Zinc Oxide Nanoparticles Inhibit Human Laryngeal Carcinoma by Regulating Redox Status

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Copyright Statement

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Dedicated to my little angel and adorable daughter Maryam Fawad Khan.

Abstract

Nanotechnology research has gained momentum in the recent years by providing innovative solutions in the field of biomedical, materials science, optics and electronics. Nanoparticles are essentially a varied form of basic elements derived by altering their atomic and molecular properties of elements. This article elaborates on the properties and applications of zinc oxide nanoparticles. Main interest in the nanotechnology permits is the controlled synthesis of materials and the Nano size of the particle which is notably smaller than most of the cells in human body. The ultra-small size is equivalent to proteins naturally present in our body. Nanoparticles applications are used in many fields such as cancer treatment. For the treatment of laryngeal cancer zinc oxide nano particles are aimed to use. The nanoparticles are synthesized, and laryngeal carcinoma is treated followed by protein expression and anti-oxidant activity determination. Genetically mutated genes in laryngeal carcinoma reportedly are caspase 3, actin, BCL2 and PARP. Caspase 3 (cysteine-aspartic acid protease) is an apoptotic protein that interacts with caspase 8 and caspase 9 to initiate apoptosis in the damaged cells. It is encoded by caspase 3 gene. Because of mutation in this gene the damaged cells remain and grow causing the cancer. In the process of cancer invasion and metastasis actin cytoskeleton and focal adhesion are involved. For the intensification the metastatic process actin proteins are involved along with then the actin binding proteins such as cofilin and N-WASP and the focal adhesion proteins such as α- and β-Parvin and PINCH are reportedly involved. They support the cell adhesion, migration, survival and regulation of actin cytoskeleton dynamics in laryngeal cancer. Our results suggest that treatment with optimized doses of zinc oxide nanoparticles up regulates PARP and Caspase-3 and down regulates Bcl-2, thus proving effective in cancer cells therapy. Key words: Caspase-3, PARP, Bcl-2, apoptosis, mutation, metastasis, momentum, proliferation, therapeutic potential.

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List of Acronyms

ZnONP	Zinc Oxide Nanoparticles
SEM	Scanning electron microscopy
XRD	X-ray Diffraction
FBS	fetal bovine serum
NC	nitrocellulose membrane
EDX or EDS	Energy-dispersive X-ray spectroscopy
FTIR	Fourier Transform Infrared Spectroscopy

1. INTRODUCTION

1.1 Laryngeal Cancer

Laryngeal cancer is a common neoplasm of the respiratory cancer, most of these tumors are squamous cell carcinomas. There are approximately 110,000 to 130,000 new cases diagnosed annually in the world (Ferris et al., 2016) the cure rate of cancer is effected by the location of the tumor. For the purposes of tumor . Laryngeal cancer can spread to adjacent structures through the process of metastasis to lymph node, or more distantly, through the blood stream most commonly to the lungs, liver and bones.(Group*, 1991)The risk of laryngeal cancer is increasing day by day because of smoking habits, alcohol consumption, asbestos and tobacco uptake (Sheu et al., 2017) other risk factors include air pollution in work place although the exact relation is yet to be explained (Phillips et al., 2018) Human papilloma virus (HPV) are also associated with the worsening of a laryngeal cancer patient, it is also an imperative risk factor for causing benign and malignant laryngeal carcinoma (Gillison et al., 2012)

1.2 Daignosis of laryngeal cancer

The diagnostic techniques for laryngeal carcinoma include MR imaging and CT to recognize the stages of laryngeal cancer MR imaging has high contrast resolution for sift tissue and is a conventional technique to diagnose laryngeal carcinoma (H Kuno et al., 2018) dual-energy CT imaging technique is used to detect cartilage invasion by laryngeal squamous cell carcinoma (Hirofumi Kuno et al., 2012) Apart from other treatment techniques Nanoscience and nanotechnology are vigorously emerging fields of scientific interest. They have already become the research and development priorities. Nanotechnology is often known as enabling technology.(Bystrzejewska-Piotrowska, Golimowski, & Urban, 2009)

1.3 Nanobiotechnology

There are various treatment techniques to cure cancer but the most emerging fields nanotechnology to cure cancer through nanoparticles nanoparticles. The diameter of nanoparticles ranges from 1 to 100 nanometers, according to the requirement. A nanoparticle is a combination of ions, organic and inorganic molecules. Inside the nanoparticle our required drug is loaded to cure the cancer it as a very efficient and sight specific treatment of cancer. Nanomaterials are well-defined by their small size (<100nm) and their innovative physicochemical properties, which are increasingly imposed on different research fields. Different nanometals are also studied as a potential hope and are widely used. the risk of their reactivity with their respective bulk material are also ideally studied so that the therapy could be made more effective (DEMİR, Kaya, & KAYA, 2014)

Nanotechnology research has gained momentum in the recent years by providing innovative solutions in the field of biomedical, materials science, optics and electronics. Nanoparticles are essentially a varied form of basic elements derived by altering their atomic and molecular properties of elements. This article elaborates on the properties and applications of zinc oxide nanoparticles. Main interest in the nanotechnology permits is the controlled synthesis of materials and the Nano size of the particle which is notably smaller than most of the cells in human body. The ultra-small size is equivalent to proteins naturally present in our body (McNeil, 2005) Nanoparticles application are used in many fields such as cancer treatment. For the treatment of laryngeal cancer zinc oxide Nano particles are aimed to use. Zinc oxide (Zno) Nano powders are available in both the powder and dispersion form it has exhibition of antibacterial, anti-corrosive, anti-fungal and UV filtering properties Some of the synonyms of zinc oxide nanoparticles are oxydatum, zinci oxicum, permanent white, ketozinc and oxozinc.(Kołodziejczak-Radzimska & Jesionowski, 2014)

Nano-sized zinc oxide (ZnO) is used in industrial products including cosmetics, paints and medical materials. As a well-known photocatalyst, ZnO has received much attention in the degradation and complete mineralization of environmental pollutants(Rangel et al., 2017)

2. LITERATURE REVIEW

2.1 Laryngeal cancer

Globally laryngeal carcinoma is the most accruing cancer in male population it is the 11th most cancer in male inhabitants. There are about 9500 to 11,000 laryngeal carcinoma cases accruing each year in united states. It is one of the most common caused malignancy in Europe (Ampil et al., 2004) in northern and southern Europe the estimated occurrence for male population is about 18 in 100,000 and for women it is 6 in 100,000. For men comparatively to women the incidence is higher. (J. Li, Yang, Wang, & Tang, 2011) different epidemiological distribution has been observed according to the types or sites of laryngeal carcinomas. In Southern Europe supraglottic carcinoma transpires commonly than glottic carcinoma where as in northern Europe the glottis carcinoma risk is protruding(Rafferty, Fenton, & Jones, 2001)

Patient diagnosed with early stage of cancer could be treated with surgery sometimes along with the combination of radiotherapy or chemotherapy. Depending on the staging of cancer nearly 80% patients could be treated. Most commonly used chemotherapeutic agents are cisplatin or carboplatin in combination with 5-fluorouracil or taxanes. Depending upon the case and condition the dose, intensity and duration of chemotherapy is decided(Su et al., 2018) regardless of the significant advances in the field of therapy little improvements has been gained in case of Laryngeal carcinoma in the last three decades thus novel therapies need to be appraised for the sake of treatment.(Reuter, Morgan, & Eckardt, 2007)

laryngeal caner also known as laryngeal carcinoma or simply cancer of larynx is usually squamous cell carcinoma. laryngeal cancer can develop from any part of larynx such as the glottis, supraglottic also known as epiglottis or the sub glottis. But most of the tumors appear in the glottis region. Supraglottic

and subglottic are least common. Laryngeal cancer can frequently metastasize to other distinct parts through lymph nodes. The treatment of laryngeal cancer depends upon the location of the tumor.(Remmelts et al., 2013)

There are many risk factors of laryngeal cancer some of which are alcohol and tobacco consumption these are the major risk factors of laryngeal carcinoma in more than 95% cases patients diagnosed with laryngeal carcinoma have tobacco and alcohol history. Other aspects being the environmental factors such as uv exposure , mutation (chromosomal damage), gastroesophageal and biliary reflux and viral infections and exposure to cancer causing agents.(Hellquist et al., 2018) laryngeal carcinoma can metastasize to other part of body through lymphatic system. Invasive carcinoma of the supraglottic region metastasize in 25-75% cases. However early stage glottic carcinoma have low incidence of metastasizing. Reputedly approximately 32% are metastasis from skin, 27% from renal cell carcinoma, 11% from gastrointestinal tract, and 105 from breast.(Kandukuri et al., 2017)Cervical lymph nodes are at high risk of spreading the cancer. Primary sub glottic carcinoma first spread to paratracheal and recurrent lymph nodes. Distinct metastases in laryngeal carcinoma is significantly less than other cancers.(Gupta et al., 2011)

Genetically mutated genes in laryngeal carcinoma reportedly are caspase 3, actin, BCL2 and PARP.

Caspase 3 (cysteine-aspartic acid protease) is an apoptotic protein that interacts with caspase 8 and caspase 9 to initiate apoptosis in the damaged cells. It is encoded by caspase 3 gene. Because of mutation in this gene the damaged cells remain and grow causing the tumor or cancer. (Peiró, Diebold, Baretton, Kimmig, & Löhrs, 2001)

In the process of cancer invasion and metastasis actin cytoskeleton and focal adhesion are involved.(Kojc, Zidar, Vodopivec, & Gale, 2005)

For the intensification the metastatic process actin proteins are involved along with then the actin binding proteins such as cofilin and N-WASP and the focal adhesion proteins such as α- and β-Parvin and PINCH are reportedly involved. They support the cell adhesion, migration, survival and regulation of actin cytoskeleton dynamics in laryngeal cancer.(Yamaguchi & Condeelis, 2007)

BCL-2 is the B cell lymphoma protein encoded by the BCL2 gene known as the apoptotic regulator protein.

It is localized in the peripheral membrane of mitochondria and plays an important function in promoting cell survival and inhibiting the action of pro- apoptotic proteins for cell survival that includes the Bax and Bak (Trask et al., 2002). these pro-apoptotic proteins promote the permeability of mitochondrial membrane and the release of cytochrome C and ROS (reactive oxidative species).(S. Li et al., 2017) which are the important signals in apoptotic process as a result the pro-apoptotic proteins are activated and are inhibited by the function of BCL-1 and BCL-XL any mutation in these genes can lead to tumor formation.(Yao et al., 2017)

Another important gene in the laryngeal carcinoma is the PARP gene (poly ADP- ribose polymerase) it is the family of proteins that regulates many cellular process such as the DNA repair, genomic stability and apoptosis, activation of inflammatory genes. PARP protein have domains like DNA-binding domain, caspase binding domain and acatalytic domain.(H.-X. Wang & Tang, 2017) PARP protein is found in the cellular nucleus that helps to perceive and initiate any cellular responses to metabolic, chemical, or radiation induced DNA damage and signaling to the repair machinery of the cell.(Tangutoori, Baldwin, & Sridhar, 2015)

The actions of these proteins in the process of laryngeal carcinoma could be elucidated through western blotting. a protein assay that is used for the detection the amino acid sequence of proteins.

2.2 Nanobiotechnology

Nanobiotechnology and nanomedicines are the emerging fields in modern sciences. This new fields of science provides and innovates novel and assorted applications in the field of new science and technology(Sirelkhatim et al., 2015). The synthesis characterization and exploration of these particles are in the Nano scale that is also reasonable for the cells in human body. The functions of nanomedicines is not only use full in medical techniques but also in agriculture, food processing and innovative compounds in fabric production not only that but also this field is embraced by industrial and electronic sector(Kalluri & Kodali, 2014) the Nano structures are designed in Nano scale size that has a larger surface area as compare to that of macro sized particles. their size ranges from 1-100nm. They might show size related properties as well. Their surface area are kept larger for the induction of drug for treatment purposes(Ashe, 2011) Nano particles are defined to be the particles with controlled or deployed particles at a Nano level for the use of drug release. Their distinct properties from other , small size and efficient working procedures allow their unique applications in the fields of biosensors, nanomedicine, and bio nanotechnology(Rasmussen, Martinez, Louka, & Wingett, 2010)

2.3 Metal Nano particles

Metallic nanoparticles have rivetted scientist for over a century and are now heavily exploited in biomedical sciences and engineering. They are a focus of interest because of their huge potential in nanotechnology. Nanotechnology is defined as the branch of science and engineering. They have been uses more effectively in efficient technologies. In metal Nano particles zinc oxide Nano particles are preferred due to their unique properties and innovative functioning. Zinc oxide nanoparticles has their own unique physical and chemical properties. Such as high chemical stability, high electrochemical coupling co efficient, broad range of radiation absorption and high photo stability (Segets, Gradl, Taylor, Vassilev, & Peukert, 2009) zinc oxides are classified as semiconductor they have high thermal and

mechanical stability at room temperature this quality might make them useful in the fields of electronic optoelectronic and laser technology(Bacaksiz et al., 2008) because of its hardness, rigidity and piezoelectric constant, biodegradability it has a great importance in biomedicines(Ludi & Niederberger, 2013) zinc nanoparticles are known to have broad range of functioning such as strategic, promising and versatile inorganic material with efficient range of function that also holds a exclusive optical chemical sensing, semi conducting properties, electric conductivity and piezoelectric effects(Fan & Lu, 2005) the conductivity of zno particles could be increased by doping it with other materials it has very strong ionic bonding with in the zn-o. it can stay for longer duration, it has higher selectivity, and it is highly heat resistant(Padmavathy & Vijayaraghavan, 2008) it could also ac as an anti-bacterial agent and anti-fungal agent. The applications of zn-o nanoparticles are the result of the potential biocompatibility of theses nano materials over other metal oxides their solubility in alkaline medium and terminated polar surfaces are another additions (Mishra et al., 2012) these nanoparticles could be synthesize through various monitoring process such as by controlling their synthesis parameters in different ways. Their properties could be tailored in various ways such as shape, size, structural properties, solvent type, pH precursors, temperature and conjugating drug in order to increase its efficiency of application. An assortment of ZnO nanostructures with different growth morphologies such as nanorods, nanosphere, nanotubes, nanowires, nanoneedles and nanoring's have been successfully synthesized(Yahya et al., 2010) the shapes of zno nanoparticles could be reformed such as ZnO spirals, drums, polyhedrons, disks, flowers, stars, boxes, and plates. Different chemical techniques are used to alter the size and morphology successfully. Each nanostructure has specific optimized structural, optical, electrical, and physicochemical properties(Ramirez-Vick, 2012) zinc nanoparticles are reportedly nontoxic to human cells. This aspect of nanoparticles is very useful and can be used in other ways such as an antibacterial agent, lethal for microbes, nanoparticles hold good compatibility with human cells

2.4 Synthesis of Zinc oxide nanoparticles

Zno nanoparticles have potential compatibility to human cells over other metal oxides. Zno nanoparticles can be synthesized using methods by controlling the parameters like shape size, quantity of drug , combination with other substances, temperature, pH and solvent types (B. Wang et al., 2008) zinc oxide nano particles could be used for the therapy of laryngeal cancer recent studies suggested that zno nanoparticles exhibit greater cancer cell selectivity as compare to other chemotherapeutic agents.(H. Wang, Wick, & Xing, 2009)

Zno nanoparticles could also be used as the biomarker for cancer diagnosis, screening and imaging techniques. zno nanoparticles when coated with polymethyl methacrylate could be used to detect cancer cells as they form ligand conjugation with the tumor cells.(Shen et al., 2008)

Zno nanoparticles have been used as a therapy for several types of cancers such as glioma, breast cancer, bone cancer, colon cancer, leukemias and lymphomas. (Hua Wang et al., 2009) At physiological PH the positively charged ZnO nanoparticles interact with cancer cell membrane by electrostatic interaction thereby promoting cellular uptake, phagocytosis and cytotoxicity. through the mechanism of photoactivation of zno nanoparticles it has been prophesied that the zno nanoparticles could lead to greater level of ROS release that could efficiently target the cancer cells and cause selective demolition of cancer or tumor cells. They could also be conjugated to the chemotherapeutic drugs and enhance the anti-cancer efficacy and photoactivation of zno particles could also be useful in targeting the cancer cells.(Fakhar-e-Alam et al., 2012) these photoactivated particles have the tendencies to conjugate to tumor cells, this attribute could be used to extinguish the cancer cells.(Liu, Zhang, Wang, Pope, & Chen, 2008) zinc oxide with a potential band gap energy of (3.2 eV) has been reported as a successful therapeutic agent in many cancers cell lines including melanoma cell lines, human carcinoma cell lines. (Theogaraj, Riley, Hughes, Maier, & Kirkland, 2007) So in this study we would investigate the possible

therapeutic redox reaction and photocatalytic effects on the human laryngeal cell lines to appraise the effect of this novel treatment pathway.

3. MATERIALS AND METHODS

3.1 Chemicals and reagents

Sodium hydroxide (NaOH) and zinc nitrate tetrahydrate [Zn(NO3)2.4H2O)] were bought from Sigma Aldrich for synthesizing zinc oxide nanoparticles.

3.2 Synthesis of zinc oxide nanoparticles

ZnONPS were synthesized from aqueous solutions of Zn (NO3)2.4H2O and NaOH. In brief, a 0.9 M solution of NaOH (100 mL) was heated in a flask at 55°C and the equal volume of a 0.45 M solution of Zn (NO3)2.4H2O was added drop-wise with vigorous stirring. After complete mixing, the flask was closed until the ZnONP precipitated out of the solution. The nanoparticles were washed with water and ethanol twice each time to remove impurities. The particles were then dried in an oven at 67°C for 2 hours. Before application, the ZnONPs were dissolved and suspended by sonication in de-ionized water (DIW).

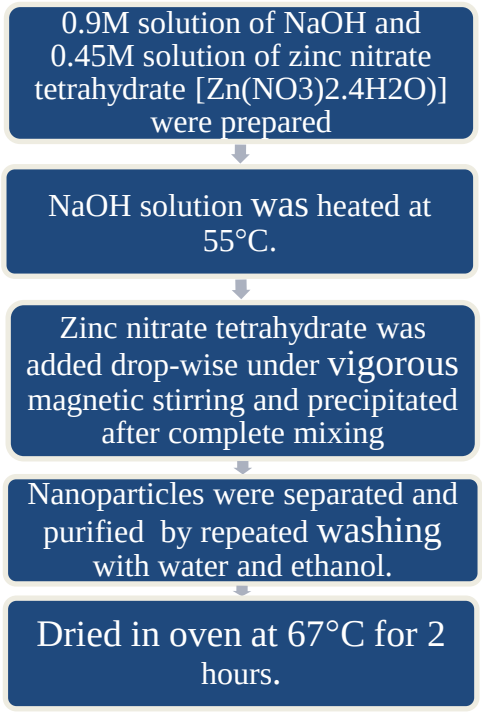

Figure 3.2 Synthesis of ZnONPs

3.3 Characterization of zinc oxide nanoparticles

Ultraviolet–visible spectroscopic measurements of the ZnONP ethanolic suspension were performed with the Jasco V-650 spectrophotometer in the 300-800 nm range and measured relative to pure ethanol.

Scanning electron microscopy (SEM) analysis of the ZnONP was performed using S-4800 and EDX-350 (Horiba) FE-SEM (Hitachi, Tokyo, Japan). Samples were dispersed in pure ethanol by ultra-sonication, dropped onto a glass plate fixed onto a brass holder, and coated with osmium tetra oxide (OsO4) using a VD HPC-ISW osmium coater (Tokyo, Japan) prior to FE-SEM analysis. FE-SEM analysis results were also utilized to calculate the particle size. For this purpose, the size of 50 nanoparticles was measured using ImageJ software and the particle size distribution graph was plotted. The average particle size was calculated from the total size distribution.

X-ray Diffraction (XRD) patterns of the ZnONP sample were recorded with an X-ray diffractometer (X'Pert-APD Philips, The Netherlands) containing an X-ray generator (3 kW) and anode (LFF Cu). The Cu Kα radiation was administered at a wavelength of 1.54 Å. The X-ray generator tension and current were 40 kV and 30 mA, respectively. The angle of scanning varied from 10º to 80°.

The FTIR analysis of the sample was done in both powdered and suspension form.

3.4 Activity of synthesized zinc oxide nanoparticles

3.4.1 Cell culturing

Media containing fetal bovine serum (FBS) 10% and penicillin 1% was kept in water bath for half an hour at 37°C. Cells were transferred through micropipette to 3ml media contained in six well plates. These plates were then incubated at room 37°C for full growth. Later on the cells were treated with different concentrations of the drug to check the anti cancer activity.

3.4.2 Drug treatment

The cultured cells were treated with different doses of zinc oxide nanoparticles and then western blot analysis was done to study the protein expression in different sample groups.

3.4.3 Dose dependent treatment (MTT assay)

In dose dependant assay, seven sample groups were made out of which one served as control and the other six groups were treated with different drug concentrations i.e 25, 50, 100, 150, 200, 300µg/ml respectively and their MTT assay was performed. These concentrations of the drug were obtained by serial dilutions of the stock solution 1mg/ml of the drug (all the dilutions were done in deionized water).

MTT assay is a procedure done in research to find out the effect of any chemical or drug on cells. It is a cell viability assay which is performed to determine living and dead cells count. Cells were cultivated in 96 well plates and were administered with drug in the following manner

Serial no.	Drug conc. (in µg/ml)	Time
1	Control (untreated)	24 hours
2	25	24 hours
3	50	24 hours
4	100	24 hours
5	150	24 hours
6	200	24 hours
7	300	24 hours

Table 3.4 Sample groups of study for MTT assay

All the sample groups were cultured and treated in triplicate in order to increase accuracy. Each sample group, except control, were administered with 10µL MTT reagent and were incubated at 37ºC for 24 hrs. This process involves the change in color of MTT reagent corresponding to living or dead cells, this change in color can be detected at 570nm wavelength by spectrophotometer. The untreated sample groups serve as control.

3.5 Time dependent treatment

The results obtained from MTT assay led us to calculate IC50 concentration i.e 400µg/ml. Cells were then treated with 400µg/ml of the drug in a time dependent manner for 24, 48 and 72 hrs to check the effect of the drug on cell viability with reference to time.

Serial no.	Drug conc.	Time
1	400µg/ml	24 hrs
2	400µg/ml	48 hrs
3	400µg/ml	72 hrs

Table 3.5 Time dependent assay

For each sample, control was left untreated and all experiments were performed in triplicate.

3.6 Protein Extraction

The cell lines used were adherent cell lines so the media in six well plates was homogenized by vigorous pipetting to detach the cells from plate. All the media (containing hep-2 cells) of each well was transferred to falcon tubes and were labeled accordingly. Control was also labeled and kept separate. For each sample, the experiment was performed in triplicate. To each sample, 500µl of 1X PBS was added followed by centrifugation at 3000rpm for 10 minutes. Supernatant was discarded and cell pellet was washed with 1X PBS, centrifuged at 3000rpm again, medium discarded and cell pellet kept. To this pellet 200µl RIPA buffer was added (now the tubes were continuously kept in ice), the tubes were vortexed for 10 seconds after every 10 minutes (this process was repeated six times). After this, the

samples were centrifuged at 12000rpm 15 minutes which resulted in proteins at the surface. The supernatants were then transferred to eppendorf tubes and stored at -20ºC

3.7 Protein Quantification

Protein quantification is done through a process called Bradford assay. In this process, Bovine serum albumin (BSA) was used as a standard for the preparation of samples, which were then analyzed by UV spectrophotometer. The spectrophotometer measures the varying change in absorbance with the change in concentration of the sample. First of all cuvettes were washed with 70% ethanol to ensure de-colorization, and then samples were poured in them. For each analysis, the cuvettes were loaded with 1mL of sample. As BSA is light sensitive, so the experiments were performed in a dark environment. Samples were prepared and vortexed to experience the color change due to Bradford reagent, stored at room temperature for 10 minutes and were then analyzed at 595nm by the UV spectrophotometer. The standard BSA curve was obtained by plotting absorbance against concentration. A sample containing water and Bradford reagent was used as blank. Samples were made in the following manner.

BSA+Sample	Water	Bradford reagent	Total Volume	Absorbance
0 µl	800 µl	200 µl	1000 µl	0.00
20 µl	780 µl	200 µl	1000 µl	0.063
40 µl	760 µl	200 µl	1000 µl	0.091
60 µl	740 µl	200 µl	1000 µl	0.192

80 µl	720 µl	200 µl	1000 µl	0.220
100 µl	700 µl	200 µl	1000 µl	0.251
24 hrs control (2 µl)	798 µl	200 µl	1000 µl	052
24 hrs treatment (2 µl)	798 µl	200 µl	1000 µl	0.96
48 hrs control (2 µl)	798 µl	200 µl	1000 µl	0.52
48 hrs treatment (2 µl)	798 µl	200 µl	1000 µl	1.21
72 hrs control (2 µl)	798 µl	200 µl	1000 µl	0.52
72 hrs treatment (2 µl)	798 µl	200 µl	1000 µl	1.30

Table 3.7 Samples for BSA

This experiment gives a curve of the corresponding absorbance values for different sample concentrations, which are then used to calculate the sample loading values for western blot analysis. For this experiment, the loading values were calculated to be 2.60, 2.05, 2.69 and 2.73 for control, 24, 48 and 72 hours treatment respectively. The loading values were derived through a curve equation for the obtained curve and is written as y=0.025x-0.0044. The following standard curve was obtained.

3.8 Western Blot Analysis

3.8.1 Gel preparation

Western blotting or immuno-blotting was done to check the expression of different proteins. SDS polyacrylamide gel was prepared by the standard procedure used for its preparation. First of all, 12% separation gel was prepared and vortexed prior to loading between the glass plates to mix it properly. Then, isopropanol was poured on top of separation gel so as to avoid any air bubbles which could effect the process negatively. The gel was left for setting for about 20-25 minutes and isopropanol was removed with the help of blotting paper. Meanwhile, 5% stacking gel was prepared, vortexed and loaded on top of separation gel. Now, gel comb was introduced in between the plates to form wells for sample loading and the gel was left for solidification. After about 10 minutes the gel was solidified, and the comb was removed.

3.8.2 Sample loading calculation

Sample loading values were calculated according to the equation obtained from BSA standard curve. Following calculations were done to obtain loading values of each sample.

Samples	Absorbance	2µg	1µg	50µg Loading	Water	4X buffer	Total

Control	0.52	30.45	15.22	2.45	3.55	1	7
24hrs Treatment	0.96	48.21	24.10	2.11	3.89	1	7
48hrs Treatment	1.21	50.46	25.23	2.59	3.41	1	7
72hrs Treatment	1.30	53.72	26.86	2.70	3.30	1	7

Table 3.8.2 Sample loading calculation

3.8.3 Sample preparation

Protein samples and 4X buffer were used to prepare samples for loading. To facilitate protein denaturation, samples were kept in hot water bath for 10 minutes after preparation. After this, the samples were centrifuged to avoid any type of membrane formation. When the samples were ready, they were loaded into the wells through micropipette, without touching the walls of the wells. Then the plates were set into the tank and the leads were attached to the battery, after this 1X buffer was poured into the tank upto the mark for one gel. Samples were loaded in the following manner:

4X Buffer	Protein marker	Sample 1	Sample 2	Sample 3	Sample 4

Table 3.8.3 Samples loading

3.8.4 Running gel

Gel was run for 120 minutes at 90V in the presence of 1X running buffer. The gel was stained with a commercially available gel staining solution called coomassie blue to visualize protein bands, to confirm the presence of protein content in the samples. At the end of electrophoresis the proteins appear in the form of bands which are separated on the basis of their molecular weight.

3.8.5 Transfer to NC membrane

At the end of electrophoresis, the protein bands were transferred to nitrocellulose membrane (NC). The gel was covered with blotting papers on both side and then the blotting papers were covered with sponges on both sides, this assembly was then kept in the transferring apparatus containing 1X transfer buffer and was run for 25 minutes at 90V.

3.8.6 Ponceau Band Detection

After the completion of bands transfer onto the NC membrane, it was dipped in Ponceau solution to visualize the protein bands. This step was done to confirm the transference of the protein bands onto the NC membrane. Then proteins were marked according to protein marker bands.

3.8.7 Blocking with non-fat milk

Blocking of the NC membrane was done to avoid non-specific protein binding. After ponceau staining, the membrane was washed with distilled water and dipped in 5% non-fat milk for 1 to 2 hours at 4ºC.

3.8.8 Treatment with primary antibodies

The NC membrane, after blocking with non-fat milk was treated with primary antibodies. Primary antibodies were diluted with 1X TBS in the ratio of 1:1000 and then the NC membrane was dipped in the primary antibodies solution and shaken for about 18 hours or overnight at 4ºC.

3.8.9 Washing

After the treatment with primary antibodies, washing was done with TBST solution. This step was carried out five times so as to completely remove any unbound primary antibodies, in order to avoid any false results. Washing was done for 5, 5, 10, 10 and 10 minutes consecutively.

3.8.10 Treatment with secondary antibodies

Like primary antibodies, secondary antibodies were also diluted with TBST but in the ratio of 1:2000. The NC membrane was dipped and incubated in secondary antibodies solution for 2 to 3 hours at 4ºC under gentle shaking condition followed by five times washing for 5, 5, 10, 10 and 10 minutes with 1X TBST solution.

3.8.11 Detection of Protein Bands

After washing step, the NC membrane was exposed to 500µl of liquid NBT substrate, which was bought from Sigma Aldrich. Treatment with NBT substrate caused the protein bands to appear.

3.9. Free Radical Scavenging Activity

1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) possesses free radicals which are used to calculate the antioxidant activity of any compound with which it reacts. The free radical scavenging activity of zincoxide nanoparticles was measured by DPPH. For this assay, 1mM DPPH was prepared using ethanol as a solvent and sixconcenterations of zincoxide nanoparticles were prepared i.e 25, 50, 100, 150, 200, 300µg/ml respectively. All these concentrations were used to compare the antioxidant activity

of each sample. Ascorbic acid was used as a standard. DPPH solution is purple in colour and due to its light sensitive nature, it was kept in dark. The volume of each reaction mixture was kept 1.50ml by adding water.Composition of reaction mixtures is given in the following table

Reaction	**DPPH soln.**	**ZnO nps conc.**	**Final volume**
1	500µl	25µg/ml (500µl)	1.50ml
2	500µl	50µg/ml (500µl)	1.50ml
3	500µl	100µg/ml (500µl)	1.50ml
4	500µl	150µg/ml (500µl)	1.50ml
5	500µl	200µg/ml (500µl)	1.50ml
6	500µl	300µg/ml (500µl)	1.50ml

After mixing the compounds in the above-mentioned proportions, the mixtures were kept in dark for about 30-35 minutes for the reaction to take place. The change in colour from dark purple to light yellow marks the end of the reaction. This mixture was then observed using UV-VIS spectrophotometer (UV-2800) at 517nm. For blank ethanol was used and control contained equal volumes of DPPH and ethanol. The percentage antioxidant activity was calculated as follows

%AA=100 – [(Abs of sample) – (Abs of blank)/Abs of control] × (100)

4. RESULTS

4.1. Zinc oxide nanoparticles characterization results

4.1.1 UV-VIS Spectrophotometry

After the synthesis of zincoxide nanoparticles, the first step was to confirm their synthesis and for that purpose the initial confirmatory test was UV-VIS spectrophotometry. The UV-VIS spectrum of the synthesized nanoparticles (obtained through UV-2800) was studied in the wavelength range of 350 to 800 nm, which gave a central peak at 529nm, the characteristic peak for ZnONPs. Thus the presence of ZnONPs was confirmed in the sample. Following is the peak obtained.

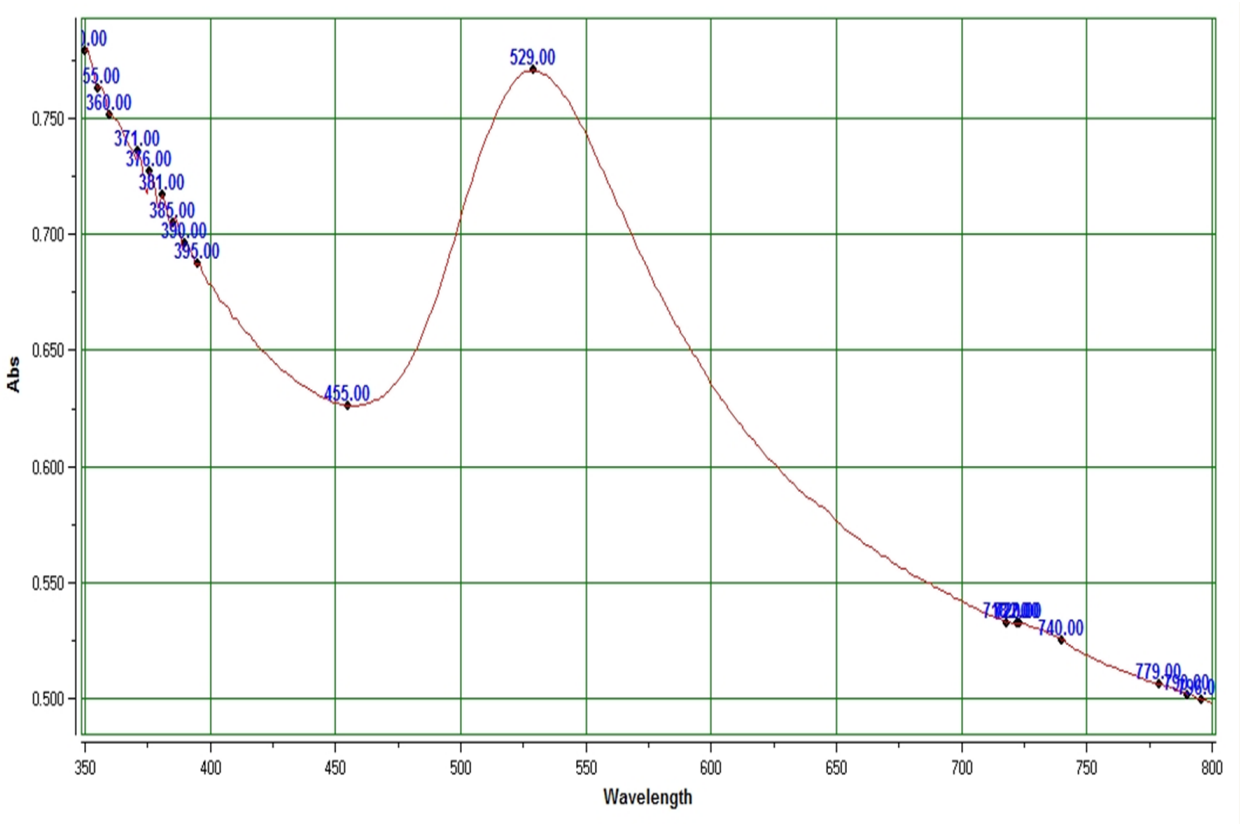

Figure: 4.1.1 UV-VIS Spectrum of ZnONPs

4.1.2 Scanning Electron Microscopy

For particle size determination Scanning Electron Microscope, JSM-6490, was used. This was also a confirmatory test for ZnONPs size determination, to know whether these particles were nanoparticles or not. Ideally the size of nanoparticles should be between 1-50nm. The average particle size of these synthesized ZnONPs was 39nm which was calculated by ImageJ software, so this test also confirmed the presence of nanoparticles.

Following are the SEM results of the synthesized ZnONPs.

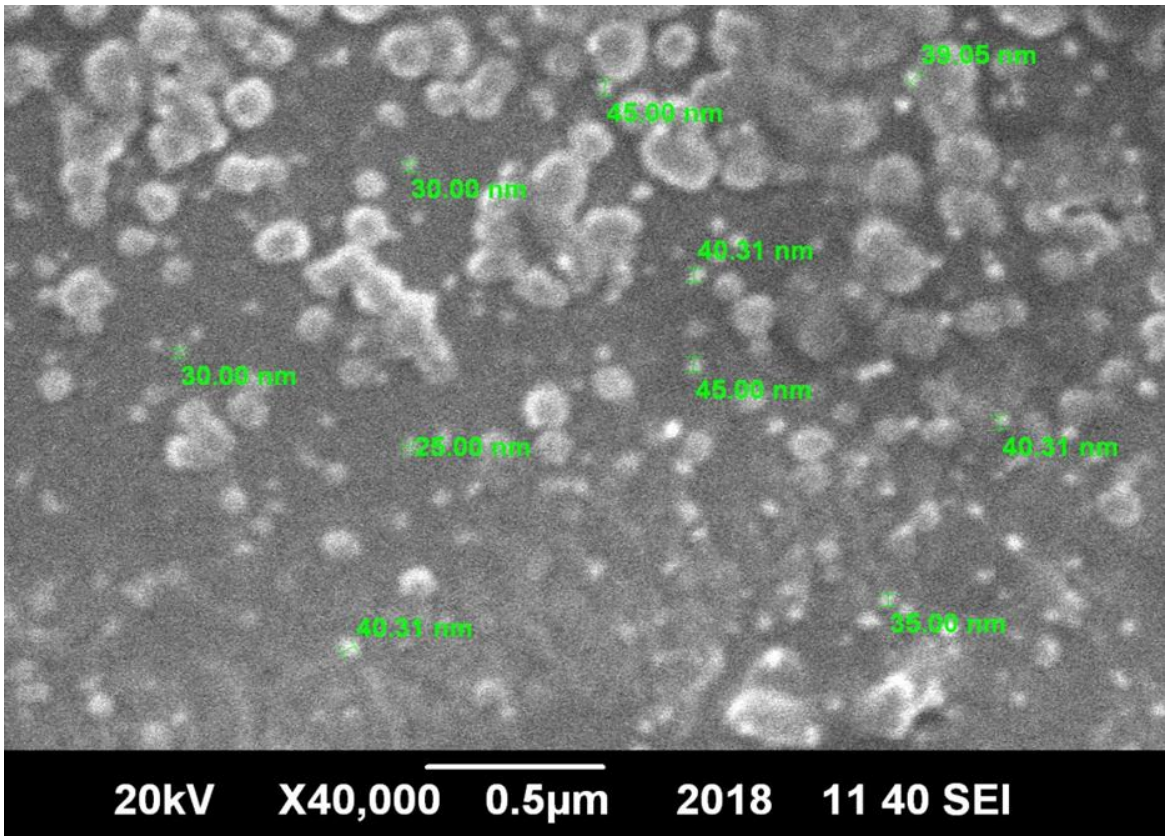

Figure: 4.1.2A SEM of ZnONPs

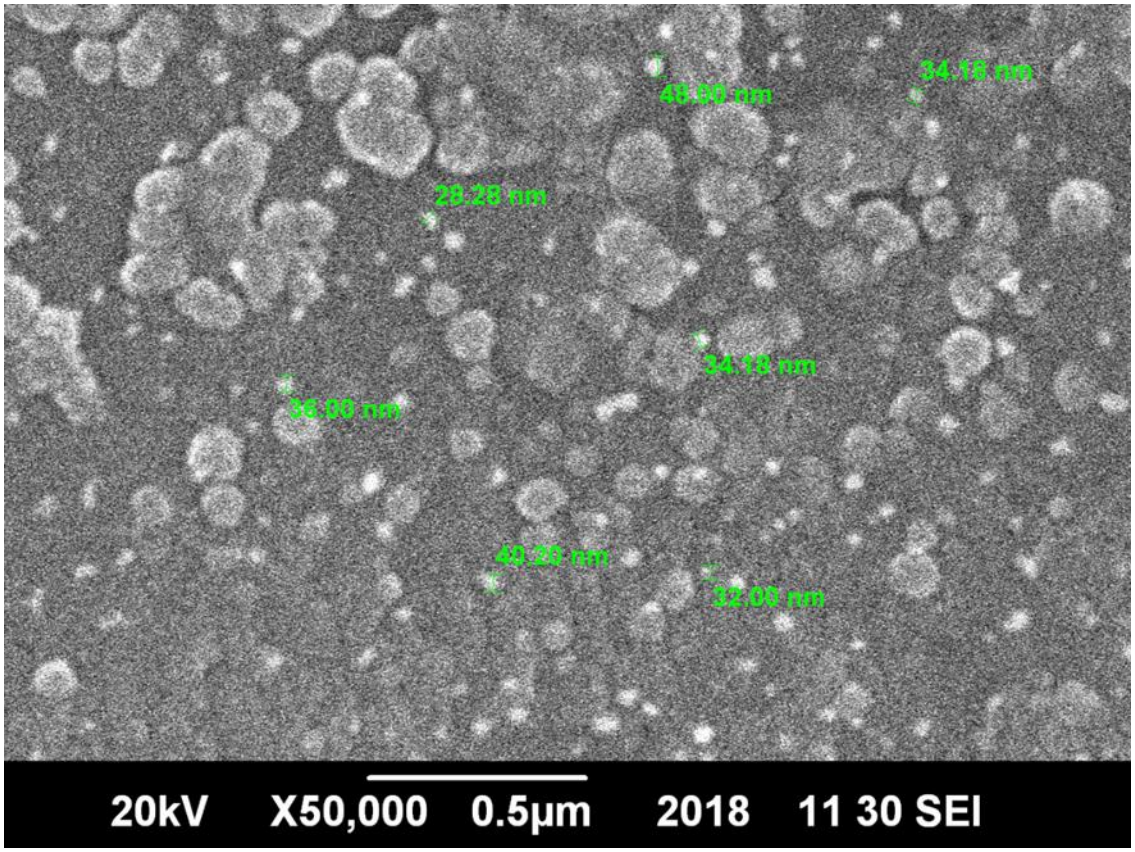

Figure: 4.1.2B SEM of ZnONPs

4.1.3 Energy-dispersive X-ray spectroscopy

Energy-dispersive X-ray spectroscopy (EDX or EDS) is used in conjunction with SEM to find out the types of elements present in a sample or compound. EDX is performed by energy and intensity distributions of X-ray signals generated by focused electron beam on a specimen. EDX characterization of our sample has shown absorption of strong zinc oxide and is shown as follows.

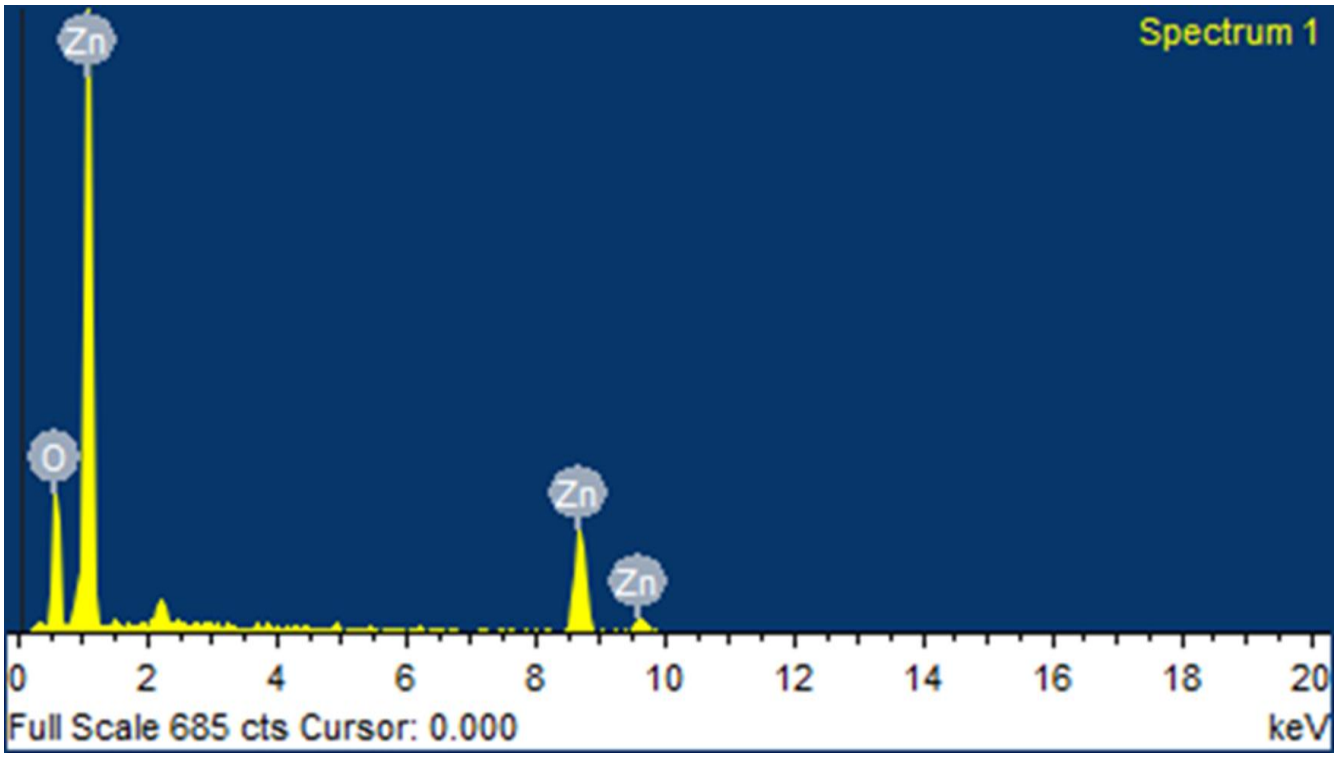

Figure: 4.1.3 EDX of ZnONPs

4.1.4 X-Ray Diffraction (XRD)

The particle size and crystalline nature of ZnONPs was determined by XRD. This was done by an X-Ray diffractometer, by using powdered sample of ZnONPs. The angle of scanning varied from 10º to 80°. The diffraction pattern showed three characteristic ZnO peaks in between 30º-40º, which confirmed hexagonal ZnO phase (Wurtzite Structure) and can be seen as follows.

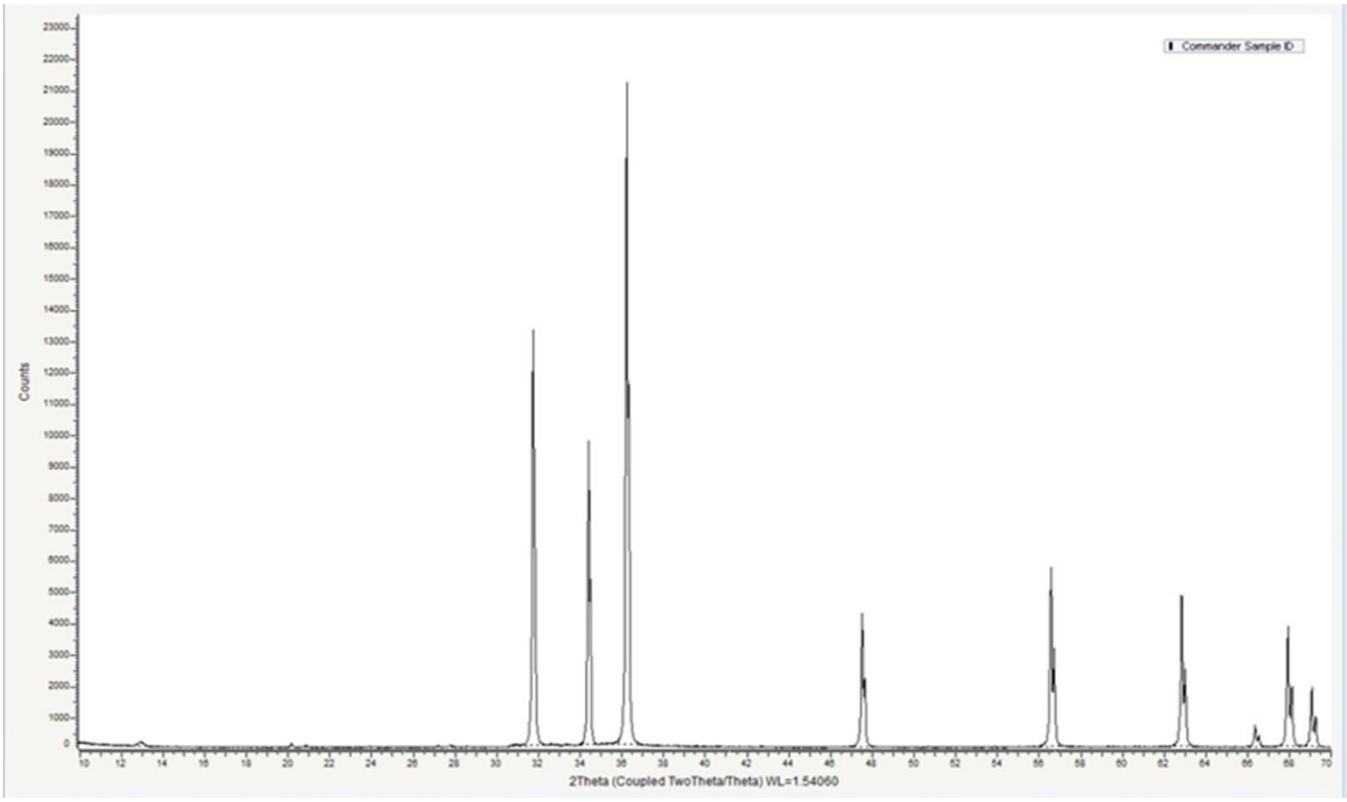

Figure: 4.1.4 XRD of ZnONPs

4.1.5 Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR Analysis) is an analytical technique which is used to identify any material. In this technique, infrared radiations pass through the sample and determine its chemical properties. The FTIR analysis of the sample was done in powdered as well as in solution form and characteristic peaks were obtained.

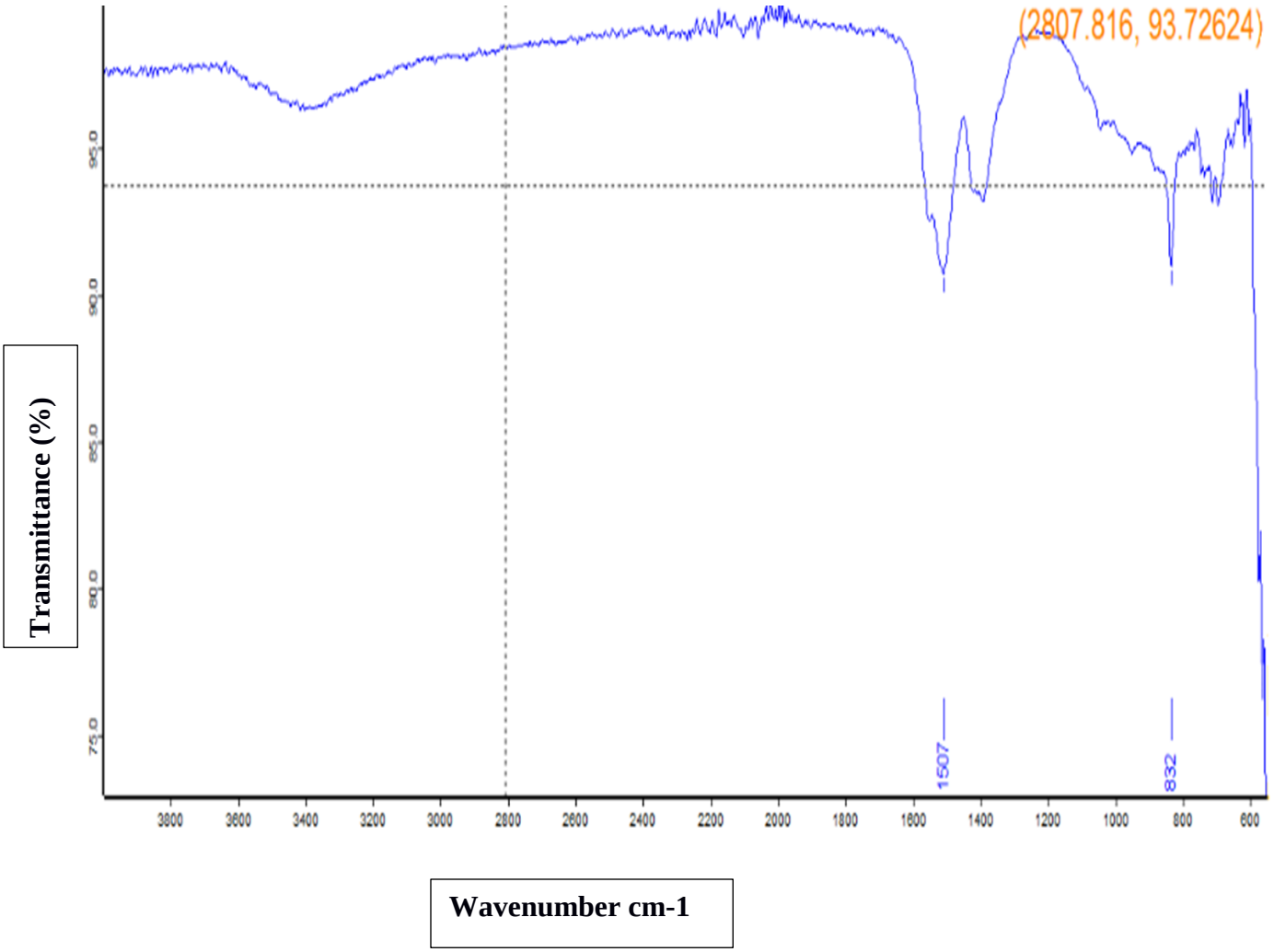

Figure: 4.1.5A FTIR of ZnONPs (powder)

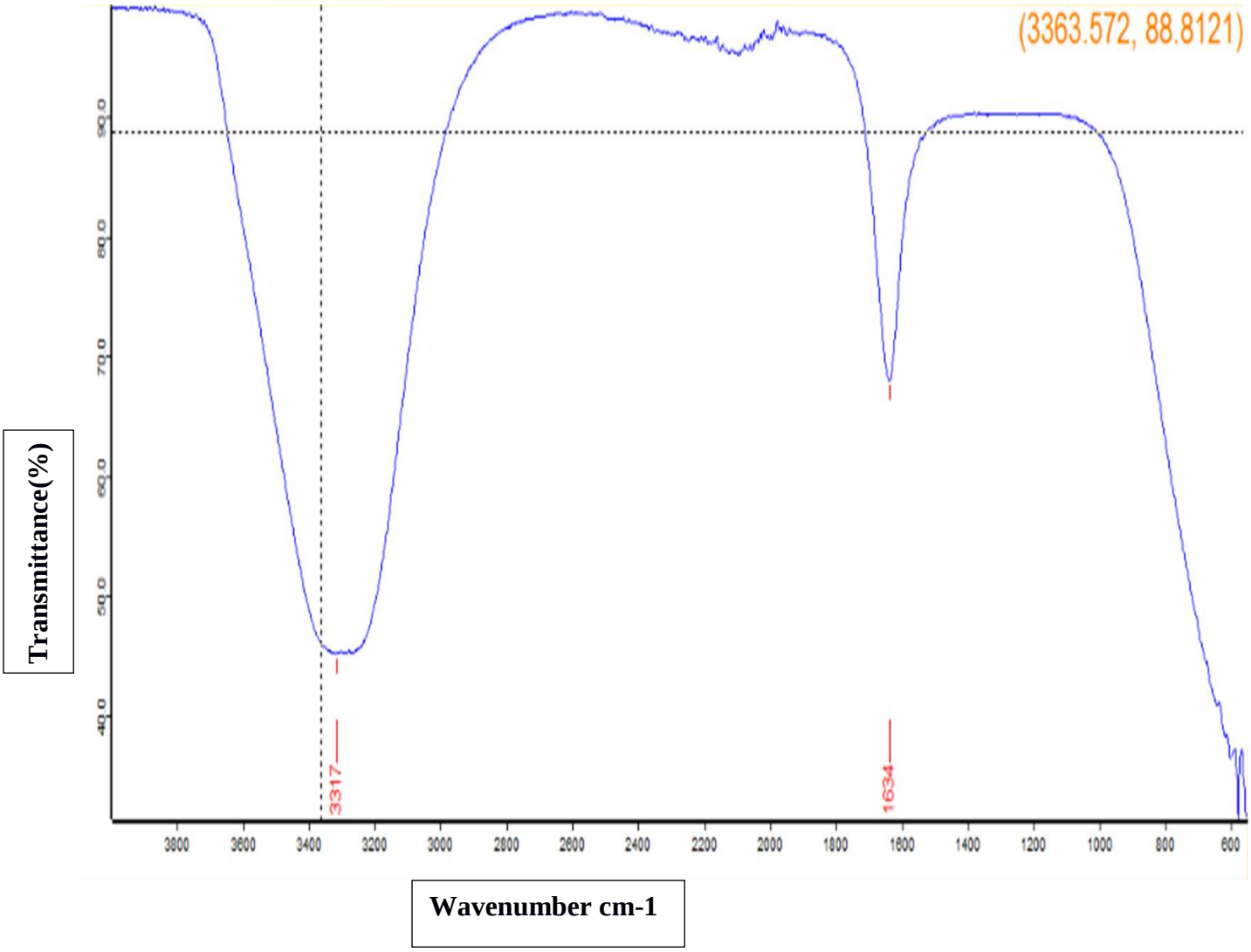

Figure: 4.1.5B FTIR of ZnONPs (solution form)

4.2 Cell viability assay results

MTT assay or cell viability assay is used to find out the count of living or dead cells in a sample. Through this assay, the effect of zinc oxide nanoparticles was studied to determine the effect of these

nanoparticles on cell viability. The effects of six different drug concentrations were studied on HEp-2 cell lines in order to determine the efficacy of the nanoparticles against laryngeal carcinoma. The MTT assay result showed that with increasing drug concentration, the number of viable cells decreased. So for 300µg concentration of the drug, the number of viable cells considerably decreased which shows that this drug can be used for cancer treatment by regulating the dose. Following results were obtained at the end of the assay.

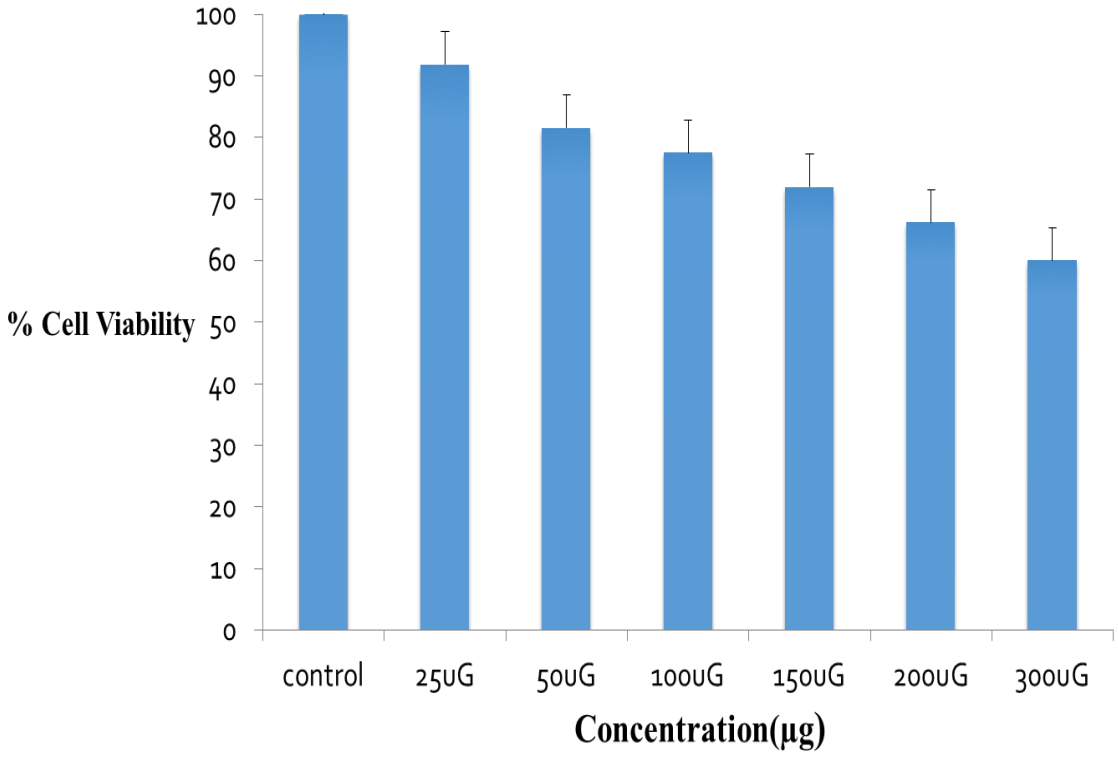

Figure: 4.2 MTT assay result

4.3 Effect of Zinc oxide nanoparticles on HEp-2 cell lines

Western blot analysis showed the effects of ZnONPs on HEp-2 laryngeal carcinoma cell lines. Actin protein is expressed in the same manner in all the groups. PARP and Caspase-3 are up-regulated in all the treatment groups whereas, Bcl-2 is downregulated in a descending order from 24 hours to 72 hours.

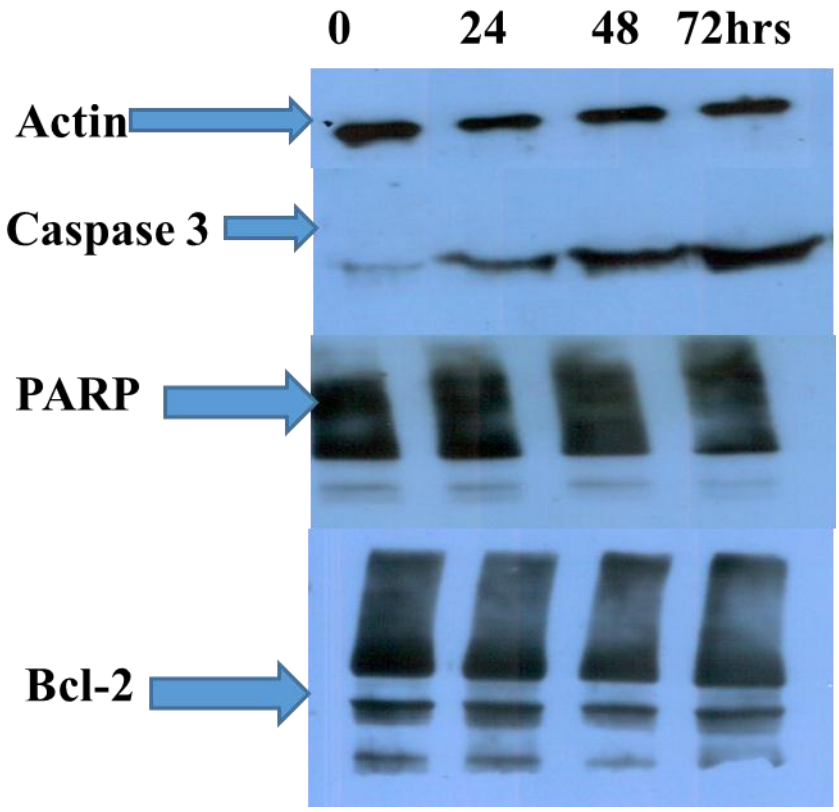

Figure 4.3 Western blot analysis of proteins

4.4 Anti-oxidant activity of Zinc oxide nanoparticles

The anti-oxidant activity of different concentrations of ZnONPs were checked through DPPH. Initially, the reaction mixture was violet in colour which gradually changed to yellow at the end of the reaction. This experiment was carried out in dark due to light sensitive nature of DPPH. The free radical scavenging activity of six different concentrations of ZnONPs were studied i.e 25, 50, 100, 150, 200 and 300µg/ml respectively. Ascorbic acid was used as a standard in the process. At 300 µg/ml, highest anti-oxidant activity was observed, with the least activity at 25 µg/ml.

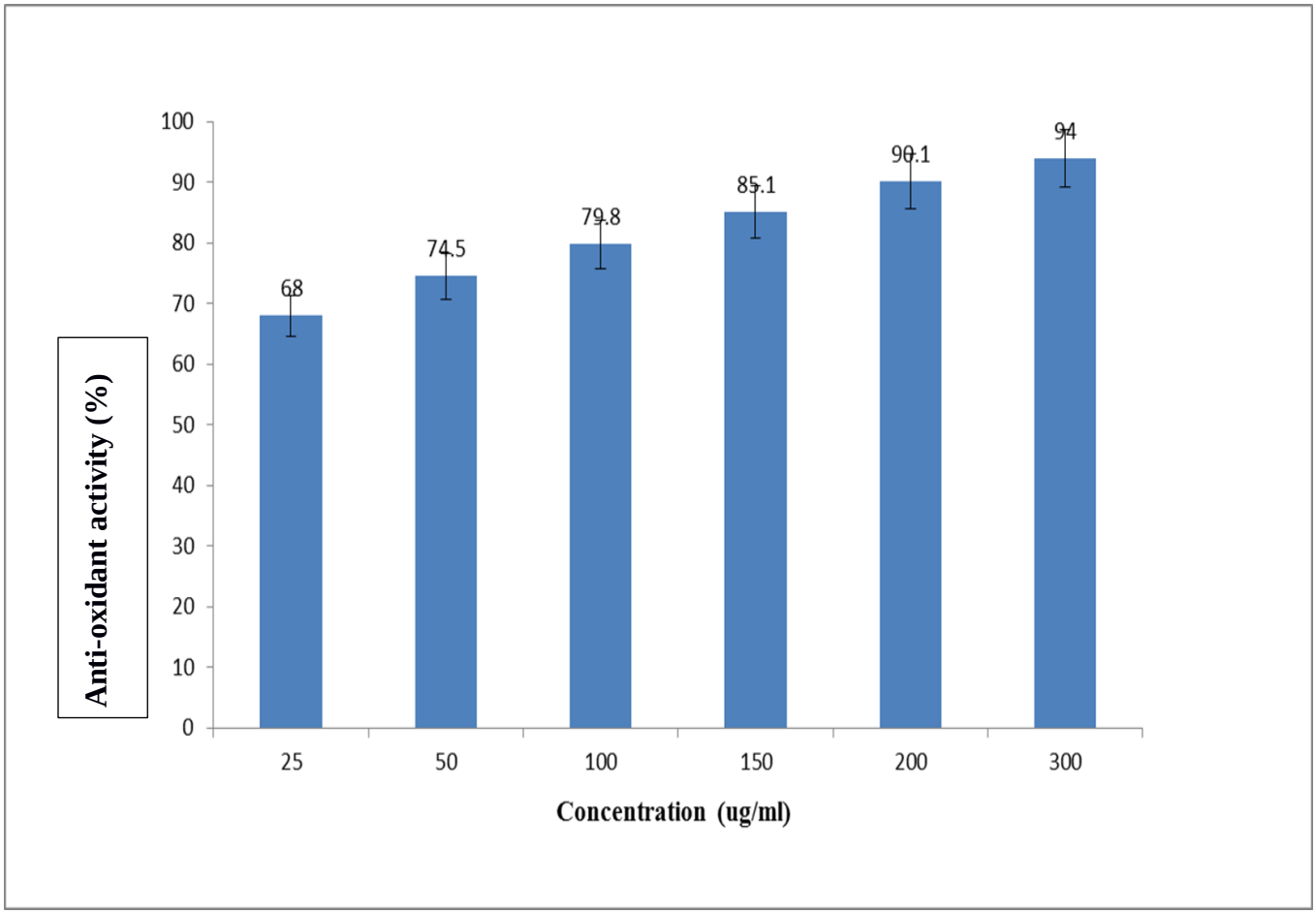

Figure: 4.4 Anti-oxidant activity and its variation with drug concentrations

5. DISCUSSION

5.1 Up regulation of PARP

Poly (ADP-ribose) polymerase Parp-1 is involved in base excision repair (BER), single- and double-strand break repair, and chromosomal stability. It is also involved in transcriptional regulation through protein-protein interaction, which explains why Parp deficiency enhances carcinogenesis. Since Parp-1 functions as both a caretaker and gate-keeper of the genome, insufficiency of Parp-1 may also enhance carcinogenesis in humans. Studies have shown that low formation of poly(ADP-ribose) induced by bleomycin treatment in peripheral lymphocytes from laryngeal cancer patients, suggest that low PARP activity is directly related with a higher risk of laryngeal cancer.(Rajaee- Behbahani et al., 2002)

This means that efficiency of Parp-1 is required for normal cell cycle regulation and cell survival. ZnONPs treatment caused HEp-2 laryngeal carcinoma cell lines to up regulate Parp, thus proving this drug efficient in cancer treatment.

5.2 Up regulation of Caspase-3

Apoptosis is a distinct genetic and biochemical pathway of cell death necessary for cell growth, development and maintenance of homeostasis in organisms. Caspase-9 was activated with the elevated level of cleaved caspase-9, which in turn cleaved caspase-3 and ultimately induced apoptosis.(Schultz & Harringto Jr, 2003; Tomita, 2010)

The results from western blot analysis showed that ZnONPs enhanced activation of cleaved caspase-3 and caspase-9, resulting in increased PARP cleavage in HEp-2 cells. Furthermore, Bax activation was confirmed to be increased for ZnONPs treatment in HEp-2 cells. These results suggested that ZnONPs treatment-triggered apoptosis was modulated through caspase activation.

The caspase-3 and PARP cleavage were highly improved, contributing to the death of HEp-2 cells and leading to cell apoptosis and cell death due to ZnONPs treatment. The results above suggested that ZnONPs suppressed human laryngeal cancer development and progression through apoptosis induction. It was also found that ZnONPs administration downregulated NF-κB phosphorylated activity, leading to the upregulation of caspase-9, caspase-3 and PARP cleavage, indicating that ZnONPs could inhibit human laryngeal cancer via AKT-mediated NF-κB signaling pathway.

5.2 Down regulation of Bcl-2

It is known that Bcl-2 protein is also a key regulator for apoptosis and its tumorigenic potential is supported by the finding of overexpression of Bcl-2 in various types of tumor, which is related to the activation of AKT(Sheppard et al., 2013). AKT is a key player in regulating cell signals that are important for cell death and survival. Activation of the AKT pathway promotes cell survival and is involved in the upregulation of Bcl-2(Kim, Moretti, Mitchell, Jung, & Lu, 2009; Levine, Sinha, & Kroemer, 2008)

In this study, we found that with the altered trend of AKT expressed levels, Bcl-2 was reduced in ZnONPs treatment, thus indicating that cell death occurs through ZnONPs treatment.

6. CONCLUSION

As a result of the experimentation carried out, it was concluded that zinc oxide nanoparticles have good therapeutic potential in case of laryngeal carcinoma, as these nanoparticles increased the expression of caspase-3 and PARP, thus treating cancer. It was also found that Bcl-2 expression decreased as a result of nanoparticles treatment. Collectively, our findings above demonstrated that zinc oxide nanoparticles prevented human laryngeal cancer proliferation, invasion and migration by PI3K/AKT and p38 suppression, resulting in caspase activation and also increasing PARP expression.The present study indicated that the use of ZnONPs might be a potential therapeutic strategy for human laryngeal carcinoma treatment. The results above suggested that ZnONPs suppressed human laryngeal cancer development and progression through apoptosis induction.

Following conclusions were made at the end of the experimentation.

- ZnONPs can be easily synthesized by chemical method.
- MTT assay results show that ZnONPs have good anticancer properties.
- ZnONPs are selectively cytotoxic towards rapidly proliferating cancer cells , hence have greater specificity.

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