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Enhancing the Anticancer Activity of Vincristine with chemically synthesized Zinc Oxide Nanoparticles

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I. Introduction:

Cancer emerges as the leading cause of death and its rising prominence has become a barrier to the increasing life expectancy in every country of the world. According to GLOBOCAN 2020, 19.3 million new cancer cases and 10 million cancer deaths have been estimated to have occurred worldwide. Globally, Female breast cancer has emanated as the most commonly diagnosed cancer, with an estimated 2.3 million new cases (11.7%) in 2020. (**Global Cancer Statistics 2020) .** The treatment for various cancers encompass Surgery, radiotherapy, hormone therapy and chemotherapy or combination of these [1]. Conventional chemotherapy due to nonspecific targeting, lack of solubility and inability to enter the core of the tumors may cause serious side effects like damaging the other non-targeted organs and immune system [2]. Employing targeted nanoparticles for the delivery of chemotherapeutic agents helps in overcoming the problems associated with conventional chemotherapy [3]. Nanoparticles can be adopted as pharmaceutical carriers to carry the chemotherapeutic drug, which would help in accomplishing increased drug localization (bio-availability) and cellular uptake by having a direct access to the cancer cells. Nanoparticles could thus be consecutively employed to enhance the *in vivo* antitumor efficacy of drugs. Biocompatibility and biodegradability are the main reasons that make Zinc Oxide nanoparticles a legitimate choice for the drug delivery system. Zinc Oxide also has an inherent preferential cytotoxicity against the cancer cells *in vitro* and it has also been recognised as a GRAS (Generally recognized as safe) substance by the United States Food and Drug Administration (FDA). Embodiment of the chemotherapeutic drug to Zinc Oxide nanoparticles may curtail the drug's adverse effects to normal body cells [4]. Vinca alkaloids like vincristine, termed as Microtubule targeting agents (MTA) are crucial chemotherapeutic agents used in the treatment of various cancers. MTAs exhibit their anti-tumour activity by interfering with microtubule dynamics which is extensively involved in mitotic spindle formation. Vincristine causes microtubule depolymerisation through their binding to the tubulin protein. This arrests the cell cycle in metaphase state, leading to cell death [5]. PEGylation (Coating the surface of nanoparticles with polyethylene glycol (PEG)) has been discovered to enhance the efficiency of the incorporated drug by prolonging the systemic circulation time and its delivery to the target cells and tissues. PEG can either be adsorbed or covalently attached to the molecule or drug of interest [6]. Folic acid is considered as a promising ligand that can target cancer cells due to the over expression of folate receptors on their surface [7]. Coupling of PEG and folic acid to the synthesized metal oxide nanoparticle would enhance the efficiency of the targeted drug. This study aims to enhance the efficacy of the anti-cancer drug vincristine with the chemically synthesized Zinc Oxide Nanoparticles.

II. Materials and Methods:

Zinc acetate dihydrate and urea from Himedia Laboratories were used as the introductory materials to synthesize the Zinc Oxide Nanoparticles.MCF-7 Breast cancer cell lines used for the MTT assay were sourced from Bharathiar University. Commercially available Vincristine was purchased from the local pharmacy. Poly Ethylene Glycol and Folic acid were obtained from Himedia Laboratories.

2.1 ***Chemical Synthesis of Zinc Oxide Nanoparticles***

Synthesis of Zinc oxide nanoparticles was performed by co-precipitation method using Zinc acetate and urea as precursors. Aqueous solution of Zinc acetate dihydrate (1 mM) and urea (1M) were prepared with deionized water. 0.5 M sodium hydroxide solution was added to the Zinc acetate-urea solution with vigorous stirring in an oil bath, until a milky white precipitate was obtained at pH 12 [8]**.** The solution was then centrifuged at 5,000 rpm for 10 minutes and washed twice with distilled water and once with absolute alcohol at room temperature and the precipitate was collected. The collected precipitate was subjected to calcination at 350ºC for 4 hours using TECHNICO muffle furnace.

2.2 ***Preparation of Poly Ethylene Glycol- Zinc Oxide Nanoparticle (PEG-ZnO NP)***

0.05mg/ml Poly Ethylene Glycol (PEG) was dissolved in Milli-Q water and heated at 80^0C for 1 hr. The nanoparticles were then added to the PEG solution and the mixture was stirred for 5 hours in the dark. The PEG-ZnO nanoparticles were collected by centrifugation at 10,000 rpm for 15 min and rinsed several times with water [9].

2.3 Preparation of Vincristine- Poly Ethylene Glycol- Zinc Oxide Nanoparticle (VCR-PEG-ZnO NP)

1mg /ml PEG-ZnO nanoparticle was added to 2mg/ml of Vincristine aqueous solution and the mixture was sonicated for 2 minutes. This mixture was then centrifuged at 10,000 rpm for 15 minutes. The supernatant was collected and used for further characterization [10].

2.4 Preparation of Folic Acid- Vincristine- Poly Ethylene Glycol- Zinc Oxide Nanoparticle (FA- VCR-PEG- ZnO)

0.5mg/ml of folic acid was dissolved in milli-Q water and 1mg/ml PEG-coated nanoparticle was added to the solution. The reaction was allowed to proceed overnight in the dark at Room Temperature. The mixture was then centrifuged at 10,000 rpm for 15minutes. The collected nanoparticles were rinsed several times with water [11].

2.5 Characterization of the Synthesized Zinc Oxide Nanoparticles:

2.5.1 Ultra Violet- Visible (UV-Vis) spectroscopy

The particles were sonicated in distilled water for the proper distribution of nanoparticles. The concentration of the particles were measured using UV-vis spectrophotometer. Distilled water was taken as a reference for the UV- visible spectroscopic analysis. The spectral analysis of the zinc oxide nanoparticles was carried out by measuring the optical density (OD) using JASCO V-750 Spectrophotometer scanning UV- vis spectrometer operated at a resolution of 1nm between 200 and 900nm.

2.5.2 Infrared spectroscopy

Distilled water was taken as a reference for the infrared spectroscopy. The spectral analysis of the zinc oxide nanoparticles was carried out by measuring the optical density(OD) using a scanning infrared spectrophotometer operated at a resolution of 1nm between 700nm and 1mm.

2.5.3 Scanning Electron Microscopy and Energy Dispersive X-Ray (EDAX) Analysis

Scanning Electron Microscopic (SEM) analysis and EDAX analysis were carried out using FEI-QUANTA 250 (FEG) machine equipped with thermos EDAX attachments. A very small quantity of nanoparticle powder was dispersed in a small volume of relatively fast-drying solvent. Solvent should be selected in such a way that there is no chemical reaction between the solvent and the nanoparticle under study. Ethanol was used as a medium in our studies. After dissolving the nanoparticle in ethanol, sonication of the suspension was done to break up the agglomerates. A micro-pipette was then used to deposit a small droplet on the tungsten filament and the solvent was allowed to evaporate. The FE-SEM instrument was operated at an accelerating voltage at 10 kV.

2.5.4 X-ray Diffraction (XRD)

The X-ray Diffraction (XRD) pattern of Zinc Oxide Nanoparticles was recorded using a PANalytical PW3050/60 X-ray diffractometer equipped with Cu-Kα radiation source (λ = 1.5406 Å) at 2θangle, operated at 40 kV and 40 mA. The particle size and nature of the Zinc Oxide nanoparticles were analysed using X-ray Diffraction.

2.6 Invitro Cytotoxicity Assay

MCF-7 cell line was used for identifying the cytotoxicity of the Zinc Oxide nanoparticles. The cells were cultured in Dulbecco's Modified Essential Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) under 5% CO_2 at 37ºC.

In vitro cytotoxicity of ZnO-NPs was investigated by the MTT assay. Cells were seeded at a concentration of $1.0x10^5$ cells/ml in a flat bottom 96-well plate. To each well,100µl of the diluted cell suspension (approximately 10,000 cells/well) was added and incubated overnight. After 24 hours, when the cell population was found adequate, the cells were centrifuged and the p different concentrations (0, 7.8, 15.6, 31.2, 62.5, 125, 250 μg/ml) . The plates were then incubated at 37ºC for 24hours and 48 hours in 5% CO_2

atmosphere. Microscopic examination was carried out and the observations were recorded every 24 hours. At the end of incubation period, 20 µl of MTT solution (2mg/ml in the medium) was added to the wells. The plates were gently shaken and incubated for 2 hours at 37ºC in 5% CO_2 atmosphere. Then 100µl of DMSO was added and the plates were gently shaken to solubilise the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540nm.The process was carried out in triplicates. The percentage cell viability was calculated using the following formula and concentration of drug or test samples needed to inhibit cell growth by 50% values (IC50) were generated from the dose-response curves [12].

$$\text{Percentage of Cell Viability} = \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

III. Results and Discussion

3.1 Chemical Synthesis of Zinc Oxide Nanoparticles and their Characterization:

Fig 1. Milky white Zinc Oxide powder

A milky white Zinc Oxide powder was obtained after the calcination of the precipitate at 350ºC for 4 hours.

3.2 Ultra Violet- Visible (UV-Vis) spectroscopy

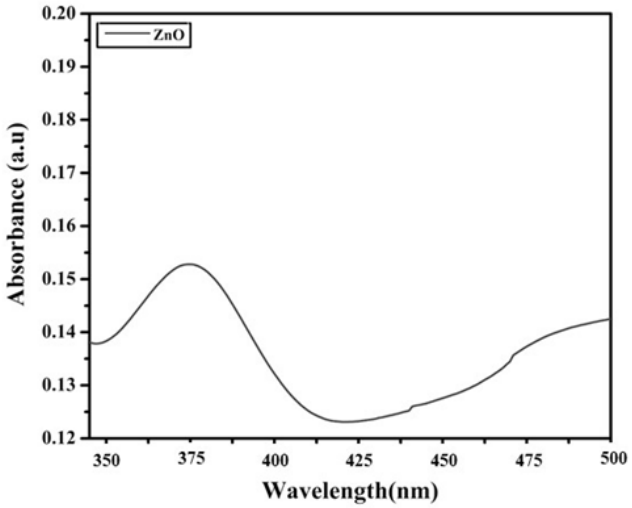


Fig 2. UV-visible spectrum of Zinc Oxide Nanoparticle

UV-visible spectroscopy is the preliminary technique used to monitor the formation of nanoparticles and their optical properties. Figure 2 shows the UV-Visible spectra of Zinc Oxide nanoparticles with an absorption edge around 375nm, which lies below the band gap wavelength of 388nm of bulk zinc oxide. This confirms that the prepared ZnO particles were in the nano range. These supporting data confirm the presence of ZnO NPs. The shift in the absorption maximum is due to the decrease in the size of ZnO nanoparticles. This clearly indicates that in the case of semiconductors, the distance between HOMO and LUMO increases when the particle size decreases. Hence, a shift towards lower wavelength is observed. Similar result of absorption band that represent ZnO NPs was also obtained from previous research in which the range of absorption band was around 355nm as reported by Satyanarayana et al.,2012 [13].

3.3 Infrared Spectroscopy

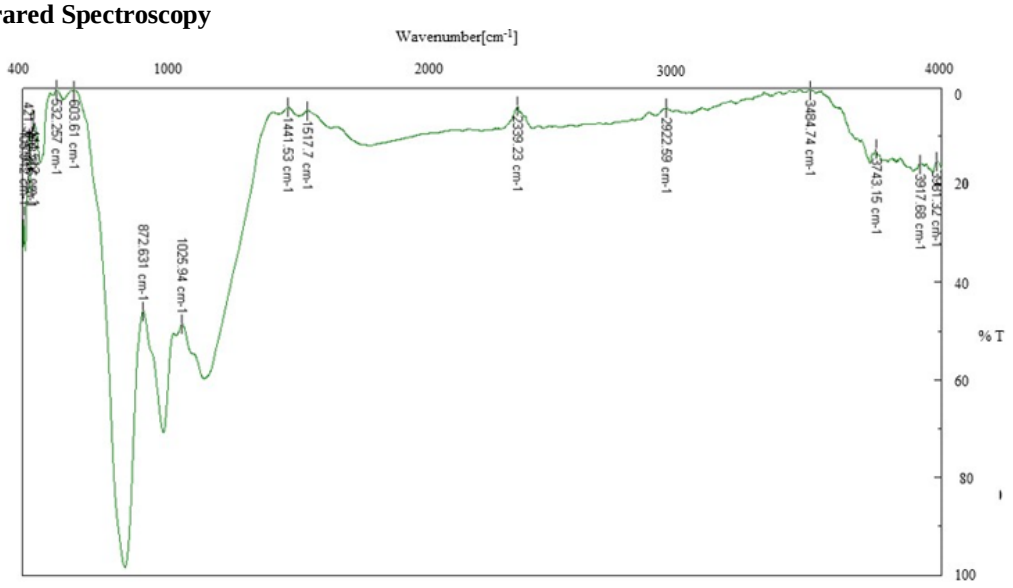
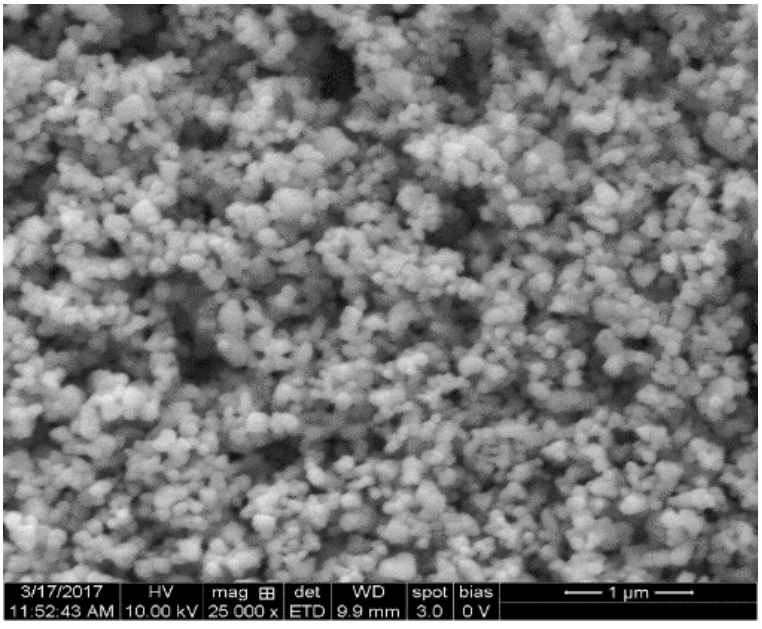
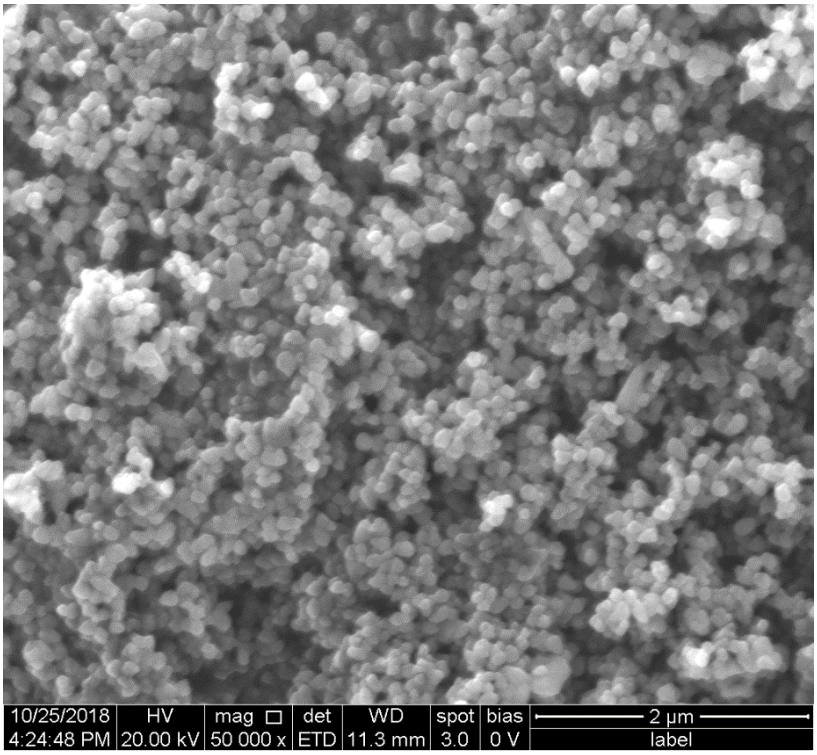


Fig 3. Infrared spectrum of Zinc Oxide Nanoparticle

Infrared spectral studies were carried out in order to ascertain the purity and nature of metal nanoparticles. The IR spectra of the zinc oxide nanopowders are given in the figure 3. The peaks observed at 1517 cm^{-1} and 1441 cm^{-1} may be due to C=O stretching and bending vibrations. The metal oxygen frequencies observed for the respective metal oxides are in accordance with literature values. The band located at 532 cm^{-1} and 606 cm^{-1} is correlated to metal oxide bond (ZnO), which confirms the formation of Zinc oxide nanoparticles. The region between 3484 cm^{-1} -3917 cm^{-1} is attributed to water adsorption onto the metal surface. The small bond located near 2399 cm^{-1} can be attributed to the C=O residue probably due to atmospheric CO_2. All the observed peaks were mentioned in previous literatures and it correlates to our findings. Similar discoveries were also summarized from previous studies related to ZnO NPs synthesis and characterization [14].

3.4 Scanning Electron Microscopy and Energy Dispersive X-Ray (EDAX) Analysis





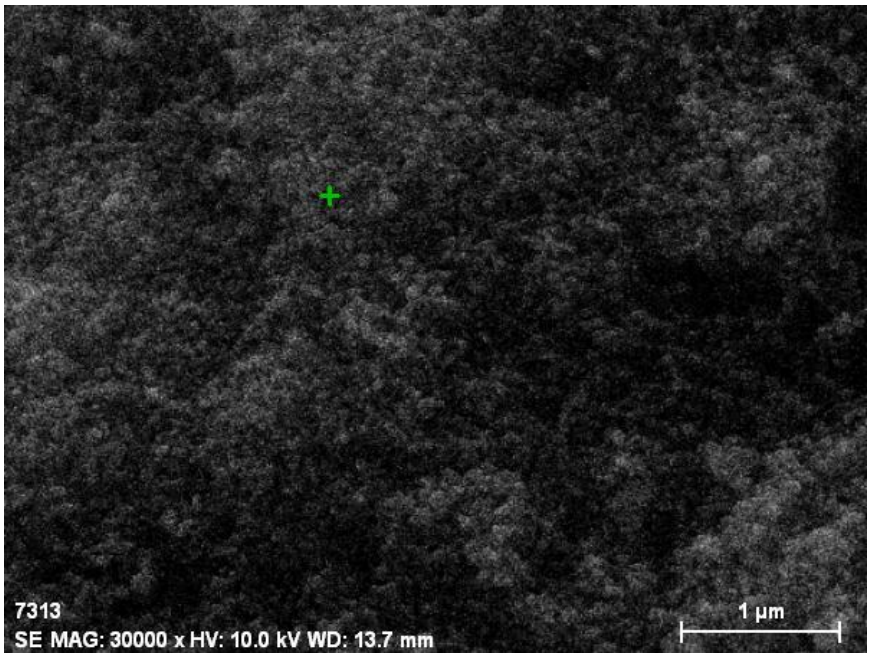


Fig 4. Scanning Electron Microscopic analysis of Zinc Oxide Nanoparticle

The surface morphology and approximate size of the prepared zinc oxide powders were determined by SEM observations (Fig 4). SEM observations along with EDAX of ZnO nanoparticles are given in figure 5. From the SEM image, the particles were found to be uniform spheres possessing size approximately in the range of 42 to 74nm [15]. Results obtained from the optical images were in agreement with the SEM images.

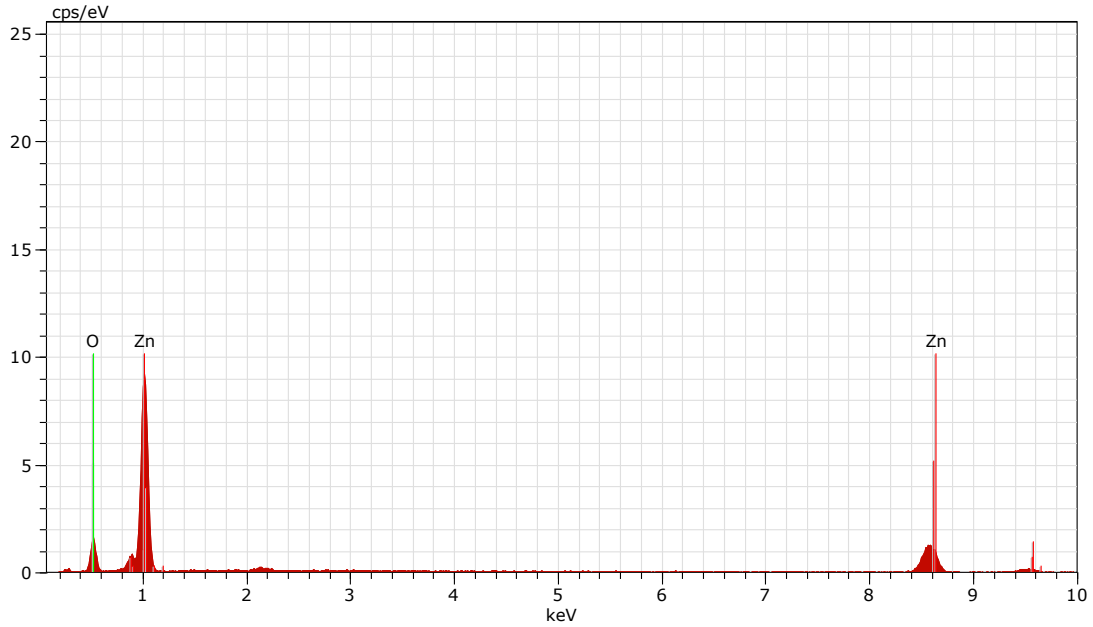


Fig 5 . Energy Dispersive X-Ray (EDAX) analysis of Zinc Oxide Nanoparticle

El	AN	Series	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]	Error (1 Sigma) [wt.%]
Zn	30	L-series	69.75	77.69	46.01	4.30
O	8	K-series	20.03	22.31	53.99	2.73
		Total:	89.78	100.00	100.00	

Energy dispersive X-ray analysis is an analytical technique used for the elemental analysis (chemical characterization) of the sample. Figure 5 shows the EDAX spectra of Zinc oxide nanopowders which shows clear peaks of zinc (Zn) and oxygen (O). Zinc and oxygen occupies 77.69 and 22.31 wt% respectively. The compositional analysis by EDAX confirms that the sample is purely ZnO without any further impurities as no other peaks of impurities were observed.

3.5 X-ray Diffraction (XRD)

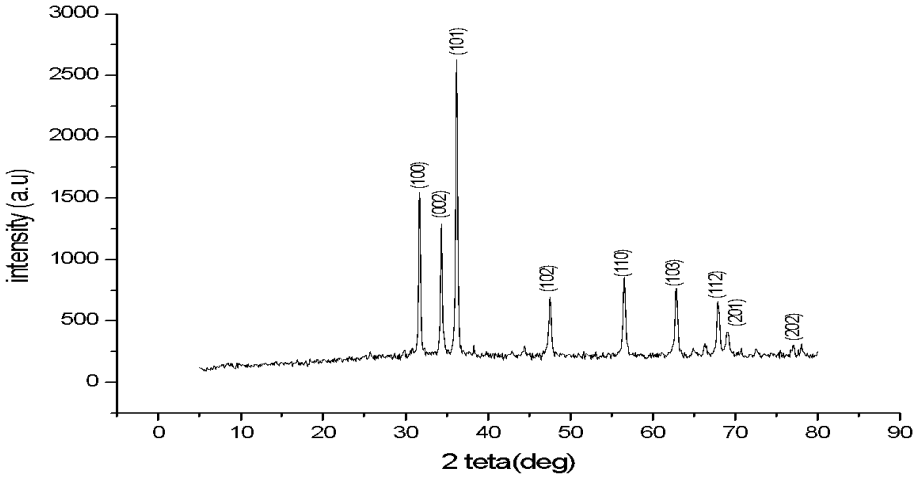


Fig 6. X-ray Diffraction analysis of Zinc Oxide Nanoparticle

XRD patterns of the ZnO nanoparticles prepared at pH 12 is given in Figure 6. The figure shows that the diffraction patterns correspond to Bragg reflections with 2θ values of 31.71^0, 34.37^0, 36.19^0, 47.49^0, 56.55^0, 62.81^0, 67.90^0, 69.07^0 and $78.06^{0.}$ Locations of the characteristic Bragg reflections were indexed to (100), (002), (101), (102), (110), (103), (112), (201), and (202) respectively. Highest intensity peak indicates that the prepared ZnO nanoparticles are in hexagonal wurtzite structure which is in agreement with the JCPDS (891397) data.

3.6 Enhancement of Vincristine Activity by chemically synthesized Zinc Oxide Nanoparticles:

3.6.1 Preparation of Folic Acid- Vincristine- Poly Ethylene Glycol- Zinc Oxide Nanoparticle (FA- VCR-PEG- ZnO)

(a)

(b)

Fig 7. a. PEG capped Zinc Oxide Nanoparticle b. PEG capped Zinc Oxide Nanoparticle with Folic Acid and Vincristine

3.7 Fourier Transform Infrared Spectroscopy (FTIR) of PEG capped Zinc Oxide Nanoparticle and PEG capped Zinc Oxide Nanoparticle loaded with folic acid and Vincristine

Infrared spectroscopy gives information about the attachment of various molecules to the nanoparticle. Here the peak values manifest the adsorption of PEG and vincristine to ZnO nanoparticle. Figures 8,9 show the FTIR spectra of PEG coated ZnO nanoparticle and PEG- coated ZnO loaded with Vincristine and Folic Acid respectively. According to the previous study by Sudha M et al., 2013, the peaks at $1626.33cm^{-1}$ and $1370.53cm^{-1}$ in the spectrum represent the formation of PEG on the surface of ZnO nanoparticle. The peak between $3200cm^{-1}$ and $3500cm^{-1}$suggests the presence of a hydroxyl (-OH) group. The strong band of the hydroxyl group in PEG capped ZnO nanoparticle shifts towards lower wavenumber $3456cm^{-1}$ which reflects the formation of hydrogen bond at ZnO-PEG interface. Carboxylic acid shows a strong, wide band for the O-H stretch. The carboxylic acid O-H stretch appears as a very broad band in the region $3300cm^{-1}$ to $2500cm^{-1}$ due to the presence of COOH group in folic acid. This confirms the attachment of folic acid to PEG-ZnO nanoparticle. Shifting in peaks at $1607.89cm^{-1}$, $1517.70cm^{-1}$due to Amide II N-H deformation and C-N stretching vibration could be a consequence of loading vincristine in nanoparticles [16].

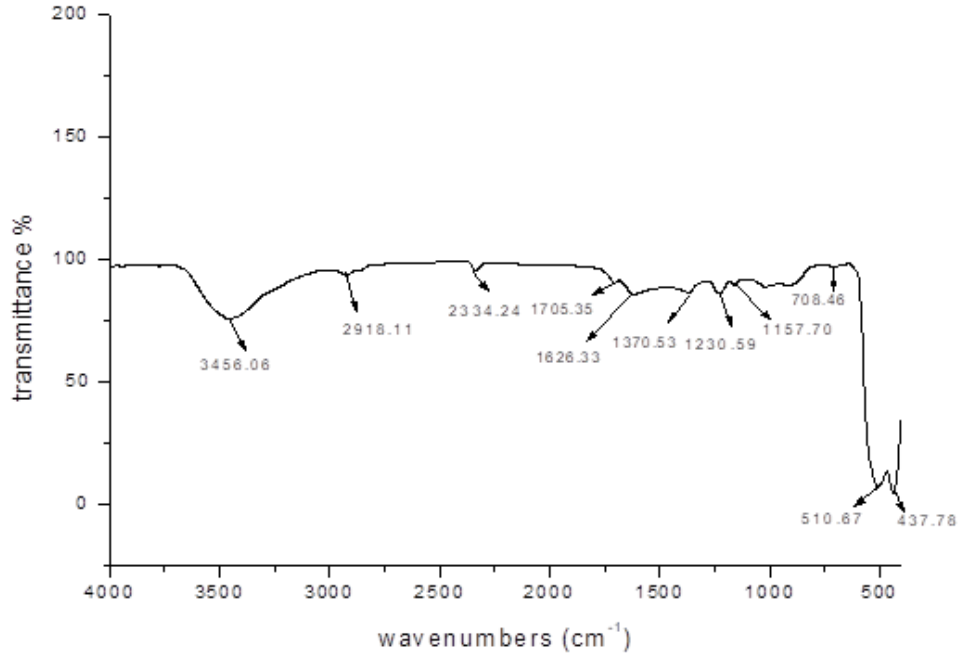


Fig 8. FTIR spectrum of PEG capped Zinc Oxide Nanoparticle

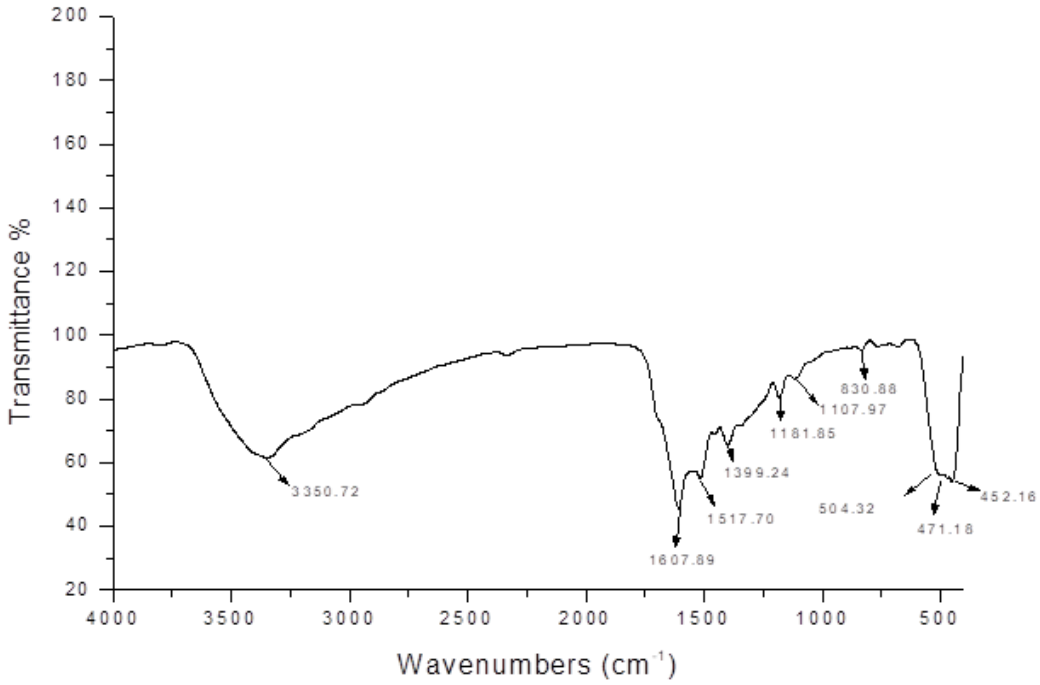


Fig 9. FTIR spectrum of PEG capped Zinc Oxide Nanoparticle loaded with folic acid and Vincristine

3.8 Cytotoxicity Assay

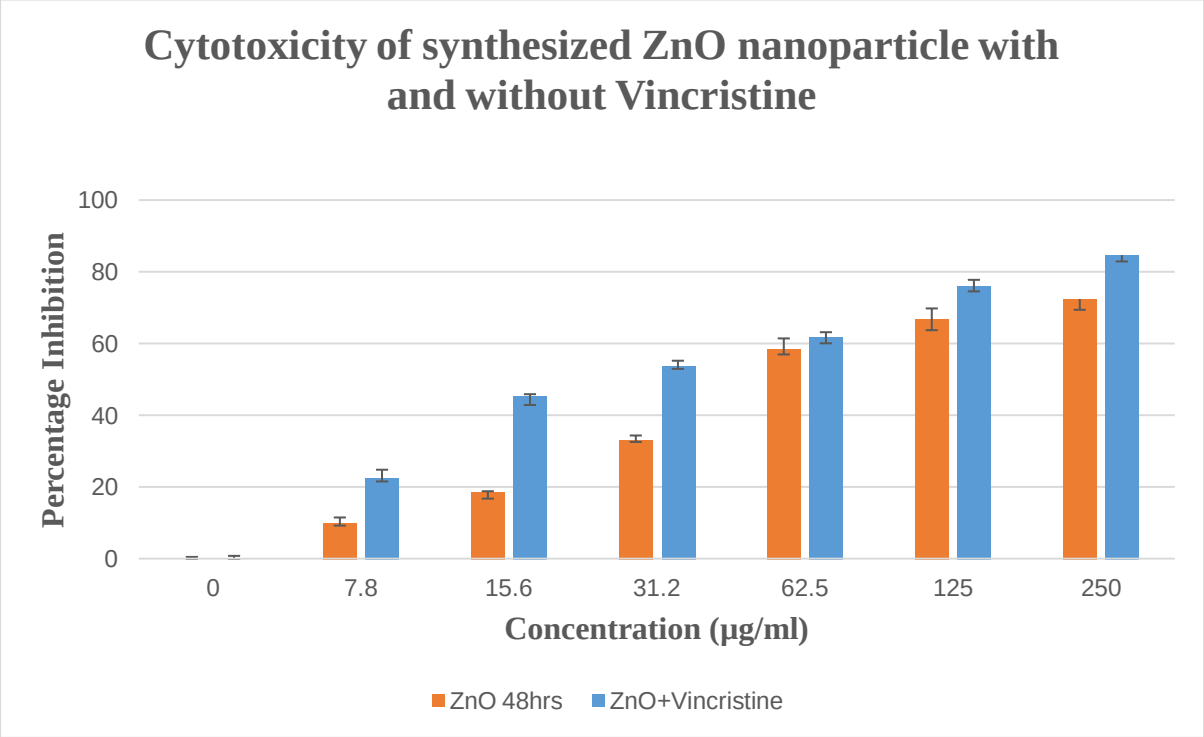
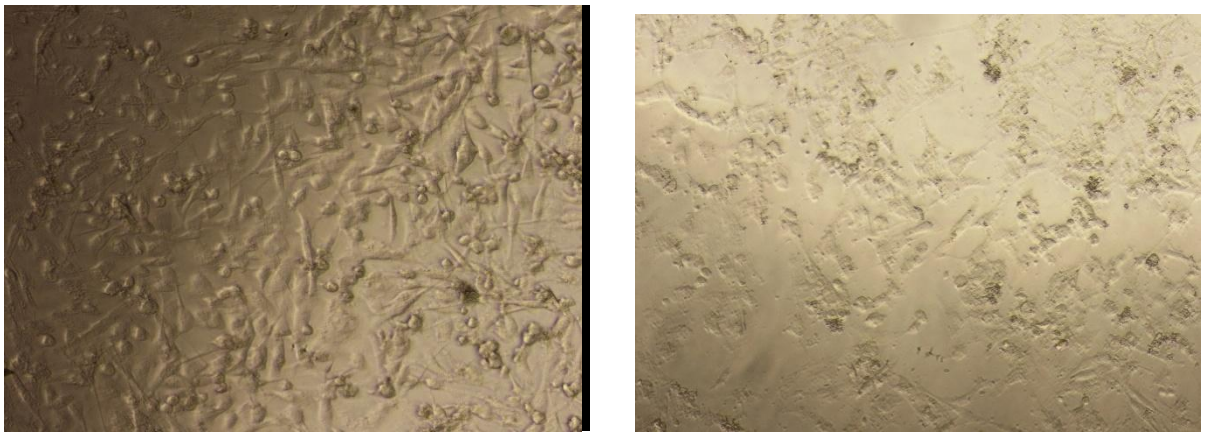


Fig 10. Cytotoxicity of synthesized ZnO nanoparticle with and without Vincristine using MTT Assay

The cytotoxicity of ZnO-NPs on cells was expressed as IC_{50} values (the drug concentration, reducing the absorbance of treated cells by 50% with respect to untreated cells). This experiment was carried out in triplicate. Untreated cells were used as negative control. Comparative analysis of synthesized ZnO nanoparticle with and without vincristine showed cytotoxicity in a concentration-dependent manner. When the MCF-7 cell line was treated with synthesized ZnO-NP, the inhibition percentage was about 72% in 48 hours while the ZnO-NPs loaded with vincristine showed about 84% growth inhibition after 48 hours of treatment. IC_{50} value of MCF-7 cell line was calculated by plotting a graph between the different concentrations of compounds (synthesized ZnO-NP and ZnO-NP conjugated to vincristine) against Percentage inhibition. This value was evaluated as 33.94 µg/ml for the synthesized ZnO-NP and 23.8µg/ml for the ZnO-NP conjugated to vincristine. Zinc-dependent protein activity disequilibrium has been delineated due to the increase in intracellular zinc concentration, thus resulting in cytotoxicity to the cell when treated with Zinc Oxide. Oxidative stress established due to the acceleration of ROS concentration by soluble zinc ions generates cytotoxicity in cancer cells [17].

(a) (b)



(c)

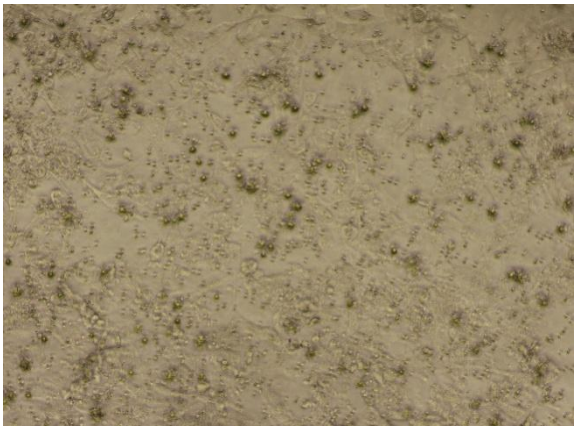


Fig 11. Morphological alteration of MCF-7 cells exposed to the IC50 (48 hours) concentration of ZnO-NP and ZnO-NP conjugated with Vincristine compared with control
(a) Untreated MCF-7 cells (b) Morphology of MCF-7 cells after ZnO-NP treatment with IC50 dose (IC50=33.94 µg/ml) (c) Morphology of MCF-7 cells after ZnO-NP+Vincristine treatment with IC50 dose (IC50=23.8 µg/ml)

IV. Conclusion:

Co-precipitation method was employed to generate ZnO nanoparticles.The synthesized nanoparticles had particle size in the range 37-68nm which is considered ideal for carying the anti-cancer drug Vincristine. Vincristine-loaded nanoparticles (PEGylated and non-PEGylated), along with the untreated ZnO-NPs, were analysed for the cytotoxic activity *in vitro* using MCF-7 cell line. Cells incubated with both ZnO-NPs and Vincristine loaded ZnO NPs manifest significant cytotoxicity at various concentrations. These findings indicate that ZnO-NPs have anticancer potential that can be enhanced by subsequent PEGylation and coupling with the anti-cancer agent Vincristine. Such modified drug-loaded nanoparticles can be probed for cancer treatment.

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