation has been suggested as the inexpensive method to remove the organic dyes and pollutants from waste water by using metal oxide semiconductors like ZnO, TiO₂, CuO and NiO [3,4]. Among the semiconductors in nanoregime, the unique features of NiO like inexpensive, non-toxic, photostability and easy availability make it to be an efficient photocatalyst for the degradation of various organic pollutants [5]. In the past few decades, the research efforts to combat the negative effects of EDCs have grown immensely, since they have to be completely removed from the sewage at water treatment plants before the final release into the environment. In recent days, EDCs like 4-CP enter waterways as...
solvents, insecticides, fungicides, herbicides and plasticizers through industrial, medical, agricultural and domestic activities, thereby causing a serious threat to the environment. Because of a new emerging pathogens and the resistance developed against antibacterial therapy, a serious research is needed for the development of efficient antimicrobial agents [6]. Development of multiple drug resistance conventionally has led to adverse side effects, hence, there is an emerging need for alternative strategies to treat bacterial diseases. Nowadays, nanoscale antimicrobial agents and nanosized carriers for antibiotics delivery have been developed and they have proven their effectiveness for treating infectious diseases in vitro and animal models [7]. NiO nanoparticle is known for its antimicrobial activity against various bacterial pathogens and also as an effective photocatalyst in the removal of dyes and organic pollutants from waste water [8]. It is also good in imparting cytotoxic activity over various cells like human airway epithelial (HEp-2) and human breast cancer (MCF-7) cells.

In recent research, nickel oxide nanoparticles have drawn a greater interest, because of its unique properties. It belongs to a wide band gap (3.6–4.0 eV) p-type semiconductor and is chemically stable with high electro-optical efficiency. Nickel oxide nanoparticles are widely applied in numerous fields as adsorbents, solar and fuel cells, catalytic agents, gas sensors, magnetic and antibacterial materials [9,10]. Since particle size, morphology and high crystallinity influence the physicochemical properties, it is of great importance to synthesize NiO nanoparticles with small particle size, preferably less than 20 nm, which could enhance the efficiency of their applications [11].

The nanomaterials having unique properties are used in various applications, such as photocatalysis, lithium ion batteries, smart windows, field emission studies, antimicrobial activity, dye-sensitized photocathodes, thermal conductivity and anti-ferromagnetic films [12–15]. Because of the peculiar physicochemical properties, the nanoparticles change the properties of the materials like its thermal, optical, electrical and mechanical properties [16]. The methods like coprecipitation, microemulsion, sol-gel method, electrospray synthesis, laser ablation and hydrothermal reaction [17–18] are used for the synthesis of nanomaterials. These conventional methods possess high cytotoxicity, low productivity and are not ecofriendly. In the past few decades, bionanotechnology is gaining momentum, as the scheme includes non-hazardous, environmentally friendly biological systems like bacteria, fungi, leaves, vitamins and yeast for the synthesis of metal oxide nanoparticles [19]. Hence in the present work, NiO nanoparticles are prepared by a novel, simple, efficient, environment friendly and green route using Aegle marmelos extract, which plays the role of reducing, capping agent and fuel. It is also reported that using leaf extract, it is possible to control the shape and size of the particles. Being biocompatible, it also hinders agglomeration of nanoparticles [20].

Aegle marmelos, commonly known as Bilva or Sriphal or Bael are indigenous to India for over 5000 years. It is found in abundance in the Himalayan regions, Bengal, Sri Lanka, Burma, Vietnam and Pakistan. The root, bark, seed and fruit of Aegle marmelos are of great value in the folk systems and the traditional ayurvedic system of medicine in India. The fruit pulp of Aegle marmelos contains bioactive compounds like phenolics, alkaloids, pectins, flavonoids, tannins, coumarins, catenoids and terpenoids [21]. The leaves also possess astringent and febrifuge properties, which are useful in treating dropsy, diarrhea, dysentery and bowel complaints. Aegle marmelos is reported to contain major phytochemicals, such as, limonene, bphellandrene, p-cymene, linalool, α-cubebene, 3,5-octadiene-2-one, trans-p-metha-2,8-dienol, citronellal, cineole, citronella, citral, cuminaldehyde, β-cubebene, β-caryophyllene, hexadecane, pulegone, α-humulene, verbeneone, karvone, Carylacetate, dihydro-β-ionone, (E)-6,10-dimethyl-5,9-undeca dien-2-one, β-ionone, caryophyllene oxide, humulene oxide and hexadecanoic acid [22].

Aegle marmelos has a broad range of therapeutic effects, namely inhibition of lipid peroxidation, anti-diarrheal, antioxidant, anti-bacterial, antiviral, anti-ulcerative colitis, cardio protective, antidiabetic, gastro protective and radio protective effects. The Aegle marmelos leaves possess antibacterial and antifungal activities in vitro. The infections in humans, especially in children caused by coxsackie viruses B1–B6 and the antiviral effects are reported by Badam et al. [23]. In recent years different types of fuels are used in the combustion synthesis which includes polyethylene glycol (PEG), poly-acrylic acid (PAA), organic polymers, starch-derived polymer and many more. During combustion these fuels decompose with the emission of large amount of heat and toxic gases. As a viable alternative to chemical synthetic procedures plant extracts are employed in the synthesis of nanomaterials, as reducing and capping agents. In our present study, we have used the leaf extract of Aegle marmelos as the fuel to synthesize NiO nanoparticles and evaluate their efficiency in terms of cytotoxicity, antibacterial activity and photodegradation of 4-CP. Since records of Aegle marmelos prove that they contain important bioactive compounds which initiated antibacterial effects on various pathogens and have also been used as an adsorbent for the removal of toxic metal in preventing the environment, it would be a promising fuel in synthesizing NiO nanomaterial.

2. Experimental Method

2.1. Preparation of Nickel Oxide Nanoparticles Using Aegle marmelos Leaves by Hot Plate Combustion Method

Aegle marmelos leaves were taken and washed thoroughly with distilled water. They were then cut into pieces and ground using pestle and mortar. From this, 30 g of leaves was stirred in 100 mL of distilled water and ethanol for 2 h. It is then kept for incubation for 24 h at room temperature. Finally, it was filtered using Whatmann No.1. filter paper. The clear solution obtained is the plant extract, which is used as a fuel to synthesize NiO nanoparticles.

The hot plate combustion method was employed to synthesize NiO nanoparticles from 0.1 mM Ni(NO₃)₂ aqueous solution (Merck chemicals, India, 99% purity), P. Aegle marmelos leaf extract (5 mL) and glycin (C₂H₄NO₂). The amount of precursor for the synthesis was estimated using the stoichiometric ratio and 1.5 g of NiO was obtained. Initially, the precursors were dissolved in a 70 mL of deionized water and kept aside for 1 h with continuous and constant stirring to attain a homogenous mixture of a solution. In the hot plate combustion method, the above homogeneous solution was placed in a hot plate heater (Barnstead Thermolyne, model no: SP46925) and uniformly heated up to 250 °C for 15 min, which leads to the volatilization of water and combustion of the reaction mixture. The black colored precipitate was then separated through centrifugation and washed multiple times with deionized water. The separated black powder was dried at 100 °C in hot air oven and subjected to annealing at 300 °C for 2 h.

2.2. Analytical Methods for Characterization

The formation of pure NiO phase was confirmed by Philips Xpert diffractrometer for 2θ = 10°–80°, λ = 0.154 nm) and Perkin Elmer infrared spectrophotometer. The morphology and particle size was observed by stereoscan LEO 440 (HR-SEM) and (JEOL 3010) (HR-TEM). Cary100 UV–Visible spectrophotometer and Varian Cary Eclipse Fluorescence Spectrophotometer were used to study the absorption and emission properties of NiO nanoparticles. Structural characterization of the synthesized nickel oxide nanoparticles prepared using Aegle marmelos leaf extract was performed using Philips Xpert diffractometer for 2θ = 10°–80° using Cu Kα radiation at λ = 0.154 nm. The surface functional group of NiO nanoparticles was determined by Perkin Elmer infrared spectrophotometer. The particle size and morphology were performed using high resolution scanning electron microscope (HR-SEM) (Stereoscan LEO 440) and high resolution transmission electron microscope (HR-TEM) (JEOL 3010). Absorption and emission characteristics of the synthesized photocatalysts were recorded using Cary100 UV–Visible
spectrophotometer and Varian Cary Eclipse Fluorescence Spectrophotometer at an excitation wavelength of 372 nm.

2.3. In-vitro Cytotoxic Activity NiO Nanoparticles by MTT Assay

Nano NiO prepared using Aegle marmelos leaf extract was evaluated for in-vitro cytotoxic activity against A549 cell lines (Life Tech Research Center, Chennai). Cells were maintained at humid atmosphere with 5% CO₂ after seeding in a 96-well plate at 37°C. It was kept for incubation for 24 h and then the culture medium was removed. The mitochondrial activity of the cultured cells were determined using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay and the degree of cytotoxicity was evaluated for various concentrations of the synthesized NiO nanomaterial and the range of extracts added to the culture medium were 1000, 500, 250, 125, 62.5, 31.2, 15.6 and 7.8 μg/mL. The mitochondrial succinate dehydrogenase enzymes in the viable cells reduced MTT, a yellow dye into purple colored formazan crystals, which were further dissolved in DMSO to attain the complete solubility. Later the optical density for various concentrations was quantified by using the spectrophotometer at specified wavelength.

2.4. Antibacterial Activity Studies

The antibacterial activity of the NiO nanoparticles was evaluated against both Gram positive (Streptococcus Pneumoniae, Staphylococcus aureus) and Gram negative (Escherichia Hermannii, Escherichia coli) bacterial strains and the test organisms were collected from American type culture collection (ATCC) and Microbial type culture collection (MTCC) Chandigarh, India. Disc diffusion method was employed to study the antibacterial behavior of NiO nanoparticles. Petri plates were prepared with 20 mL of sterile Muller Hinton agar (MHA, Himedia) and each bacterial pathogen was individually swabbed on MHA in separate plates. The antibacterial activity was tested at a specific concentration with the required quantity of the NiO nanoparticle dissolved in dimethyl sulphoxide (DMSO). The zone of inhibition levels (mm) were measured after 24–48 h and before this step, it was incubated overnight at 37°C. The sample loading was carried out by Kirby Bauer method.

2.5. Photocatalytic Reactor Setup and Degradation Procedure

The schematic diagram of the photocatalytic reactor is shown in Fig. 1. The degradation studies were done by using a photocatalytic reactor designed by us for the study. It has quartz/borosilicate cylindrical tubes with dimensions 36–1.6 cm (height-diameter). After the optimization, optimum 4-CP solution and NiO nanoparticles (photocatalysts) were taken in the above mentioned reactor tubes supported with air passage, so that the photocatalyst is exposed to the irradiated light throughout the reaction. It is irradiated with UV light using a low pressure mercury lamp that emits light of wavelength at 365 nm. In the present study, 100 mL of aqueous 4-CP was taken along with optimum NiO catalysts and kept for stirring for 30 min. It was aerated for 30 min and then subjected to UV light. Aliquots were withdrawn at specific intervals of time from the suspension, centrifuged (1500 rpm) and filtered using 0.2 μm Millipore filter paper. The rate of degradation of 4-CP was monitored using Perkin Elmer UV–Visible spectrophotometer.

The expression given below is used to calculate the degradation efficiency (η);

\[ \eta = \frac{Ci - Ct}{Ci} \times 100 \]

where Ci and Ct are the initial concentration and concentration of 4-CP after “t” minutes respectively.

3. Results and Discussion

3.1. Structural Investigations of NiO Nanoparticles Prepared Using Aegle marmelos Leaf Extract

Fig. 2 shows the XRD pattern of the synthesized NiO nanoparticles using Aegle marmelos leaf extract prepared by the hot plate combustion method. The highly crystalline NiO nanoparticles displayed sharp and intense diffraction peaks that perfectly matched well with the standard JCPDS file no: 4-0835. It shows the presence of (111), (200), (220), (311) and (222) planes. The XRD pattern confirms the formation of pure face centered cubic NiO and the broad peaks correspond to the presence of oxygen vacancies and local lattice disorder in the sample [24]. The leaf extract used acts as a fuel to produce nanoparticles in nanoregime having high surface area and good dispersity. The crystallite size was estimated from the plane (200) and is found to be 8.15 nm, and this may be due to the presence of various photochemicals in the Aegle marmelos leaf extract, which plays the role of fuel, capping and reducing agents so that nanometal oxides are formed [25]. This is the favorable condition towards their biological and photocatalytical activity.

The surface morphology of the as-synthesized NiO nanomaterial prepared using Aegle marmelos leaf extract was studied using HR-SEM images as shown in Fig. 3. The particles were cubic and spherical in shape with slight agglomeration. The formation of nanoparticles is due
to the large volume of heat produced during the combustion process. The amount and nature of the fuel used forms the size, shape, morphology, degree of agglomeration and properties. The formation of cubic and spherical nanoparticles which are slightly agglomerated and porous was observed in HR-TEM images (Fig. 4). The corresponding selected area electron diffraction (SAED) shows polycrystalline nature.

The particle size was in the range of 8–10 nm and it agrees well with the average crystallite size calculated from the XRD pattern. The agglomeration of particles confirms the presence of polymeric adherence and magnetic interaction among the particles [26]. The *Aegle marmelos* leaf extract, which acts as a fuel is capable of producing NiO nanoparticles and therefore act as a promising fuel to prepare the nanomaterials. The purity of NiO nanoparticles was confirmed by Energy Dispersive X-ray analysis (EDX), which accurately quantified the Ni and O contents by showing only Ni and O peaks without any other impurity (Fig. 5). It is the supporting evidence for the formation of pure NiO along with XRD and FT-IR studies.

### 3.2. Photoluminescence Investigations of NiO nanoparticles Prepared Using *Aegle marmelos* Leaf Extract

The nanostructured materials possess high density of crystal defects than their bulk. The PL spectra was recorded to understand the effects of quantum size and crystal defects. Fig. 6 shows the PL spectra with intense emissions peaks at 363 nm, 412 nm and broad emission near 440 nm. The radiative recombination of the excited electrons with the holes in the valence band is confirmed by the UV emission at 363 nm. Due to the presence of $Vo$ and Ni(in) defects there is an emission peak at 412 nm, which in turn confirms the intrinsic defects. The presence of shoulder peak near 440 nm [27,28] corresponds to the surface defects. Hence the *Aegle marmelos* leaf extract used in the present study was able to synthesize NiO nanomaterials with intrinsic defects, oxygen vacancies and surface defects, which will usually enhance the photocatalytic and biological
activity. Hence, it is believed that the synthesized NiO nanomaterial with reduced particle size, high surface defects and high surface area will possess enhanced activity.

3.3. DRS Studies

Fig. 7 shows the optical diffuse reflectance spectra (DRS) recorded at room temperature using Teflon as a reference sample to calculate the energy band gap of NiO nanoparticles. There is a small band at 247 nm (5.02 eV) and a broad band in the range of 269–346 nm (4.60–3.58 eV). Generally NiO displays an optical band gap of 3.5 eV and the absorption peak at 355 nm. Hence, the absorption peaks at 303 nm, 371 nm and 341 nm may be due to the quantum confinement. The electronic transition from the ionized oxygen vacancies to the valence band of NiO originated from the surface defects occurs below 300 nm. Therefore the increased band gap of the synthesized NiO nanomaterial than that of the bulk indicates the reduced particle size of the NiO nanomaterial and the existence of quantum confinement effect. However, the absorption peak of the NiO nanomaterial is at lower energy in the UV region than that of the bulk, hence it could be potentially used as a photocatalyst,
since the excitation of electrons from the valence band to the conduction band is possible through UV light excitation.

3.4. Fourier Transform Infrared Spectroscopy (FT-IR) Studies

Fig. 8 shows the FT-IR spectra of NiO nanoparticles prepared using Aegle marmelos leaf extract. The strong intense peaks at 482 cm\(^{-1}\), 580 cm\(^{-1}\) and 900 cm\(^{-1}\) confirm the presence of metal oxygen stretching frequency of Ni-O in the synthesized nanoparticles [29]. The moderate peak observed at 1018 cm\(^{-1}\) corresponds to single C–O bond stretching mode. Absorption peaks observed at 1186 cm\(^{-1}\), 1356 cm\(^{-1}\) and 1518 cm\(^{-1}\) corresponds to C=C aromatic stretching vibration band, which may be due to various phytochemicals present in the Aegle marmelos leaf extract. The broad band at 3400 cm\(^{-1}\) is because of the stretching vibration mode of the chemically bonded hydroxyl group, and it is due to the water molecules adsorbed on the surface of NiO. The adsorption of water molecules by the nanoparticles indicate that the synthesized nanomaterial possess high surface area [30]. The presence of carbonyl group in the leaf extract is confirmed by the strong intense peak at 1750 cm\(^{-1}\) and the moderate peak 3380 cm\(^{-1}\) is due to the N–H (amine) stretching frequency [31]. Thus, the results of FT-IR spectrum coincide with the XRD data and in turn confirm the formation of highly pure single NiO nanoparticles.

3.5. Magnetization (VSM) Studies

The magnetization behavior of the synthesized NiO using Aegle marmelos leaf extract was investigated by sweeping the external magnetic field between +20 kOe to –20 kOe at 300 K. Fig. 9 shows the plot between magnetization (M) Vs magnetic field (H). The NiO exhibited slight curves in the low magnetic field, and a linear portion at higher magnetic field regions. It was also observed that the sample never attained saturation even at higher magnetic field of about (20 kOe), which confirms the presence of super paramagnetism. If there is a reduction in the particle size, the magnetic property will be changed from diamagnetism to super paramagnetism [32]. Hence the super paramagnetic behavior exhibited is due to the reduced particle size (8.15 nm) [33], which are also proven by the obtained XRD results. As the magnetic behavior is sensitive towards the magnetic impurities, the existence of super paramagnetic behavior even at higher magnetic field proves the purity of the sample. The methodology employed in synthesizing NiO nanoparticles using phytochemicals in the present work has resulted in reduced particle size with super paramagnetic behavior, and in turn proves to be a promising material for various magnetic applications.

3.6. Evaluation of In Vitro Cytotoxicity Activity Using NiO nanoparticles prepared using Aegle marmelos leaf extract

The cytotoxic effect of the synthesized NiO nanoparticles was evaluated for A549 cell line. The diluted ranges of synthesized NiO extracts added to each plates were 1000, 500, 250, 125, 62.5, 31.2, 15.6 and 7.8 μg/mL. The test samples was taken along with known quantity of the cells, and the % cytotoxicity for each concentration was tested by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) assay. The absorbance of the purple solution obtained by mixing the above crystals with dimethyl sulfoxide was measured at 540 nm using spectrophotometer [34]. The cytotoxicity was recorded using (IC50 value) the formula given below as the drug concentration causing 50% growth inhibition of the tumour cells.

\[
\text{% cell viability} = \frac{OD \text{ sample mean}}{OD \text{ control mean}} \times 100
\]

Table 1 shows the variation of cell viability against various concentrations of the NiO nanoparticles. In general, the cell viability decreases with an increase in the concentration of nanoparticles and the percentage of reduction was dose dependant. In MTT assay, A549 cell viability was observed to be 25%, when treated with 1000 μg/mL of NiO nanoparticles prepared by Aegle marmelos leaf extract, and increased gradually to the maximum of 64.6% at the concentration of 7.8 μg/mL. The variation of the percentage cytotoxicity and optical density against various concentrations of NiO nanoparticles is shown in Fig. 10. The absorbance increases from 0.103 OD to 0.331 OD, since the NiO nanoparticle concentration is decreased from 1000 μg/mL to 7.8 μg/mL respectively, whereas for the controlled cells, the absorbance was 0.512 OD. From the results, it is confirmed that NiO nanoparticles have imparted a remarkable effect over A549 cells in cell damage, and it agrees well with the literature [35]. Fig. 11 shows the cell morphology of A549 cell lines treated against different concentration of NiO nanoparticles. From the image, it is clear that the morphology of A549 cell lines on treatment with the synthesized NiO nanoparticles has undergone cell swelling and cell damage as a result of apoptosis. In controlled cells, a smooth and regular morphology was observed and the intensity of cell line damage was dose dependent. It is evident from the figure that the cell line treated with 1000 μg/mL has a severe swelling and damage in the cell line. The mechanism for the influence of specific factors on the cytotoxic effects is not clear yet. There are few reports, which suggests that the metal ions released from the nanometal oxides play a major role in nanocytotoxicity [36]. The release of Ni\(^{2+}\) ions will be higher for nanosized NiO, which will induce a higher cytotoxicity than the micron sized particles. Hence, the higher cytotoxic activity of the synthesized NiO nanoparticles over A549 cell lines may also be attributed to their reduced particle size (8.15 nm). It favors the adsorption of proteins and salts on the surface and it can be related to the cellular influence [37,65–68]. The particle size of 8.15 nm achieved holds good agreement with TEM and XRD results. Metal ion release also supports the movement of the exogenous materials inside the cells and

<table>
<thead>
<tr>
<th>S. No</th>
<th>Concentration (μg/mL)</th>
<th>Dilutions</th>
<th>Absorbance (O.D)</th>
<th>Cell viability (%)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1000</td>
<td>Neat</td>
<td>0.103</td>
<td>20.11</td>
</tr>
<tr>
<td>2</td>
<td>500</td>
<td>1:1</td>
<td>0.187</td>
<td>36.52</td>
</tr>
<tr>
<td>3</td>
<td>250</td>
<td>1:2</td>
<td>0.225</td>
<td>43.94</td>
</tr>
<tr>
<td>4</td>
<td>125</td>
<td>1:4</td>
<td>0.264</td>
<td>51.56</td>
</tr>
<tr>
<td>5</td>
<td>62.5</td>
<td>1:8</td>
<td>0.293</td>
<td>57.22</td>
</tr>
<tr>
<td>6</td>
<td>31.2</td>
<td>1:16</td>
<td>0.312</td>
<td>60.93</td>
</tr>
<tr>
<td>7</td>
<td>15.6</td>
<td>1:32</td>
<td>0.324</td>
<td>63.28</td>
</tr>
<tr>
<td>8</td>
<td>7.8</td>
<td>1:64</td>
<td>0.331</td>
<td>64.64</td>
</tr>
<tr>
<td>9</td>
<td>Cell control</td>
<td>-</td>
<td>0.512</td>
<td>100</td>
</tr>
</tbody>
</table>

Fig. 9. Magnetic hysteresis (M-H) curves measured at 300 K for NiO nanoparticles.
induces oxidative stress directly or indirectly. Different mechanisms were proposed based on the metal ions released. This includes the activation of calcium dependant and stress-inducible signaling cascades, which interferes with DNA repair pathways and causes epigenetic changes [38,39]. The excess production of reactive oxygen species ROS and oxidative stress can cause DNA damage and apoptosis. The oxidative stress may be because of the electrons transferred from the ROS like \( \cdot \text{OH}, \text{H}_2\text{O}_2^{-}, \text{O}_2^{-} \) [40]. The above mentioned mechanism for the various damages due to the cell lines is shown in Fig. 12. The ROS produced damages cell wall of, DNA, single and double strand breaks and leads to severe cell death [41,69–71].

Thus the synthesized NiO nanoparticles using Aegle marmelos leaf extract as the fuel possess high cytotoxic activity and the results from Table 1 and Fig. 10 proves that at increased concentration of NiO nanoparticles, the absorbance and cell viability decreases. The reduced particle size, specific surface area and release of more Ni\(^{2+}\) ions cause maximum cytotoxic effect in the present study. Hence, NiO nanoparticles are promising biomaterial, since it induces cytotoxicity and apoptosis in A549 cell lines, which occurs through the generation of ROS and oxidative stress.

### 3.7. Evaluation of Antibacterial Activity

Antibacterial activity of the NiO nanoparticles synthesized by Aegle marmelos leaf extract was evaluated against two Gram positive and Gram negative strains. Various zones of inhibition (mm) are presented Fig. 10. MTT assay measurement on variation of % cell viability and optical density against various concentrations of NiO nanoparticles.

![MTT assay](image-url)

![Effect of A549 cell morphology on treatment with NiO nanoparticles under various concentration.](image-url)
Hermannii (6 mm). It is a well-known fact that the antibacterial activity that were more effective over Gram positive bacteria than the micron sized particles. There are also few reports on NiO nanoparticles for long term activity. In general, the effective antimicrobial activity depends on the particle dosage, methodology used and treatment time [45].

The exact mechanism of antibacterial activity is not reported yet, but there are studies that the nanoparticles interact with the bacteria by causing a change in the morphology of the membrane, thereby increases the permeability. It interrupts in the regular transportation through the plasma membrane, thus leading to cell death [46]. As the synthesized NiO nanomaterial has reduced particle size accompanied with large surface to volume ratio, it facilitates the interaction and enhances the antibacterial activity than the micron sized particles. Sulphur and phosphorus in the DNA of the bacteria and the functional groups of the protein interacts with NiO nanoparticles and leads to protein leakage and cell death [47].

The formation of ROS enhances the antibacterial activity and they are the natural by products of metabolism [48]. The cells are destroyed, because the induction of ROS that leads to the formation of highly reactive radicals, thereby damage the proteins, cell membranes, DNA, and intracellular system [49]. The schematic representation for the ROS-formation is shown in Fig. 14. When the light is incident on NiO nanomaterial, whose photon energy is higher than the band gap $E_g$, because of the photoexcitation there will be a formation of holes ($h^+$) in the valence band and electrons ($e^-$) in the conduction band. They have high redox properties and hence react with molecular oxygen (O$_2$) and hydroxyl ions ($\cdot$OH) to generate various ROS•. When molecular oxygen traps the electron, it generates superoxide anion ($O_2•^-$) and hydroxyl radicals (•OH). Superoxide anion reacts with water molecules and there will be absorption of electrons from water and hydroxyl ions from the holes for the mineralization of bacterial cells. The production of ROS• is mandatory and they are the dominant species to decide the cell death, since it induces oxidative stress, and lead to the damage of cell membrane, protein deactivation, interrupt in the electron transport, damages DNA and mitochondria, and thus results in cell death [50].

Hence, in the present study, the antibacterial activity is enhanced, because of the use of NiO having high stability, smaller size and spherical morphology and this is because of the usage of Aegle marmelos leaf extract by a green route. The sufficient amount of Ni$^{2+}$ ions released penetrate into the cell wall, which later disturb the electron transport and affect DNA, protein and mitochondria, which finally leads in Table 2 and Fig. 13. It was observed that the as-synthesized NiO nanoparticles were more effective over Gram positive bacteria than the Gram negative bacterial strains Staphylococcus aureus (16 mm), Streptococcus Pneumoniae (12 mm), Escherichia coli (11 mm) and Escherichia Hermannii (6 mm). It is a well-known fact that the antibacterial activity will be more pronounced for Gram negative bacteria than Gram positive bacteria, because of the thin layer cell wall with single layer of peptidoglycan, whereas Gram positive bacteria possess multilayered thick peptidoglycan. There are also few reports on NiO nanoparticles that were more effective towards Gram positive bacterial strains [42,43].

The enhanced antibacterial activity is due to the smaller particle size of the NiO nanoparticle, as it enhances the dispersibility, and permits the inference of the extracellular Ni$^{2+}$ with the intracellular Ca$^{2+}$ metabolism, thus resulting in the cell damage. When the size of the nanoparticles decreases, the electrostatic interaction between the microbial cell membrane and Ni ions released from NiO nanoparticles becomes strong and hence increases the antibacterial activity [44]. The activity also depends on the stability, morphology, and shape of the nanoparticles for long term activity. In general, the effective antimicrobial activity depends on the particle dosage, methodology used and treatment time [45].


table2

Antibacterial activity of NiO nanoparticles against pathogens.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>16</td>
</tr>
<tr>
<td>Streptococcus Pneumoniae</td>
<td>12</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>11</td>
</tr>
<tr>
<td>Escherichia Hermannii</td>
<td>6</td>
</tr>
</tbody>
</table>

Fig. 12. Schematic mechanism of toxicity effect of NiO nanoparticles against bacteria. NiO nanoparticles and their ions generate ROS, and induce oxidative stress resulting in the damage of mitochondria, DNA, proteins and cell membrane with consequent cell death.

Fig. 13. Results of inhibition zones of antibacterial activity by NiO nanoparticles (a) Staphylococcus aureus - 16 mm (b) Streptococcus Pneumoniae - 12 mm (c) Escherichia coli - 11 mm and (d) Escherichia hermannii - 6 mm.

Fig. 14. Schematic representation of the light irradiated NiO nanoparticles and the generation of ROS causing bacterial death.
to the cell death. As a result of natural metabolism, there is a generation of ROS under light irradiation. Oxidative stress is induced inside the cell that results in cell death. Since the activity and stability of NiO nanoparticles are effective, they could be employed in as antimicrobial coatings that will be helpful for various environmental and biomedical applications.

3.8. Photocatalytic Degradation of 4-CP

3.8.1. Effect of the Concentration of 4-chlorophenol

50–250 mg/L of the 4-CP was considered at a neutral pH to study the effect of initial concentration on the photocatalytic degradation (PCD) (Fig. 15). The PCD efficiency decreased from 93% to 74%, when the initial concentration of 4-CP is increased from 50 to 250 mg/L. It may be because of the adsorption of more and more 4-CP molecules on the surface of NiO and the amount of \( \cdot \text{OH} \) and \( \cdot \text{O}_2^- \) radicals may be insufficient to degrade higher concentration of 4-CP [51]. There may be a production of more intermediates, which will compete to react with \( \cdot \text{OH} \) formed from photo generated holes [52]. Hence, under the given set of conditions, the maximum concentration of 4-CP that could be photodegraded by 30 mg/100 mL of NiO nanoparticles is found to be 100 mg/L. Thus, 100 mg/L 4-CP was fixed as the optimum concentration for the study of other parameters.

3.8.2. Effect of the Catalyst Dosage

In order to investigate the optimum amount of catalyst needed for the efficient PCD of 4CP, the catalysts dosage was varied as shown in Fig. 16. The results indicate that a maximum PCD occurs for 30 mg/100 mL, which confirms that the rate of photodegradation is affected by the total number of available active sites of the catalyst and photo absorption ability. With increased catalyst dosage, the PCD is decreased because it prevents the illumination of UV light and affects the production of hydroxide radicals, which are primary oxidants in the photo degradation process and high catalyst dosage also induces scattering effect. Thus, 30 mg/100 mL of NiO nanoparticle was fixed as the optimum catalyst dosage to study the mineralization of 4CP.

3.8.3. Effect of Initial pH

The PCD efficiency was studied under different pH [4,6,8,10] under the optimized experimental conditions (100 mg/L 4-CP solution and 30 mg/100 mL NiO nanoparticle loading) shown in Fig. 17. When pH = 2, the PCD of 4-CP was found to be very low and with an increase in pH the efficiency of PCD also increased and maximum PCD is observed at pH = 8, which later decreased at pH = 10. In acidic medium, the reduced PCD of 4-CP is due to the significant loss of NiO to Ni\(^{+2}\) [53] which in turn reduces the production of \( \cdot \text{OH} \). In alkaline medium, there will be a production of more \( \cdot \text{OH} \) radicals, the primary oxidizing agent which will enhance PCD [54]. Also the stability of NiO nanoparticles was not disturbed under the alkaline conditions, which favors the adsorption of 4-CP on the surface and leads to increased PCD efficiency. At pH = 10, the decrease of PCD is attributed to the negative charge on NiO surface, which reduces the concentration of 4-CP. The formation condition occurs at a pH, where the surface charge becomes zero so that there will be a generation of more \( \cdot \text{OH} \) radicals and adsorption of more 4CP molecules on the catalyst surface [55]. Consequently, the PCD was effective till pH = 9 and decreased at pH = 10. Hence, the optimum pH is considered as pH = 8.

3.8.4. Photocatalytic Mineralization of 4-CP

The photocatalytic degradation of 4-CP was carried out under optimized conditions (4CP = 100 mg/L, photocatalyst = 30 mg/100 mL, pH = 8, NiO nanomaterial) at the wavelength of 365 nm. The irradiation of UV light on the photocatalyst promotes the electrons to the conduction band with high kinetic energy and there is a formation of holes in the valence band. The effect of time on PCD efficiency of 4-CP
mineralization is observed at 210 min. The presence of few long lived intermediates would have caused the slow degradation initially. Later, the PCD is initiated by branch dissociation, where ·OH reacts with the organic molecules (4-CP) on the catalytic surface [56]. The possible degradation mechanism scheme is shown in Fig. 20. When NiO is irradiated by light having the greater band gap energy, there will be an electron excitation from the valence band [VB] to a conduction band [CB], thereby leaving a hole in the valence band. The electrons at the conduction band absorb molecular oxygen and produces ·O$^{2-}$ and the holes in VB forms ·OH by reacting with water [57]. OH$^-$ being the powerful oxidizing agent degrade the organic pollutants on the photocatalytic surface. In the present work, the efficient and complete mineralization of 4-CP occurs, which is due to the reduced particle size of NiO [58-62]. Photocatalytic nanoparticles with smaller particle size possess more active sites and exhibit high efficiency in surface charge carrier transfer [63-64]. Furthermore, the synthesized nanomaterial was capable of absorbing light on irradiation with the production of sufficient amount of hydroxide, superoxide and peroxide radicals to mineralize 4-CP completely to CO$_2$ and H$_2$O. Thus the synthesized NiO nanomaterial using $Aegle marmelos$ leaf extract possess unique features like spherical self-assembled morphology, reduced particle size inherent with surface defects, high crystallinity and stability. From all the above results, it is concluded that NiO nanoparticles prepared in the present study can be used in waste water treatment and it helps to reduce the environmental pollution.

4. Conclusion

$Aegle marmelos$ leaf extract is used as a reducing, capping agent and fuel for the synthesis of NiO nanoparticles through a green route in the present study. XRD and FT-IR confirms the formation of pure and crystalline NiO nanoparticles, which showed superior performance in cytotoxic activity, antibacterial studies and photocatalytic activity. The cytotoxic activity studies over A549 cell lines were carried out and it showed effective activity. From the antibacterial studies, it is found that NiO activity was more pronounced for Gram positive bacterial strains than Gram negative bacterial strains. The photocatalytic degradation studies confirms its effectiveness towards the complete degradation of 4-CP at pH 8 under the optimized conditions. The mechanism is also proposed wherever required. Thus, we have proposed a green protocol to synthesize an effective biomaterial (NiO nanoparticles) with high purity and crystallinity, reduced particle size, more surface defects and self-assembled morphology, which are the advantageous factors to be used for various environmental and applications.

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