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BIOINSPIRED NANOTHERANOSTIC AGENT: ZINC OXIDE; GREEN SYNTHESIS AND BIOMEDICAL POTENTIAL

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Extraordinary biocompatibility and low toxicity has proved ZnO as potential nanotheranostic agent**.** *Eucalyptus globulus* leaf extract facilitates green synthesis of zinc oxide nanoparticles under ambient conditions for estimation of antibacterial, antioxidant and cytotoxic activity on MCF-7 cell. Samples were characterized by Transmission electron microscopy, X-ray Diffraction and UV-Vis Spectrophotometrically. Grains shape was spherical with mean size of 12.63 nm. *Streptococcus pseudopneumoniae* gave best zone of inhibition with *Eucalyptus globulus* ZnO Nanoparticles. DPPH assay showed 73% radical scavenging at 250 g/ml. while cytotoxic effect was in between 29-95%. It was concluded that synthesis of ZnO nanoparticles via *Eucalyptus globulus* is simple, ecofriendly and capable for strong antioxidant, antibacterial, cytotoxic and antitumor effect.

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Keywords: Green synthesis, *Eucalyptus globulus*, Cytotoxic, Biocompatibility

1. Introduction

Nanotechnology is providing a wide area to determine the properties of material in a new way of ecofriendly and biosynthetic route. Zinc oxide nanoparticles (ZnO NPs) is more famous for its nontoxic, biocompatible and stable effects. It can serve as anticancer agents [1] drug carriers, antimicrobials [2], cosmetics [3], and biosensors [4] due to its unusual physic-chemical properties. Zinc is biocompatible that's why attributed as second most common trace element in human and plays various activities in human body. There are many predefined production methods of metallic oxide nanoparticles are mostly toxic, unfriendly chemicals and expensive [5]. Modification in metals oxide nanoparticles might be attained by switching particular atoms, which can boost their electrical, and optical behavior due to change in their surface properties chemically [6].

Recently ZnO NPs gained more attention because of their unusual nature of n-type semiconductor hexagonal phase and wurzite structure having 3.37 eV band gap [7]. ZnO has a wide applications especially in the field of solar cells, liquid crystal devices, piezoelectric and as semiconducting diodes [8, 9]. ZnO NPs synthesized through green method is cost-effective, feasible and provide good yield. The physical appearance is better as compare to titanium, silver, nickel and gold NPs [10]. Literature validated that ZnO NP has potential to serve as antibacterial agent. It disrupted cell membrane provide extended spectrum of beta lactamase of bacteria *Klebsiella pneumonia* and *Escherichia coli* [11]. It could hinder the growth of *E. coli* by disturbing cell membrane of bacteria which cause the increase in permeability of NPs and leading to gathered in cytoplasm and finally cell disruption [12].

It is well known that medicinal plants are rich in phenolic compounds, terpenoides and vitamins which have antioxidant potential [13]. Moreover it is reported that NPs synthesized by medicinal plants showed more antioxidant behavior *in vitro*. ZnO NPs also investigated for its cytotoxic activity against normal cells and cancerous cells especially for lungs and lens epithelial cells [14]. Most of the studies of ZnO NPs using different plants (*Vitex negundo* [15], *Costus pictus D. Don* [16], *Cassia auriculata* [17] and *Pongamia pinnata* [18]) have been claimed for cytotoxic activity.

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Traditionally *Eucalyptus* leaves contain phytochemicals like alkaloids, flavonoids, saponins, tannins and terpenoides that helpful in wound healing and curing fungal infections. Besides that they serve as food and now a days it also serve in cosmetics. Literature shows that plant extracts can serve as antibacterial and antioxidant agent [19] and granular from *E. perriniana* [20] and macro carpals from *E. macrocarpa* [21] efficient against (*Bacillus subtilis, Staphylococcus aureus).*

This work repots the cost-effective and simple green synthesis of ZnO NPs with *Eucalyptus* leaf extract as capping agent. Antioxidant, antibacterial and cytotoxic effect of *Eucalyptus globulus* ZnO NPs with leaf extract was determined which provide ecofriendly effect. The anti-bacterial effect of four selected pathogens (*S. pseudopneumonie, S. aureus, E. coli*, and *K. pneumoniae*) was under observation. The anti-oxidant property was observed *in vitro* by free radical scavenging assay. Except that investigation of cytotoxic activities against breast cancer lines MCF-7 was also under study.

2. Experimental

This study was completed in chemistry laboratory, Department of Chemistry, University of Management and Technology, Lahore. During experimental process analytical grade solvents, reagents and glassware were used.

2.1. Plant collection

Fresh *Eucalyptus globulus leaves* were collected from home garden. The plant was identified by Dr. Zaheer-u-din Khan (Department of Botany, GC University, Pakistan). The voucher specimen was deposited under number GC. Herb. Bot.3630. *Eucalyptus globulus* was shade dried for three weeks. These leaves were grinded in mixer grinder and then used for extraction.

2.2. Extraction of plant material

Plant material was extracted in methanol for 48 hrs. using Soxhlet extractor. After extraction it was concentrated in rotary evaporator and stored at 4°C in air tight bottle.

2.3. Synthesis of ZnO nanoparticles: (ZnO-nps)

0.05M Zn $(NO_3)_2.6H_2O$ solution was prepared in deionized water and incubated at 37^{o}C for 30 min. 2.0 M NaOH was added with constant rate to adjust the pH at 11. Reaction mixture was refluxed at 60^{o}C for 6hrs. White precipitates were washed with deionized water and ethanol for several times and then dried for 3hrs at 50°C.

2.4. Synthesis of *eucalyptus globulus* ZnO nanoparticles: (*e. Globulus* zno-nps)

Firstly, *E. globulus* leaf extract was refluxed for 10 min then 0.3 M zinc nitrate was added drop wise with constant stirring until pale white precipitates were obtained. Precipitates were centrifuged for 15 mins at 4500 rpm. After filtration, dried at 80 °C.

2.5. Anti-bacterial study

Antibacterial effect was evaluated on two gram negative bacteria *Escherichia coli* (ATTC-11303), *Klebsiella pneumonia* (ATCC- BAA-1705) and two gram positive bacterial species were selected *Staphylococcus aureus* (ATTC-6538), *Streptococus pseudopneumonie* (ATTC-BAA-960). 1mg/mL gentamycin was used as positive control and DMSO served as negative control. Agar diffusion method [22] was selected for the study of antibacterial activity having 25, 50, 75 and 100 μg mL^{-1} of ZnO NPs. Disc with diameter of 7mm was filled with different concentrations to determine antibacterial activity.

2.6. Antioxidant activity

2.6.1. DPPH free radical scavenging activity

The DPPH free radical scavenging activity was performed as defined by Lee *et al*. [19] Different concentrations (250, 125 and 60 µg/ml) of *E. globulus* leaf extract were taken in vials. Then 3ml methanolic solution of DPPH (0.01g/ 250ml methanol) was mixed with sample solution and kept at room temperature for 1h. Absorption capacity was calculated by spectrophotometer at 517 nm. Lesser the absorption capacity of photometer indicates high level of free radical controlling capacity. Percentage of DPPH scavenging of representative is estimated by following formula:

$$Percentage(\%) of\ DPPH\ scavenging\ inhibition = \frac{A_{Blank} - A_{Sample}}{A_{blank}} \times 100 \quad (1)$$

A= Absorbance

2.7. Anticancer activity

ZnO NPs and *E. globulus* ZnO NPs were tested for MCF-7 (breast cancer cells) by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) colorimetric assay. Firstly, ZnO NPs were weighed and further diluted to 1, 5, 10, 15, 30, 60 and 120 µg/mL in cell culture media. Prepared dilutions of ZnO NPs were mixed with culture and left for 24h in incubator at room temperature. Cells without nanoparticles served as control. After incubation cells were washed with phosphate buffer solution. Well was filled with 100 µL of Tetrazaolium MTT solution and further incubated for 2-3 h at room temperature for the reduction NADH to NADPH (MTT dye) by living normal cells than 100 µL of DMSO was added. The effect of cytotoxicity on MCF-7 cells was estimated at 595 nm by measuring its optical density. Percentage of cell viability was estimated by comparing sample and control with the help of following formula:

$$\%\text{Cell viability} = \frac{(A_{treatment} - A_{blank})}{(A_{control} - A_{blank})} \times 100 \quad (2)$$

A= Absorbance

2.8. Characterization

ZnO NPs and *E. globulus* ZnO NPs were characterized from Ibn-e-Sina institute of Science and Technology, Islamabad. X-ray diffraction technique (XRD, PanalyticalX' PertPro) was used to determine crystallinity of prepared samples using Cu Kα radiation (k = 1.54056 Å) at 40 kV and 30 mA. TEM analysis was performed to analyze the grain size of synthesized sample. UV-Vis spectroscopy (UV–Vis, JESCO, 200–800 nm) was used to analyze the absorbance to determine its antioxidant and anticancer activity.

3. Results and discussion

3.1. XRD analysis

XRD analysis of *Eucalyptus globulus* ZnO NPs is shown in Fig 1. XRD profile of ZnO NPs demonstrated nine pronounced diffraction peaks in plane (100), (002), (101), (102), (110), (103) ,(200), (112), (201) respectively at the angle of 32^o, 35^o, 37^o, 47^o, 57^o, 63^o , 66^o, 67.5^o, 70^o. Obtained results indicating that synthesized ZnO NPs has polycrystalline wurtzite hexagonal shape without any effect on phase of ZnO NPs crystallinity and in agreement to (Zincite, JCPDS 5-0664). There is no distinughshing peak of impurity which is suggesting that synthezied *Eucalyptus globulus* ZnO NPs were highly pure. Scherrer's formula was used to estimate mean crystallite size of ZnO NPs [23, 24]. Modified ZnO NPs showed broading of peak in XRD pattern, which shows relativly small size of particles. However, sharp peaks showed that Crystallinity of NPs is good.

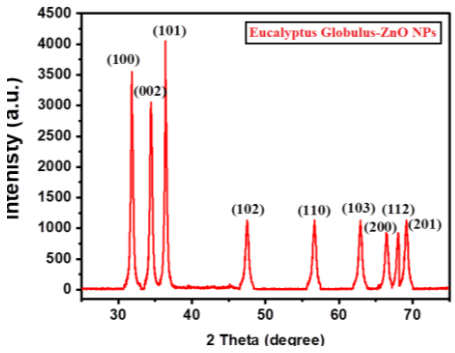
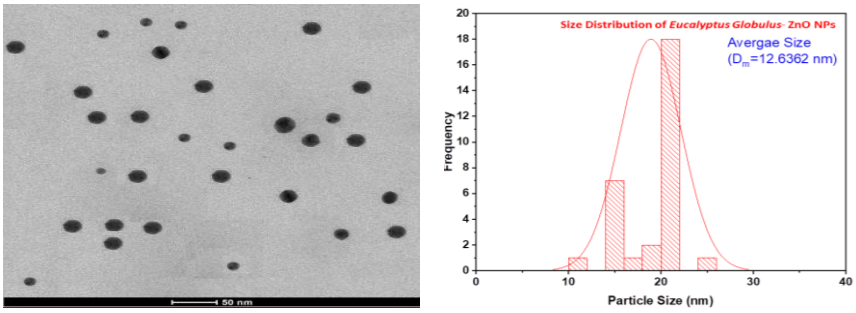


Fig. 1 XRD pattern of Zinc oxide nanoparticles.

3.2. TEM analysis

The transmission Electron Microscope used to verify diameter, morphology and dispersion of modified ZnO NPs. Transmission electron microscopy (TEM) results was analyzed at 120 kV by the LEO system (model 912 AB). TEM image of modified ZnO NPs was shown in Fig. 2a. Results shows that NPs are uniformly distributed with no agglomeration while the morphology of ZnO NPs is spherical. The size is in between 10-30 nm and average size of nanoparticles were 12.64 nm as shown in Fig. 2b.



(a) *(b)*

Fig. 2. (a) TEM image of modified Eucalyptus globulus- ZnO NPs (b) Size distribution of modified Eucalyptus globulus- ZnO NPs.

3.3. Anti-bacterial activity of *Eucalyptus globulus* ZnO nps:

Anti-bacterial activities of ZnO NPs and *Eucalyptus globulus* -ZnO NPs were estimated by disc diffusion assay and shown in Fig. 3. Gentamycin is used as control. The order of reactivity is found to be *E. coli* < *K. Leb* < *S. Aureous* < *S. pseudo.* Gram negative bacteria show highest antibacterial activity at all tested concentrations as compare to gram positive bacteria, as they have high inhibitory zone of inhibition. As the concentration of ZnO NPs increases, zone of inhibition also increases i.e. high antibacterial activity. This activity is high in gram negative bacteria due to the presence plant bio actives as compare to the ZnO NPs synthesized by chemical method as shown in Fig 3.

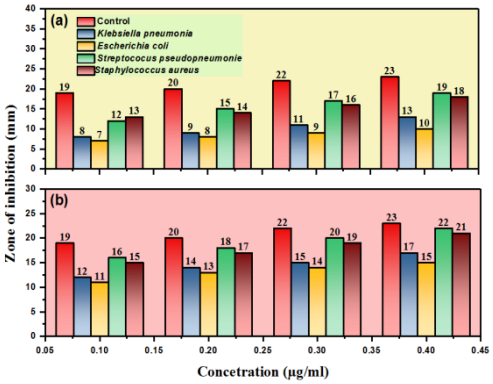


Fig. 3 (a) Antibacterial activity of ZnO NPs (b) Antibacterial activity of Eucalyptus globulus- ZnO NPs.

Inhibitory effects of *Eucalyptus globulus*-ZnO NPs of 0.4 µg/ml against *S. aureus* and *S. pseudopneumonie* have less significant change as compared with gentamycin and provide significant changes in the result of *E. coli*, and *K. pneumonia* (Fig 3). Even lower concentrations of *Eucalyptus globulus*-ZnO NPs gives sensible inhibition against all tested bacteria. The *Eucalyptus globulus* leaf extract indicated moderate to mild activity while ZnO NPs exposed mild activity as compare to *Eucalyptus globulus*-ZnO NPs. Results illustrate that although both sample have significant antibacterial effect but *Eucalyptus globulus*-ZnO NPs have more potential which is provide extra strength to Zn ion of ZnO NPs and gram negative bacteria showed less potential which could be due to presence of thick layer of peptidoglycan in cell wall of this group. Similar study was done by Sinha *et al.* they studied mesophilic and halophilic potential of ZnO NPs on selected bacterial species and conclude that *Entero* bacteria is extra sensitive to prepared NPs than *B. subtilis.* Reason of good resistance of gram positive bacteria is the presence of thick layer of peptidoglycan in cell wall [25]. Elham Zare *et al* also studied antibacterial effect of Cumin seed with ZnO NPs and found potential of antibacterial effect on gram-negative bacteria and summarized that presence thick layer of peptidoglycan in cell wall is responsible for such behavior that is quite similar to present study. It is already conveyed that ionic form of zinc can bind to cell membrane of bacteria and produce reactive oxygen species in cell which results in disorder to the cell [26].

3.4. Antioxidant activity of *eucalyptus globulus*-ZnO nps and ZnO nps:

Parashant et al exposed that presence of phenolic compounds in plants are responsible to give the potential of antioxidant activity and provide such a great importance to green synthesis of NPs [27]. Pharmaceutical science and nanoscince provide a wide plate form to investigate antioxidant properties of green synthesized NPs. A comparison of antioxidant activity of bulk and nanosized material was studied by Banerjee et al. [28] and concluded that antibacterial and antioxidant activities were enhanced in green synthesized ZnO NPs because most of the chemical compounds were cover the surface of NPs [29]. It is also investigated that β-Sitosterol and cuminic aldehyde are the main phytochemicals of plant extracts which subjects the antioxidant activity [30], while ethyl palmitate and esters are responsible for anti-inflammatory effect [31]. Edris et al. reported that *E. globulus* has two major polyphenols (thymol and 1, 8 cineole) are responsible for durable antioxidant activity [32]. Dasa et al. also compared the nanosized and bulk ZnO materials and concluded that higher free radical scavenging is due to high surface to volume ratio [33].

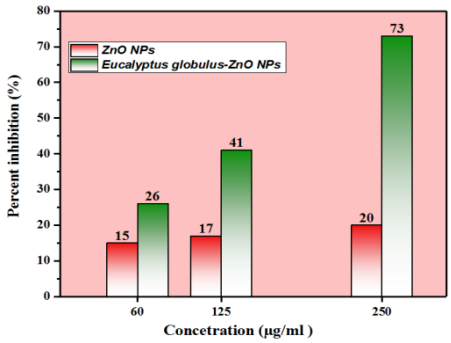


Fig. 4. Comparison of Free radical scavenging activity of the ZnO- NPs and Eucalyptus globulus-ZnO NPs.

Antioxidant activity of ZnO-NPs and *Eucalyptus globulus*-ZnO NPs samples with various concentrations are shown in Fig. 4. All the sample were run in triplicate to obtained concordant results. In this study different concentration (60, 125, 250 µg/ml) of ZnO-NPS and *E. globulus* ZnO NPs were prepared to investigate the antioxidant activity. Change in color from blue to yellow was observed by UV-Vis spectrophotometer at 517 nm. BHT serve as standard. Results indicated that ZnO NPs provide 20% scavenging at with 250 µg/ml, on the other hand *Eucalyptus globulus*-ZnO NPs provide 73% scavenging with the same concentration and enhanced the radical scavenging property of ZnO NPs. Moreover, it was concluded that all tested concentrations active and radical scavenging activity increases with increasing concentration This effect might be because of reduced grain size (12.63 nm) of ZnO NPs and may also be because of transfer of electron density to odd electron of nitrogen atom present in DPPH from oxygen atom which decrease the transition intensity due to transition of n π^* [34].

Mechanism of DPPH scavenging is shown in Fig 5. When different concentration was added to DPPH solution, it turn form unstable blue color to stable yellow color due to donation of electron from oxygen atom to odd electron of nitrogen and as a result a stable complex of DPPH molecule produced [35, 36]. In short, antioxidant activity is directly proportional to hydrogen donation capacity and due to good ratio of electrons and holes pair formation of ZnO NPs it provides a good redox potential which helps in splitting of water into its radicals [37].

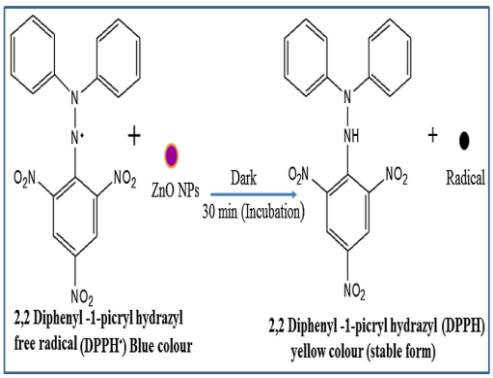


Fig. 5. Mechanistic representation of interaction between DPPH free radicle and ZnO NPs forming stable DPPH molecule.

3.5. Anticancer activity of *eucalyptus globulus* ZnO nps:

Cytotoxicity investigated by MTT assay and shown in Fig 6. *Eucalyptus globulus* ZnO NPs was studied for (MCF-7) breast cancer cell line estimation of anticancer activity. Results were analyzed after 24 hrs. at different concentration (1, 5, 10, 15, 30, 60, 120 µg/mL) taking cell line as control and check its cytotoxicity on the bases of percent viability. Cell viability

corresponding to rates of survive cell were 95, 92, 77, 60, 50, 35 and 29% respectively. Although all the concentration are active against MCF-7 cells when compared with control ($P \leq 0.05$, $n \geq 3$, Fig 6) but 120 µg/mL ZnO NPs showed highest anticancer activity as compare to other selected doses this effect is due to high concentration of NPs. The results validated that dealing with ZnO NPs protected the cell growth significantly ($P \leq 0.05$, $n \geq 3$, Fig. 6) and concentration of modified ZnO NPs is directly proportional to the anticancer activity as increases the concentration, cell death rate of cancerous cells also increased.

ZnO NPs have specific properties like smooth synthesis, biocompatibility and cytotoxicity which provide it strength to use as anticancer agent [38]. One of the closer mechanism of cytotoxicity effect of ZnO NPs is the ability to evoke oxidative stress in cancer cell. This effect is due to semiconductor nature of ZnO, uptake of electricity took that path where unzipped electrons are present in valance band [39].

Conversely, in the case of nano scale ZnO NPs, when there is no UV irradiation, additional electrons jumps to the conduction band [40]. Recombination of electrons and holes is take place and they only respond to adsorbed species without reacting to the surface of NPs [41]. As a result a variety of electrons and holes pair raised up on the surface of NPs convinces to reactive oxygen species generation and this will leading to oxidative stress and finally cellular death [38].

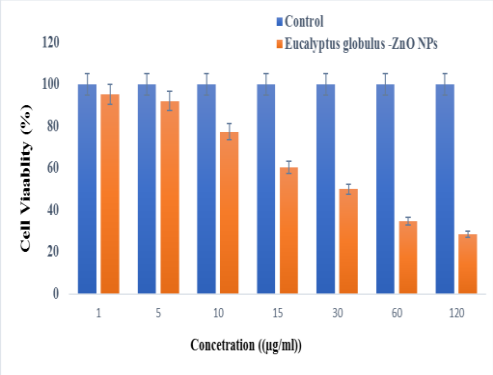


Fig. 6. Cytotoxicity effect of using MTT assay of Eucalyptus globulus ZnO NAPs against MCF-7.

4. Conclusion

Green synthesis of ZnO NPs with the help of plants extract is taking more attraction in the field of nanotechnology due to its stability and biocompatibility. It has a wide applications especially in the field of biosensors, biomedical and cell imaging. *Eucalyptus globulus* leaf extract facilitated the green synthesis of ZnO NPs under ambient conditions. X-ray Diffraction (XRD) study provide intense, narrow width and strong peaks which confirms purity and crystallinity of bioactive compounds which help in stabilization of ZnO Nanoparticles. Transmission electron microscopy (TEM) results illustrate that grains are regular spherical in shape with mean grain size of 12.63 nm.

Moreover, antibacterial activity of *Eucalyptus globulus* ZnO nanoparticles were demonstrated on selected bacterial strains (*Klebsiella pneumonia, Escherichia coli, Staphylococcus aureus, Streptococus pseudopneumonie*) which gives remarkable zone of inhibition. *S. pseudopneumonie* gives best zone of inhibition with *Eucalyptus globulus* ZnO Nanoparticles i.e. 22 mm at 0.4 µg/ml concentration. DPPH assay shows 73% radical scavenging at 250 g/ml. Percentage of cell viability of zinc oxide nanoparticles by green method ranges from 29-95 %. The same is the case with anti-cancer activity of ZnO NPs. 120 µg/ml concentration of ZnO NPs gives high percentage of cell viability (95%). Cell survival rate is high at low concentration and only 29% (1µg/ml) of cell was viability. It is concluded that green synthesizes of ZnO NPs via *Eucalyptus globulus* is simple, ecofriendly, stable and can serve as strong antioxidant, antibacterial and cytotoxic agent.

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