Antibacterial and anticancer activity of hydrothermally-synthesized zinc oxide nanomaterials using natural extracts of neem, pepper and turmeric as solvent media

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Abstract

A novel and simple wet chemical hydrothermal synthesis method was employed in the preparation of zinc oxide (ZnO) nanoparticles using neem (N), pepper (P) and turmeric (T) extracts as solvent media. The structural and optical properties as well as the antibacterial and anticancer properties of all the samples (ZnO, N/ZnO, P/ZnO, T/ZnO and NPT/ZnO) were characterized and analyzed. Solvent media was found to have an effect on both the size and the morphology of the nanoparticles, which in turn affected their optical and cytotoxic properties. The colony forming unit (CFU) assays were done for E. coli, S. aureus and S. typhi in which T/ZnO (~2) and P/ZnO (~3) showed a remarkable effect on S. aureus for 100 μg ml⁻¹ and nearly zero for 150 μg/ml. The zone of inhibition (ZoI) was measured for S. agalactiae, S. dysgalactiae and S. pyogenes. The results showed that S. dysgalactiae is more sensitive to N/ZnO. Finally, the anticancer properties of these compounds towards prostate cancer cells was investigated. Among the active compounds T ZnO showed the highest activity with low IC₅₀ value (37.75 μg/ml) followed by P ZnO (45.68 μg ml⁻¹).

1. Introduction

Nanotechnology has been playing a vital role in the biomedical and pharmaceutical areas [1–6]. Nanoparticles (NPs) can be synthesized with desired physical and chemical properties [7–11]. In general, there are two types of nanomaterials: (1) organic and (2) inorganic; in which the organic nanomaterials are often less stable at high temperature and pressure compared to inorganic nanomaterials. In general, the inorganic nanomaterials such as metals and metal oxides are preferred as bactericidal and cancer targeting materials for the new era of biomedicine[12]. Among these metal oxides, nanoparticle zinc oxide (ZnO) had been playing a leading role in a lot of bacterial and cancer research due to its high surface-to-volume ratio of nanoparticles which allow for better interaction with bacteria and cancer cells [13–15]. ZnO is a bio-safe material that provides photo-oxidation and photo catalysis on both chemical and biological species. ZnO-NPs antibacterial and anticancer activity includes testing methods, impact of UV illumination, ZnO particle properties (size, concentration, morphology, and defects), particle surface modification, and minimum inhibitory concentration[16, 17]. Prior studies have also clarified the mechanisms of these properties which focus on generation of reactive oxygen...
species (ROS) including hydrogen peroxide ($H_2O_2$), hydroxyl radicals ($OH^-$), and peroxide ($O_2^{2-}$). These have led to mitochondria weakness, intracellular outflow, and release in gene expression of oxidative stress which caused eventual cell growth inhibition and cell death [18, 19]. There has been a long tradition to use plant extracts as medicine for various illnesses – termed herbal remedies. Such natural products, such as neem (Azadirachta indica), pepper (Piper nigrum), turmeric (Curcuma longa) are widely used in Asian countries to increase the immune efficacy and fight against bacterial and fungal infections. Recent studies have demonstrated antimicrobial and anticancer activities of various nanoparticle materials, including organic and inorganic [20, 21]. This study provides the detailed anti-bacterial and anti-cancer behaviour of ZnO nanoparticles synthesized using extracts as solvent media from natural products: neem (leaves), pepper (black seeds) and turmeric (fresh rhizome).

2. Materials and methods

Zinc acetate dihydrate and sodium hydroxide, were purchased from Merck with 98% and 97% purity, respectively. Neem leaves, turmeric rhizomes and black pepper seeds were obtained and used as natural products. Double distilled (DD) water (with neem, turmeric and pepper extract) was used for the solvent extraction in the reaction scheme detailed below.

2.1. Synthesis of ZnO by hydrothermal technique with various solvent extracts

2.1.1. Preparation of extract

50 grams of turmeric stem was cut into small pieces and 500 ml of DD water was added to it followed by heating at 100 °C for 2 h. Then the solution was allowed to cool overnight. Finally, the heated solution was filtered and the extract was further used as a solvent in the synthesis of ZnO.

Similarly, 50 g of neem leaves and pepper seeds was used for the preparation of extract. For the combination of turmeric, neem and black pepper extract 25 ml from each extract is added.

2.1.2. Synthesis procedure

3 gram of zinc acetate dihydrate and 2.4 gram of sodium hydroxide (NaOH) of was dissolved in 75 ml of DD water separately. Then the NaOH solution was added by dropwise into the zinc acetate dihydrate solution under constant stirring. A colloidal solution was obtained which was set aside under stirring for half an hour. After that, the solution was transferred to the autoclave and kept in oven for 2 h at 160 °C. Next it was washed with DD water 5 times using the centrifuge. The obtained precipitate was dried in a hotplate at 120 °C and ZnO nanopowder were obtained.

The same method in used for synthesis the zinc oxide with the natural products, but the DD water is replaced with the different solvents such as neem (N), turmeric (T) and black pepper (P) extract to make the five ZnO nanomaterials studied here: ZnO, N/ZnO, T/ZnO, and P/ZnO.

2.2. Nanomaterial characterization

The structure of ZnO samples was investigated for the crystalline purity with a Bruker X-ray diffractometer (XRD) (Model AXS D8 Advance using Cu Wavelength 1.5406 nm). Scanning electron microscopy (SEM) images were recorded by SEM; VEGA TESCAN 3. The thickness and diameter of the nanoplates by SEM were measured using ImageJ software (free and open source available from website http://rsb.info.nih.gov/ij). UV–vis spectroscopic measurements (UV) provides the diffuse reflectance spectrum of ZnO obtained with an Agilent 8453 UV-Visible spectrophotometer from 200 to 800 nm. Photoluminescence spectroscopy (PL) affords the emission spectrum by Agilent spectrophluorometer carry eclipse under 325 nm excitation wavelength.

2.3. Preparation of bacterial cultures

(i) The test human pathogenic bacterial cultures such as *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhi* were obtained from the Department of Microbial Technology, School of Biological Sciences, Madurai Kamaraj University, Madurai, Tamil Nadu, India. The synthesized compounds ZnO, P/ZnO, T/ZnO, N/ZnO were tested for antibacterial activity against the different human pathogens: *E. coli*, *S. aureus*, and *S. typhi* by spread plate method on Muller—Hinton agar (MHA) medium. The overnight bacterial culture was grown on nutrient broth (NB). Bacterial culture was serially diluted on an NB medium and two different concentrations of nanoparticles (100 and 150 μg ml$^{-1}$) was used and it was incubated for 4 h at 37°C in a shaker at 120 rpm. After incubation, 100 μl of the sample was spread on MHA plates [22]. The inoculated plates were incubated for 24 h at room temperature. After incubation, the results were observed; plates and colonies were counted. Distilled water and tetracycline act as negative and positive control, respectively. The experiments were performed in triplicates.
(ii) Antibacterial activity of synthesized nanocomposite against human pathogenic bacteria streptococcus was tested by well diffusion method on MHA medium amended with 5% sheep blood ([g/L]: beef extract-3, casein acid hydrolysate-17.5, starch-1.5 and agar-17)\textsuperscript{23}. The human pathogenic streptococcus species were obtained from Apollo hospital, Chennai. The above six human pathogens were inoculated in a 5 ml of sterile Todd-Hewitt broth with 1% yeast extract and incubated at 37°C for overnight. The cultures were swabbed with the help of sterile cotton buds on MHA medium amended with 5% sheep blood. A 9 mm disc borer was used to make the wells and each well 200 μl of sonicated nanocomposite (2 mg ml\textsuperscript{-1}) and tetracycline (2 mg ml\textsuperscript{-1}) as reference control was added. Inoculated plates were incubated for 24 h at 37°C. After 24 h of incubation, different levels of zone of inhibition (ZOI) were measured using a meter ruler. The experiments were performed in triplicates.

2.4. Preparation of cancer cell line and cytotoxicity assay

2.4.1. Cell line and maintenance

Human prostate cancer cell line (PC3) was obtained from National Centre for Cell Sciences (NCCS), Pune. Cells were maintained in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS), and 1% penicillin/streptomycin antibiotic mixture. Cells were maintained at 37°C in an atmosphere of 95% air and 5% CO\textsubscript{2}.

2.4.2. Cytotoxicity assay

The formulated compounds ZnO, N\textsubscript{2}, P\textsubscript{2}, T\textsubscript{2} ZnO and TNP were dispersed in DMSO. Different concentrations from 1.56 μg ml\textsuperscript{-1} to 50 μg ml\textsuperscript{-1} was prepared using DMEM medium. Cytotoxicity effects of the above compounds against prostate cancer cell line was determined as follows. PC3 cells were seeded in 96-well flat bottom micro plate and then incubated overnight at 37°C in 5% CO\textsubscript{2} for cell attachment. The medium was removed and replaced with fresh medium containing various concentrations of selected drugs. Cells were incubated at 37°C in 5% CO\textsubscript{2} for 48 h. Forty-eight hours later, 15 μl of MTT (5 mg ml\textsuperscript{-1}) solution was added to each well and then the plates were further incubated for 4 h. After incubation, 75 μl of lysis buffer was added to dissolve the formed crystal formazan and incubate at room temperature for overnight. The optical density was measured using a microplate reader at a wavelength of 570 nm and the percentage of inhibition was calculated. IC\textsubscript{50} values were calculated using graph pad prism, version 5.02 software (Graph Pad Software Inc., CA, USA).

2.4.3. Morphological change analysis

General morphological structure of cells was examined to study the effect of formulated drugs on the PC3 cell line. Briefly, cells were cultured in 12 well plates and incubated overnight at 37°C. Then the medium was removed and replaced with the drug containing medium and incubated for 48 h. Simultaneously medium alone serve as a control. After the respective incubation time, cells were washed twice with PBS and visualized under phase contrast microscopy at 40X magnification.

3. Results and discussion

3.1. Structural analysis by XRD

Figure 1 depicts the diffraction spectra of ZnO, N\textsubscript{2}/ZnO, P\textsubscript{2}/ZnO, T\textsubscript{2}/ZnO and NPT/ZnO. The peaks at 2θ = 31.76, 34.34, 36.18, 47.65, 56.53, 62.93, 67.94, 69.22 were assigned to hexagonal wurtzite structure. The zinc oxide phases matched their JCPDS card numbers 89-0510. The average crystallite size (d) of ZnO NPs was estimated by Scherrer’s formula\textsuperscript{24}:

\[
D = \frac{k \lambda}{\beta \cos \theta}
\]  \tag{1}

where k = 0.9 is the shape factor, λ is the X-ray wavelength of Cu K\textalpha\ radiation (1.54 Å), \( \beta \) is the Bragg diffraction angle, and \( \theta \) is the full width at half maximum of the respective diffraction peak. The average size of prepared sample is calculated as 21 nm (ZnO), 32 nm (N\textsubscript{2}/ZnO), 24 nm (P\textsubscript{2}/ZnO), 28 nm (T\textsubscript{2}/ZnO) and 44 nm (NPT/ZnO). All the other samples were red shifted with respect to ZnO and also no diffraction peaks of other impurities were detected, which testify that all the prepared sample is ZnO with same structure.

3.2. Optical studies

3.2.1. UV visible spectroscopy and diffuse reflectance spectroscopy (DRS)

Figure 2 depicts the room temperature absorption spectra of ZnO, N\textsubscript{2}/ZnO, P\textsubscript{2}/ZnO, T\textsubscript{2}/ZnO and NPT/ZnO, which were recorded using the BaSO\textsubscript{4} powder compact as the reference sample. The excitonic absorption peak
was observed at 355 nm, blue-shifted compared to that of the bulk ZnO (375 nm) due to the quantum confinement effect [25]. However, for the N/ZnO, P/ZnO, T/ZnO and NPT/ZnO samples, due to the presence of neem, pepper and turmeric, the absorption edge shifts towards the higher wavelength region. These have similar broad and strong absorption spectrum with an onset around 396 nm. The peak maxima of these samples were around 360–370 nm. Of all the samples, the N/ZnO and NPT/ZnO exhibited another absorption peak in the visible region, which may be due to the presence of neem. The absorption spectra of the nanocomposites show a slight red shift, which is consistent with the XRD result.

The indirect band gap of these nanoparticles (figure 2) is estimated from the graph of \( h\nu \) versus \((\alpha h\nu)^{1/2}\) for the absorption coefficient \(\alpha\) [26]. The absorption coefficient \(\alpha\) is related to the bandgap, \(E_g\), as

\[ \alpha h\nu = A(h\nu - E_g)^{1/2} \]

where, \(h\nu\) is the incident photon energy and \(A\) is a constant. The energy band gap, \(E_g\), of the nanoparticles is determined as 3.4 eV (ZnO), and for other samples, it is around 3.13 eV from the above expression. The presence of neem, pepper, and turmeric in the ZnO synthesis provided noticeable prominent change in the band gap implying that the optical properties of these materials are clearly affected by the synthesis medium.
3.2.2. PL spectroscopy
To study the electron transfer mechanism the photoluminescence (PL) characteristics of the ZnO, N/ZnO, P/ZnO, T/ZnO and NPT/ZnO nanoparticles were carried out at room temperature with an excitation wavelength of 325 nm [27]. A sharp ultraviolet (UV) emission peak centred about 368 nm is observed for pure ZnO with the absence of any emission band in the visible region is shown in figure 3. This UV emission band is attributed to the near edge band emission of ZnO [28]. For all other samples such as N/ZnO, P/ZnO, T/ZnO, NPT/ZnO, the intensity of near edge band emission is very weak and a small visible emission at about 400 nm are observed with PL measurements. The absorption edge in UV/visible spectra suggests right shift towards the visible region compared to the pure ZnO. All the samples exhibit emission peaks within the UV region, but with lower emission intensity than ZnO, which means they have lower recombination rate [29].

3.3. Morphological study by SEM
Figure 4 displays the SEM micrographs of ZnO, N/ZnO, P/ZnO, T/ZnO and NPT/ZnO. By using the neem, pepper and turmeric extracts the morphology of the nanomaterial changes. ZnO, N/ZnO and P/ZnO (0.3 to 0.5 μm) shows nanoplate-like structures. However, the T/ZnO show morphological changes as spherical particles (2.6 μm). Finally, the NPT/ZnO (0.8 μm) formed into non-uniform cuboidal structures. Not only the temperature and pressure, but also the inoculation of natural products play a vital role in the morphological changes of ZnO as can be seen in figure 4.

3.4. Antibacterial behaviour and cancer studies of ZnO nanoparticles
3.4.1. Discussion on colony forming unit (CFU)
ZnO nanoparticles have a broad spectrum of antibacterial activities [30]. Here all the synthesized nanoparticles ZnO, N/ZnO, P/ZnO, T/ZnO and NPT/ZnO were tested on three bacteria’s E. coli, S. aureus and S. typhi (table 1). All the synthesized nanoparticles showed good inhibitory effects on S. aureus compared to other nanomaterials (numerous colonies to count). This result showed that S. aureus was extremely sensitive to P/ZnO and T/ZnO. It is also clear that the nanoparticles function as bactericidal agent and not bacteriostatic by showing no recovery of the treated cells and rapid killing of S. aureus. The antibacterial activities of Ag-NPs MIC value at 100 and 150 μg ml$^{-1}$ against S. aureus and E. coli was inhibit both Gram-positive and Gram-negative bacteria [22].

Figure 5 shows the colony forming inhibition of S. aureus exposed to P/ZnO (100 and 150 μg ml$^{-1}$) and T/ZnO (100 and 150 μg ml$^{-1}$) NPs. The CFU assay results demonstrate that S. aureus colony formation were inhibited under illumination. When the quantity of P/ZnO increased it shows better activity and the colonies
Figure 4. SEM micrographs for ZnO, N/ZnO, P/ZnO, T/ZnO and NPT/ZnO.

Table 1. CFU assay on E. coli, S. aureus and S. typhi by ZnO, N/ZnO, P/ZnO, T/ZnO and NPT/ZnO.

| S. No. | Compounds  | E. coli (μg/ml) | S. aureus (μg/ml) | S. typhi (μg/ml) |
|--------|------------|----------------|------------------|-----------------|
| 1.     | ZnO        | TNTC           | TNTC             | TNTC            |
| 2.     | N/ZnO      | TNTC           | 48               | 23              |
| 3.     | P/ZnO      | TNTC           | 3                | —               |
| 4.     | T/ZnO      | TNTC           | 2                | —               |
| 5.     | NPT/ZnO    | TNTC           | 37               | 18              |
| 6.     | Tetracycline | —             | —                | —               |
| 7.     | Control    | NA             | NA               | NA              |

TNTC—Too Numerous To Count; NA—No Activity.
were reduced to 3. Overall, T/ZnO showed remarkable performance, which is due to the presence of turmeric in the solvent medium of the ZnO synthesis.

3.4.2. Discussion on zone of inhibition

Among the five nanocomposites tested for antibacterial activity against human pathogens, N/ZnO followed by P/ZnO showed more activity. The other three nanocomposites (T/ZnO, NPT/ZnO, ZnO) also have minimum inhibition activity (table 2). Among the six Streptococcus isolates tested for five nanocomposite, S. dysgalactiae is more sensitive (72%) followed by S. agalactiae (53%), S. pyogenes (45%) as compared with tetracycline, which was used as a reference control (figure 6).
The human pathogenic bacteria such as *E. coli*, *S. aureus*, *S. typhi* and *Streptococcus* spp have developed multidrug resistance activity. Several recent studies reported *Streptococcus* and *Staphylococcus* species are becoming resistant to currently available synthetic antibiotic [31], therefore we attempted natural extracts to check the antimicrobial activity and it showed significant results also in the prostate cancer cell line. Hence, we selected biosynthesis of zinc oxide nanomaterials with natural extracts of neem, pepper and turmeric effect on antibacterial activity and cellular toxicity on prostate cancer cells (*PC3*). Among five different biosynthesized zinc oxide nanomaterials efficacy against antimicrobial activity the results showed significantly reduced bacterial growth on turmeric coated zinc oxide (*T/ZnO*) at 100 μg ml⁻¹ followed by pepper (*P/ZnO*) at 100 μg ml⁻¹. Among the three different human pathogens tested *S. aureus* effectively inhibited the bacterial growth by *T/ZnO*. The *T/ZnO* antimicrobial activity is equal to commercial antibiotic tetracycline. Based on the results *T/ZnO* was superior activity against bacterial pathogens and also cytotoxic activity of prostate cancer cell line with low Ic₅₀ value (37.75, table 3). On the other hand, *N/ZnO* showed better activity against *Streptococcus* species. Hence, the results indicate that the active molecule *T/ZnO* could be used as drug or drug delivery system in medical applications.

### Table 2. Antibacterial activity of human pathogens against nanocomposite (zone of inhibition in mm).

| Bacteria name                | Pure | N/ZnO | P/ZnO | T/ZnO | NPT/ZnO | Tetracycline |
|------------------------------|------|-------|-------|-------|---------|--------------|
| *S. agalactiae* NBMKU42      | 5    | 7     | 6     | 5     | 5       | 11           |
| *S. agalactiae* NBMKU46      | 5    | 6     | 6     | 3     | 5       | 18           |
| *S. dysgalactiae* NBMKU21    | 6    | 14    | 13    | 3     | 11      | 25           |
| *S. dysgalactiae* NBMKU19    | 5    | 6     | 6     | 4     | 4       | 18           |
| *S. pyogenes* NBMKU15        | 4    | 6     | 6     | 4     | 4       | 29           |
| *S. pyogenes* NBMKU14        | 3    | 6     | 5     | 4     | 3       | 21           |

### Table 3. IC₅₀ values of selected compounds against PC3 cells.

| S. No | Name of the Compound | IC₅₀ (μg/ml) |
|-------|----------------------|-------------|
| 1     | ZnO                  | 165.89      |
| 2     | N/ZnO                | 151.38      |
| 3     | P/ZnO                | 45.68       |
| 4     | T/ZnO                | 37.75       |
| 5     | NPT/ZnO              | 115.58      |

3.4.3. Cellular toxicity studies

Anticancer activity of the selected compounds against prostate cancer was screened based on MTT cytotoxicity assay (figure 7). This is an appropriate method to analyse new compounds within a short period in order to fix their toxicity effect on cancer cells. Cytotoxicity has been defined as the cell killing property of a chemical compound independent from the mechanism of death. There are many established methods such as crystal
violet method, tritium-labelled thymidine uptake method, MTT and WST methods are used for counting the number of live cells. Among these, MTT assay is mainly used because of its high reproducibility, and it can use for both cell viability and cytotoxicity tests. This assay is based on the mitochondrial dehydrogenase activities in the living cells. From our MTT assay result, the results showed that IC$_{50}$ values of ZnO, N/ZnO, P/ZnO, T/ZnO and NPT ZnO against PC3 cells were 165.89 $\mu$g ml$^{-1}$, 151.38 $\mu$g ml$^{-1}$, 45.68 $\mu$g ml$^{-1}$, 37.751 $\mu$g ml$^{-1}$, 115.58 $\mu$g ml$^{-1}$, respectively. The results suggested that all drugs showed a cytotoxicity effect on PC3 cells except ZnO and N/ZnO.

Among the active compounds T/ZnO showed the highest activity with low IC$_{50}$ value (37.751 $\mu$g ml$^{-1}$) followed by P/ZnO (45.68 $\mu$g ml$^{-1}$).

3.4.4. Morphological change assessment
The implementation of cell death is associated with morphological changes of cells. Figure 8 shows the several morphological changes of cells with respect to the treatment. From the analysis, it was confirmed that the formulated nanomaterials induce cell toxicity via morphological changes. When compared with ZnO treated cells, the remaining formulated compound treated cells showed many morphological changes. The control cells remained confluent with spindle shape throughout the incubation period. The morphology of ZnO treated cells was similar to control cells. Whereas, PC3 cells treated with other compounds showed remarkable changes including cell shrinkage, membrane blabbing and damage. Among these compounds, T/ZnO and P/ZnO showed potential changes. In case of T/ZnO, most of the cells were shrunk and lost their proliferating ability. Meanwhile P/ZnO treated cells underwent membrane damage and thus the complete architecture of the cells was changed. Hence this study proved that the formulated ZnO compounds with natural extracts were more biologically active against prostate cancer.

4. Conclusions
In summary, the ZnO nanoparticles have been prepared using a simple and efficient hydrothermal synthesis method with the neem, pepper and turmeric extract. The structural characterization of synthesized nanoparticles is crystalline in structure and the hexagonal wurtzite structure of ZnO does not changed due to neem/pepper/turmeric, but a visible red shift is observed. The synthesized ZnO nanoparticles exhibit the absorption peak at 375 nm and it shifts towards the visible region. The synthesized ZnO nanoparticles exhibit photoluminescence within UV region and it is observed that no defect states are created in the visible region. The present work proves it is simple and low-cost method for producing ZnO nanoparticles using natural antibiotic elements such as neem, pepper and turmeric. For these samples S. dysgalactiae is more sensitive (72%) followed by S. agalactiae (53%), S. pyogenes (45%) compared with tetracycline as a reference control. The results signify that T/ZnO nanoparticles have remarkable bactericidal efficacy against S. aures and anticancer property against prostate cancer cells. T/ZnO is grabbing attention as a potentially powerful cancer fighter. In general, the
chemical composition, curcumin helps to counter the inflammation that contributes to the tumour growth. This work shows investigated the combination of turmeric and ZnO as a possible treatment for prostate cancer. Also other nanoparticles such as P/ZnO and N/ZnO plays a vital role against S.aures and S. dysgalactiae.

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Author contributions

CA—result analysis and writing (original draft preparation); R M and N D K—Cancer and cytotoxicity studies and interpretation; P S and V S—antibacterial studies and analysis; N B—Zone of Inhibition and bacterial studies against human pathogens; N M—material synthesis; P S—reviewing; J M P–Proof reading, reviewing and editing; JM and JA—Designing the experiments with suitable natural drug’s and metal oxides screen processes, review and editing.

Conflicts of interest

All the authors declare no conflict of interest.

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