CHAPTER 5
DISCUSSION

Ever rising infectious diseases and drug resistance and their diffusion are presently of great fear. This situation has led to treatment failure in many cases. It is believed that present trend can claim 10 million lives per year by 2050 due to antibiotic failure (O’Neill 2014). Thus, the hunt for new antimicrobials to replace or change the existing one has become a requisite. To dispense with the current problematic situation nanoparticles is the most bright and novel therapeutics. Nanoparticles have unique properties compared to their bulk counterparts. From centuries metals such as silver, copper, zinc etc have been utilized for treating burns, chronic injuries, making water drinkable etc. It is perceptible that some of the metallic compounds possess antimicrobial property (Rai and Bai, 2011). Metal oxide NPs like ZnO and CuO have been used industrially for several purposes like textiles, cosmetics etc. Recently, the merger of nanotechnology and biology has brought to manipulate the metals in the form of nanoparticles that could have a potential antimicrobial affects. Inorganic NPs have several benefits over conventional chemical antimicrobial agents. Presently multi drug resistance is of serious concern. But by using NPs this can be tackled. Since NPs attack multiple targets it will become difficult for microbes to develop resistance because in order to save them they have to undergo several series of mutation, which is quite not possible in the near future (Pal et al., 2007). Nanoparticles are able to kill microorganisms in ways that do not depend on metabolism-related mechanisms.

Naturally zinc oxide is known to have good inhibitory activity against wide range of microbial strains (Soderberg et al., 1990). For decades it has served as antimicrobial agent in various fields because of their antimicrobial potential. During the synthesis of nanoparticles with decrease in particle size, specific surface area is increased facilitating better material interaction with the surface in contact. Thus, for materials like zinc, copper, silver which innately possess antimicrobial potential, increase in surface to volume ratio boost the antimicrobial effect (Yamamoto, 2001; Dodd et al., 2006; Zhang et al., 2007; Nair et al., 2009). ZnO NPs are reported to have better antibacterial effect than micron scale ZnO (Jiang et al., 2009). Further several studies from the past revealed better antibacterial activity of nanoparticles than their large counterparts with similar chemistry (Jones et al. 2008; Padmavathy and Vijayaraghavan 2008).

In the present study, antimicrobial feature of undoped and doped ZnO NPs was exploited along with the studies involving combination therapy, ultra structural studies and
toxicity. This chapter compares the results obtained in the present study with other reports in the literature.

To begin with undoped and doped ZnO NPs (Fe, Mn, Co and Cu) were synthesized by wet chemical co-precipitation technique by using standard laboratory equipments and standard reagents that can be procured very easily. Zinc Chloride (ZnCl$_2$) and sodium hydroxide (NaOH) were used as precursors and polyvinyl pyrrolidone (PVP) was added as stabilizing agent. PVP solution stops the growth of nanocrystals, stabilizes them and prevents their aggregation. Similar method of nanoparticle synthesis were employed by Sartiman et al. (2013) and Kumar et al. (2015) for chromium (Cr) doped ZnO NPs and by Reddy et al. (2013) for silver (Ag) and cobalt (Co) doped ZnO NPs. Simplicity, low cost involved and efficient stabilization of nanoparticles growth favors the use of this method for the large-scale production (Mukhtar et al., 2012; Sharma et al., 2015).

Synthesized nanoparticles were further characterized by X-ray diffraction, Transmission Electron Microscope (TEM), Fourier transform infrared spectrometer (FTIR), Photocatalytic measurements. The X-Ray powder diffraction pattern of the synthesized ZnO nanoparticles was recorded on an X-Ray diffractometer using Cu (ka) radiation ($\lambda = 1.54184$ Å). Presence of broad peaks supports the formation of nanoparticles. Joint Committee on Powder Diffraction Standards (JCPDS) Card no. 36-145 depicted hexagonal wurtzite structures of the synthesised nanoparticles by interpreting the XRD peaks (Wong-Ng et al., 2001). This is in conformity with the studies of Talari et al. (2012) and Khoshhesab et al. (2011) who depicted wurtzite structure of the synthesised NPs. Hexagonal wurtzite structure have tetrahedral Zn atom linked to four oxygen atoms and vice versa (George et al., 2009). Further XRD data reveals thermodynamic stability of nanoparticles in an ambient environment similar to Moezzi et al., 2012. XRD patterns obtained are identical for all the samples without any impurity phase peaks which suggest that Mn$^{2+}$, Fe$^{2+}$, Cu$^{2+}$ and Co$^{2+}$ ions replaces the Zn$^{2+}$ ions without disturbing the crystal structure. This is in accordance with the study conducted by Karumakaramoorthy and Suresh, 2014 and Boxi and Paria, 2014. The diameter of synthesized undoped and doped ZnO nanoparticle was calculated using Debye-Scherrer formula (Cullity, 1967)

$$d = \frac{0.89 \lambda}{\beta \cos \theta}$$
where 0.89 is Scherrer’s constant, $\lambda$ is the wavelength of X-rays, $\theta$ is the Bragg diffraction angle, and $\beta$ is the full width at half-maximum (FWHM) of the diffraction peak. The estimated crystalline sizes at most intense crystallographic plane are found to be 14-26 nm for all the synthesized samples.

Images of the TEM study indicate spherical particles with average particle size of 14-26 nm which is similar to that of calculated by Scherer formula on the XRD pattern. TEM images support the formation of ZnO NPs. Similar findings have been reported by the researches of Dutta and Ganguly, 2012; Thirumavalavan et al., 2013 and Arora et al., 2014.

FTIR spectra in KBr matrix gives information about the various functional groups and chemical bonding present in undoped and doped ZnO NPs. The FTIR spectra were recorded in the range of 400–4000 cm$^{-1}$. Peaks found in FTIR supported the presence of various functional groups with characteristic peak indicating the presence of polyvinyl pyrrolidone (PVP). Similar results were observed by other researchers regarding FTIR studies (Hernandez et al., 2007; Ravichandrika et al., 2012; Rani and Kumar, 2013). According to Labhane et al. (2015) the position and number of absorption bands is determined by crystal structure, chemical composition and also by crystal morphology. According to FTIR results obtained, slight shift in the spectra with doping was seen. Substitution of $\text{Cu}^{2+}$, $\text{Mn}^{2+}$, $\text{Co}^{2+}$ and $\text{Fe}^{2+}$ ion at the ZnO lattice results in the shift of the band toward lower frequencies by changing the bond length. Similar findings have been reported by Murtaza et al. (2014).

Photocatalytic study revealed that doped nanoparticles showed better photocatalytic activity with best being shown by 10% Cu doped ZnO NPs. This is in accordance with the studies of Gupta et al., (2013) and Box and Parai (2014) where doping with silver (Ag) increased the degradation of dye and thus enhanced the photocatalytic activity of TiO$_2$, CdS and ZnS NPs respectively. This may be due to the trapping of photo-excited electrons by the dopant in ZnO NPs that extends the lifetime of charge carriers resulting in improved photocatalytic activity of ZnO NPs. Karumakaramoorthy and Suresh (2014) reported that the replacement of transition metal in Zn sites enhances the photocatalytic activity. Therefore, in our work excellent substitution of $\text{Cu}^{2+}$ with $\text{Zn}^{2+}$ in ZnO nanoparticles is responsible for the enhanced photocatalytic. It is highly possible because ionic radii of $\text{Cu}^{2+}$ (87 pm) is almost similar to that of $\text{Zn}^{2+}$ (88 pm) compared to ionic radii of other dopants used. Additionally, Zhang et al. (2011) stated that lowering the band width, resulted in formation of dopant energy levels below the conduction band which enhances the photocatalytic activity when doping with transition metal is done. Study conducted by Wu et al., (2011) under both UV
Chapter 5

Discussion

and visible-light reported enhanced photocatalytic activity of 3% Antimony (Sb)-doped ZnO nanowires than undoped ZnO nanowires. Similarly, Zhang et al., 2012 reported enhanced photocatalytic activity of Fe doped ZnO nanowires compared with pure ZnO nanowires under different light irradiation as well as different contaminants. Further photodegradation of methylene blue (MB) dye follows pseudo-first order kinetics which is in accordance with the results of Kumar et al. (2015). According to the report by a research group (Ahmad et al., 2013), accelerated transfer of photo generated electrons by the loading of metals on the ZnO NPs make surfaces negatively charged. Also oxygen reduction by transfer of trapped electrons from metal to oxygen generates superoxide anion radicals. Subsequently, generation of radicals and suppression of recombination of the photo-generated carriers enhanced the photodegradation of organic pollutant (methylene blue) by doped ZnO NPs.

The relative antimicrobial activity of undoped and doped ZnO NPs was studied qualitatively by disk diffusion and quantitatively by MIC, MBC and MFC. Several other authors have also suggested similar methodology to study antimicrobial activity (Shin and Lim, 2004; Nirmala and Pandian, 2007; Bansod and Rai, 2008; Chung et al., 2011). Antibacterial activity was further assessed by colony forming unit assay similar to the assay performed by Ashe, 2011.

With an increasing number of bacteria developing resistance to commercial antibiotics, nanoparticles hold great promise for novel antimicrobial agents in modern times. Therefore, antimicrobial activity of all nanoparticles synthesized in the present case was studied against six human pathogenic bacteria i.e., Escherichia coli, Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa, Bacillus subtillis, and Klebsiella pneumonia and two fungal species i.e., Trichophyton mentgerophytes and Cryptococcus neoformans. Antimicrobial activity of ZnO NPs against wide range of microorganisms (S. aureus, E. coli, S. typhi, L. monocytogenes and fungi Fusarium) has been reported by Sharma et al. (2010) and Brayner et al. (2006). Mechanism of action of antimicrobial activity is due to the electrostatic attraction of negatively charged cell membrane and positively charged nanoparticles (Hamouda et al., 2001; Dibrov et al., 2002; Dragievaet al., 1999) and release of reactive oxygen species as well as free metal ions including zinc (Amro et al., 2000). Nanoparticles possess positive charge. Microbial cell wall is negatively charged. Gram positive bacteria are negatively charged due to presence of teichoic acids attached to peptidoglycan, Gram negative bacteria are negatively charged due to lipopolysaccharides and chitin makes fungal cell wall negatively charged (Brogden, 2005). Many studies strongly
indicated that ZnO nanoparticles whether doped or undoped have concentration-dependent antimicrobial activity (Zhang et al., 2007; Peng et al., 2011). In the present studies, we used five different concentrations of NPs namely 100mg/ml, 50mg/ml, 25mg/ml, 10mg/ml and 1mg/ml. High concentration contributes to large zone of inhibition. With decrease in concentration zone size also decreased. This is in conformity with Rizwan et al. (2010) who depicted that enhanced growth inhibition by increased concentration is due to proper diffusion of nanoparticles in agar medium. In the present study, appreciable antimicrobial effect was exhibited by 10% Cu doped ZnO NPs where as undoped ZnO NPs were least effective. The present observation is in agreement with the findings of Poongodi et al. (2014), Nair et al. (2011) and Bhuyan et al. (2015). They too demonstrated increased antimicrobial activity of doped ZnO NPs as compared to undoped ZnO NPs. Enhanced antimicrobial activity of Mn, Fe, Co doped ZnO NPs against gram positive and gram negative bacteria is also reported by Rekha et al. (2010). Quantitative bacterial reduction was studied by colony forming unit (CFU) assay and evaluated by percentage reduction. Present study reports that CFU has reduced significantly with increasing doping percentage of ZnO nanoparticle which is supported by the percentage reduction value of bacteria obtained. For undoped the percentage reduction was in the range 31.2%-76.7%, for 1% doped ZnO NPs range was 61.2%-78.7% and similarly for 10% doped ZnO NPs range obtained was 77.6%-89.8%. The results of this percentage reduction test correspond with that of the other antibacterial tests performed in the present study exhibiting better activity by 10% doped ZnO NPs. Our results of percentage reduction are in conformity with the results of Parthasarathi and Thilagavath (2011) and also with the findings of Rajendran et al. (2010). Both reported reduction in the colonies of E. coli and S. aureus when treated with ZnO NPs as compared to untreated samples. Thus the present observation indicated that the growth rate of bacteria is very much affected by the undoped and doped ZnO nanoparticles. The growth rate of the bacteria is affected due to the interaction of the nanoparticles in the cells. Doped nanoparticles have larger surface area available for interactions which enhances the bactericidal effect than the large sized particles and hence they impart cytotoxicity to the micro organisms (Baker et al., 2005; Nagarajan and Rajagopalan, 2008). Our results are in accordance with the findings of Venkatasubramanian and Sundaraj, 2014. They reported enhanced antibacterial effect of silver (Ag) doped ZnO NPs.

Minimum inhibitory concentration (MIC) assay was done using 96 well microtitre plate and resazurin as indicator. Resazurin is blue coloured oxidation reduction indicator used
for the evaluation of cell viability. It is reduced to pink/red resorufin by enzyme oxidoreductase present in the viable cells. Resorufin is sometimes further reduced to colourless hydroresorufin (McNicholl et al., 2007). MIC was taken as lowest concentration that prevented colour change. Minimum bactericidal/fungicidal concentration (MBC/MFC) was determined by plating an aliquot on the media plates. MBC is determined as the lowest concentration that showed no bacterial growth in the fresh medium. MIC obtained in present study was in the range of 0.004 mg/ml-0.43 mg/ml. Many bacteriological tests have shown that ZnO suspensions in the lower concentration range (0.01–1 mM, i.e., 0.8–80 μg/ml) exhibit less antimicrobial activity (Padmavathy and Vijayaraghavan, 2008). MIC results of the present studies also confirm this point. Fewer Zn$^{2+}$ ions might act as nutrient supplement promoting the metabolic action of bacteria at trace concentrations (Zhu et al., 2016). Our results were better than reported by Lakshmi et al. (2012) who reported the MIC in the range of 3mg/ml-5 mg/ml. Better results obtained in the present case might be due to particle size. Particle size in our study was approximately 25nm whereas Lakshmi et al. (2012) reported particle size of approximately 100nm. Generally with decrease in particle size antibacterial activity of ZnO NPs is increased (Zhang et al., 2007; Jones et al., 2008). Many studies have shown that the antibacterial activity of ZnO NPs is particle size dependent (Yamamoto, 2001; Padmavathy and Vijayaraghavan, 2008; Raghupati et al., 2011). Similarly our results were also better than the findings of Emami-Karvani and Chehrazi (2011) who obtained MIC for E. coli and S. aureus at 3.1 and 1.5 mg/ml, respectively. Likewise, Reddy et al., (2007) reported MIC at 3.4 mg/ml for E. coli and 1 mg/ml for S. aureus.Minimum bactericidal concentration was determined by the standard plating method. MBC for undoped ZnO nanoparticles was found to be 0.56–0.63 mg/ml while for 1% to 10% doped ZnO NPs, values of MBC lie between 0.13 and 0.41 mg/ml. Similar (enhanced activity) results were observed in other bacteria as shown in table 4.9. Our results are more or less similar to the results obtained by Oves et al., (2015) who also reported improved MBC values of 5% Co doped ZnO NPs followed by lower (1%) doped ZnO NPs and lastly undoped ZnO NPs.

Undoped and doped ZnO NPs synthesised with an average size falling between 14-26 nm were evaluated for antifungal activity against Trichophyton mentegrophytes and Cryptococcus neoformans. In comparison to the antibacterial studies reported for undoped and doped ZnO NPs, only few studies pertaining to antifungal efficacies of undoped and doped ZnO NPs have been documented in the literature. To our best knowledge this is the first report of antifungal activity of doped ZnO NPs against Cryptococcus neoformans.
Admirable antifungal activity against *C. neoformans* has been obtained in our studies. Our results are in agreement with the findings of Navale *et al.* (2015), He *et al.* (2011) and Singh and Nanda, (2013) who reported good antifungal activity of ZnO NPs against species of *Aspergillus, Penicillium* and *Candida*. In the present study antifungal activity was observed only against *C. neoformans* and no activity was reported in case of *T. mentagrophytes*. This is in contrast to the studies conducted by Kim *et al.* (2008) who stated that Ag NPs have fungicidal effect on *T. mentagrophytes*. Such differences could be due to the nature of the particles used, the difference in size being particularly important. It is known that size and shape of metallic nanoparticles influence their chemical, optical, thermal and antimicrobial properties (El-Sayed, 2001). Further, the different antifungal effects may result from different cell wall compositions of these two fungi. *T. mentagrophytes* have high percentage of chitin as well as complex cell wall structure. Presence of chitin layer prevents any destruction caused by OH produced by NPs (Gajbhiye *et al.*, 2009). Another possible reason for the difference could be innate tolerance of each fungus to ZnO NPs (He *et al.*, 2011). In case of *C. neoformans* also 10% Cu doped ZnO NPs showed most effective results. This was in accordance with the study conducted by Sharma *et al.* (2015) who demonstrated enhanced antifungal activity of Fe and Mn doped ZnO NPs against *C. neoformans* as compared to undoped ZnO NPs. These findings support the fact that smaller the size of nanoparticles better is their activity (Yamamoto 2001a, Makhluf *et al.*, 2005). In our study MIC value obtained against *C. neoformans* was in the range 5.1-26.6 mg/ml which was very much higher than obtained by Ishida *et al.* (2014) (0.42-0.84 µg/ml). Type, shape and size of nanoparticle used, characteristic species tested and stabilizing agent may influence the antimicrobial activity of NPs. In our study undoped and doped ZnO NPs were functionalized with PVP (stabilizing agent), which is considered biocompatible and did not exert inhibition in *C. neoformans* (Buhler, 2005). Minimum fungicidal concentration (MFC) values obtained were larger than the MIC values as also depicted by the study of Gunalan *et al.* (2012). MFC of the doped ZnO NPs in the current study was found to be in the range of 20.1 mg/ml-41.7 mg/ml. However, MFC of magnesium (Mg) doped ZnO NPs was found to be 2000 µg/ml for *C. albicans* (Hameed *et al.*, 2015). The reason for the difference in the antifungal activity for different test microorganisms may be due to the difference in structure and thickness of the cell wall membrane (El-Diasty *et al.*, 2013).

According to the results of the present study, it can be concluded that undoped and doped ZnO nanoparticles are effective antibacterial agents on both Gram-positive and Gram-
negative bacteria. The same results were confirmed in the study of Zhongbing et al. (2008) in which Gram-negative membrane and Gram positive membrane disorganization was approved by transmission electron microscopy of bacteria ultrathin sections. Further from our studies it is evident that Gram negative bacteria were more susceptible towards undoped and doped ZnO NPs. Excellent activity of ZnO NPs against gram negative bacteria has been depicted in several studies from the past also (Kawahara et al., 2000; Li et al., 2009). More inhibitory activity against Gram negative bacteria can be due to the difference in the structure of cell wall. Bacteria have different membrane structures. Gram-negative bacteria have thin layer of peptidoglycan, whereas Gram positive have a thick layer of peptidoglycan (Sondi and Salopek-Sondi, 2004). Although there is the outer membrane of lipopolysaccharides in case of Gram negative, but it lacks strength and rigidity whereas, compact layer of peptidoglycan on Gram positive is thick and rigid preventing the penetration of nanoparticles. The rigidity and cross-linking not only provide the cell walls with less anchoring sites for the ZnO NPs but also make its penetration difficult (Le Brun et al., 2013). Further Applerot et al. (2009) also showed higher susceptibility of E. coli to ZnO NPs than S. aureus. However, according to explanation of Applerot and co-workers, the reason may be differences in the intracellular antioxidant content such as carotenoid pigments, as well as the presence of detoxification agents such as antioxidant enzymes within the bacteria.

Antimicrobials which were once developed to inhibit microbial growth are presently themselves under the attack of microorganisms. Resistance to antimicrobial drugs is ever increasing problem which has reduced their effect over the time (Hajipour et al., 2012). Apart from resistance their diffusion also causes health troubles, leading to therapeutic failure (Braga et al., 2005; Schito, 2006). Drug resistance has given rise to many other problems also like antimicrobial toxicity due to high dose of antibiotics, development of new antibiotics which demands significant economic, labor and time investments. Consequently, it has deviated minds towards combination therapeutics (Gibbons, 2005; Wright, 2005). Thus, the interaction of nanoparticles with commonly used antimicrobials was studied by checkerboard method (Jayraman et al., 2010). The checkerboard test measures the inhibitory concentration of the compound to be tested. Present study reports 100% synergistic effect with 10% doped ZnO NPs and antibiotic combination. Improved activity (synergistic effect) on combining nanoparticle and antibiotic is due to bonding reaction between the two. The antibiotic molecules contain many active groups such as hydroxyl and amide groups, which react easily with nanoparticles by chelation (Chauhan et al., 2015). Findings of the current study
Chapter 5

Discussion

further show slightly better activity with ciprofloxacin than ampicillin. Our results are in accordance with the study conducted by Banooee et al. 2010 showing better activity of ciprofloxacin nanoparticle combination as compared to ampicillin nanoparticle combination. They reported 27% and 22% increase in diameter of inhibition zones against S. aureus and E. coli, respectively when ZnO nanoparticles combine with ciprofloxacin. Similarly Seif et al. (2011) also reported better activity (4 to 32 fold increases) of ciprofloxacin coated zinc oxide nanoparticles against S. aureus and E. coli. However, our study is contradictory to the study conducted by Gaddad and coworkers (2010) who reported good activity of ampicillin and moderate activity of ciprofloxacin against S. aureus. Better activity of ciprofloxacin is due to either of any reason: suppression of ciprofloxacin efflux, increased uptake of ciprofloxacin by manipulating the activity of membrane protein Omf and stabilization of ciprofloxacin-nanoparticle combination (Banooee et al., 2010). The present study involving combined effect of antibiotics and nanoparticles also revealed better activity of doped nanoparticles than the undoped ZnO nanoparticles. Thereby further strengthens the fact that doping increases the antimicrobial activity. This may be due to the coupling or synergistic effect of Mn, Co, Cu and Fe loading as it accelerates the generation of reactive oxygen species (ROS). Relatively less enhanced or unaffected antimicrobial activity of undoped ZnO NPs in present study is due to some weak hydrogen bonds between undoped ZnO nanoparticles as well as insufficient target site for binding (Banooee et al., 2010).

In fighting fungal infections, some antifungal substances have three main disadvantages: limited range of action, self-medication, which may interact negatively with different types of antifungal agents, and the resistance of microorganisms. Further they have side effects which result in complications such as amphotericin B causes kidneys and renal failure, fever, tremble, nausea and diarrhoea, fluconazole causes liver cirrhosis, toxicity and prevents testosterone synthesis (Espinel-Ingroff, 2009). Hence, the continuous investigation of novel drugs with less complication becomes urgent requirement. Therefore combination therapy makes an effective approach as it is better to act as a group rather than acting alone towards microorganisms. Additive effect was seen for the combination of fluconazole and nanoparticles against C. neoformans in the current study. Padalia et al. (2015) also showed good antifungal activity of Ag NPs and antibiotics (Nystatin, Ketoconazole, Fluconazole and Amphotericin B) combined together rather than their individual effect against C. albicans, C. neoformans and C. glabrata. Study by Gajbhiye et al. (2009) also supported the improved combined effect of Ag NPs and fluconazole rather than their individual effect against C.
albicans. Similar to our previous assays, this study also exhibited better activity of 10% Cu doped ZnO NPs than undoped ZnO NPs. Similar study conducted by Wang et al., (2010) suggested that brain infections caused by C. neoformans can be very well treated by cholesterol-conjugated G(3)R(6)TAT (CG(3)R(6)TAT) nanoparticles. Because of their small size, nanoparticles can easily pass through the blood-brain barrier (BBB) suppressing the yeast growth in the brain tissues similar to amphotericin B but does not have any adverse effect on liver, kidney or composition of blood. Our study supports the findings of researchers who state that combining nanoparticles with antibiotics not only decreased the toxicity of both the agents but also improved their antimicrobial property (Hwang et al., 2012; Namasivayam et al., 2015). Apart from increasing the antimicrobial effect, combination therapy also reinstates antibiotic’s ability to harm microbes which had gain resistant to them. Combination therapy increases the absorption of antibiotics at the microbe-antibiotic contact and facilitates easy attachment of antibiotics to microorganisms (Allahverdiyev et al., 2011). Studies on the combined effect of doped ZnO NPs with fungicidal drugs may be particularly useful as it is well known that even fungicidal agents have limited action in immunosuppressed patients (Graybill et al., 1997). Therefore, an additive effect of doped ZnO NPs–flucanozole drug may be particularly helpful in some clinical cases.

By observing the results of the present studies it is evident that doping enhances antimicrobial activity and also higher doping concentrations are more effective than lower and undoped NPs. This fact is very well supported by the earlier studies of Rekha et al., (2010), Pongodi et al., (2014) and Sharma et al., (2015) depicting enhanced antimicrobial activity of ZnO on doping with transition metal. According to the research done by Jan et al., (2013), tin (Sn) doped ZnO NPs exhibited 37% higher inhibition zone than the undoped ZnO NPs when used against S.aureus. Enhanced concentration of dopant increases the antimicrobial activity. In our case 10% Cu doped ZnO NPs showed most enhanced antimicrobial activity. On increasing the concentration of dopant in ZnO NPs, interaction between oxygen and dehydrogenase enzyme increases which leads to enhanced antimicrobial activity (Sikong et al., 2010). Also it has been recently reported that doping increases the quantity of free Zn$^{2+}$ ions which adds to the bactericidal efficacy of doped ZnO NPs. Addition of the dopant compels the cations towards interstitial sites which favours easier release of metal ions than native sites (He et al., 2014). Another reason is the decreased aggregation of doped nanoparticles. This supports more attachment of the cell membrane and
interaction with sulphur and phosphorous containing compounds which will in turn impair the cell, normal functioning like permeability, respiration, finally causing death (Tam et al., 2008; Tariq et al., 2014). Several other studies also have reported a rise in antimicrobial activity of NPs like ZnO, TiO$_2$ and SnO$_2$ on doping (Jin et al., 2009; Zhang et al., 2011). Gold (Au) NPs doped with toludene blue O and certain antibodies are effective against MRSA (Zharov, 2006; Gil-Tomas, 2007; Perni, 2009). There is a significant difference in the antimicrobial values (Disc diffusion, MIC, MBC, CFU) of undoped ZnO nanoparticles and doped ZnO nanoparticles in the present study. This indicates the fact that dopant was interfering with the active principle as the values obtained are greater in doped ZnO nanoparticles as compared to undoped ZnO nanoparticles for different groups of bacteria and fungi. Dopant impurities like Cu$^{2+}$, Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$ are rare earth transition metals which brings about significant changes in the physical, chemical and biological properties of host material depending upon the dopant used and the dopant concentration (Peng et al., 2006; Zhang et al., 2006). Increased dopant concentration produces reactive oxygen species (ROS) which enhances antibacterial and photocatalytic activity.

Evidently, doped ZnO NPs has more surface defects with reduced particle size. Electrons, holes get trapped in these defects which prevents their recombination resulting in higher antibacterial activity of doped ZnO NPs. On the other hand, Stoimenov et al., (2002) reported that metal oxide nanoparticles bind on the surface of microorganisms due to electrostatic interaction resulting in microbial death. Electrostatic force is further enhanced by the incorporation of valence ions (Fe$^{2+}$, Cu$^{2+}$, Co$^{2+}$ and Mn$^{2+}$) into ZnO NPs resulting in stronger adsorption and attachment of nanoparticles on bacteria.

To gain direct evidence of the antibacterial behavior of doped ZnO nanoparticles, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to characterize the microbial morphology with and without treatments. On the basis of MIC, 10% Cu doped ZnO nanoparticles showing most effective results against each Gram positive bacterium (B. subtilis), Gram negative bacterium (S. typhi) and fungus (C. neoformans) were chosen for electron microscopy studies. It revealed the distribution and location of the nanoparticles, as well as the morphology of the bacteria before and after treatment with the nanoparticles (Kim et al., 2011). The difference of bacteria morphology between the untreated and treated samples is easily observed. The former keeps better cell shape and cell state; however, the latter shows altered bacterial shape, fewer number of bacteria as well as clumping of bacteria. The mechanism of inhibition by nanoparticles is not fully understood.
But literature suggests that interaction of microorganisms with nanoparticles results in altered membrane morphology which increases its permeability, and ultimately causing cell death by impairing functions like respiration etc (Danilczuk et al., 2006; Sondi and Saploek-Sondi, 2004). Heavy metals, are lethal and when they react with microbial proteins, cellular metabolism is inhibited leading to death of microorganisms (Lee et al., 2003). No clear visualization of membrane deformation was noted in our case. This similar to the case of Roselli et al., (2003) who stated that this does not affected the probability of changed cell membrane permeability and deviated intracellular metabolic functions in microbial cells caused by doped ZnO NPs. These data suggest that doped ZnO NPs break the barrier of bacterial cell membrane causing the contents of cytoplasm to ooze out. Continuous release of lipopolysaccharide (LPS) and other membrane proteins results in the development of pits in the exterior of bacteria (Amro et al., 2000). TEM micrograph in the present study shows penetration of nanoparticles within the bacterial cells. Penetration of nanoparticles inactivates cellular enzymes and releases hydrogen peroxide resulting microbial death (Kokkoris et al., 2002). Small sized doped ZnO NPs (around 20nm) can effortlessly and efficiently bind to the cell membrane, impairing the bacterial functions like respiration and permeability (Nirmala and Pandian, 2007).

To investigate the antifungal mechanism of doped ZnO NPs, SEM and TEM analysis was employed to examine the structural changes of C. neoformans. In our study, 10% Cu doped ZnO NPs were found surrounding C. neoformans cells as well as non-specifically distributed within the cell cytoplasm and in different regions of the cell wall. Similar observations were reported by Kim et al. (2009) also. Another study reports disruption of the cell wall and cytoplasmic membrane in C. neoformans treated with AgNPs at an exposure time of 72 hours (Ishida et al., 2014). From TEM studies it was clear that doped NPs accumulated on the cell wall. This strongly suggests a dynamic release of free ions like zinc (Zn$^{2+}$), copper (Cu$^{2+}$) by the nanoparticles which actively penetrate the cell (Vazquez-Munoz et al., 2014). It has also been established that damage to important biochemical processes can be caused by copper ion uptake by the bacterial cells (Kim et al., 2000). Strength of fungal cell walls is due to the presence of ergosterol in the membrane along with the various gradients between cytoplasmic membranes (Das and Marsili, 2010). Electron microscopic studies shows that the interaction between doped ZnO NPs and the membrane structure of C. neoformans cells brought significant changes to their membranes. As a consequence of interaction with nanoparticles, theses gradients and membrane conformity are destroyed.
causing cell destruction. Previous studies have mentioned that ZnO nanoparticles have fungicidal and fungistatic effect against yeasts and molds respectively (Kim et al., 2008; Kim et al., 2009). However, for molds it is contradictory to our study where only susceptibility against yeast was observed. High percentage of chitin present in the cell wall of T. mentegrophytes might be responsible for its resistance towards nanoparticles (Gajbhiye et al., 2009).

Up till now the precise mechanism of immunity to the fungus is not recognized. Reason for least studying fungus may be their complex cell wall construction. One reason proposed is the electrostatic interaction between fungal cells and nanoparticles. Chitin present in the cell wall of fungi is negatively charged to which positively charged nanoparticles attaches by electrostatic interactions resulting in inhibition of the fungal growth (Shi et al., 2010). Nanoparticles attach itself to the fungal cell wall and penetrate it. Inside the cell it attaches itself to respiratory sequence and last cell division, leading to cell death (Hassan et al., 2013). Another possible reason may be generation of reactive oxygen species (ROS) and nuclear fragmentation that leads to cell death. Similar antifungal mechanism has been reported for silver nanoparticles (Ag NPs) against C. albicans by Hwang et al. (2012). Some researchers also suggest that release of proteins, carbohydrates and lipids through the damaged cell membrane results in reduction in the amount of protein, carbohydrates and lipids in fungal cell leading to the death (Peral et al., 2002; He et al., 2011).

Advent of every new technology has its pros and cons. Nanoparticles on one hand has pushed advancement in many fields to heights on the other hand similar nanoparticles are threat to human race and environment. The clinical use of nanoparticles as an antimicrobial agent depends upon its negative effect on the mammalian cells. In other words, the amount/quantity of nanoparticles employed to inactivate/kill microorganism should not affect the functioning of mammalian cells. The ZnO and doped nanoparticles of the current study presented homogeneity and purity of good scale. Therefore, the toxicity established in the present study is solely due to the nanoparticles themselves. It might involve particle size or mineral element composing nanoparticles. Our study is consistent with other studies of oral intake of ZnO NPs resulting in symptoms like lethargy, nausea, vomiting, and diarrhea in the mice. Similar findings regarding mineral intake has been reported by Lock and Janssen, 2003 and Piao et al., 2003 as well. Since no death or other life threatening effect has been reported in our study we can state that the current study proposes moderate intestinal effect of undoped and doped ZnO NPs rather than molecular ZnO.
Diseases of heart, liver, kidney, etc are diagnosed by blood biochemical tests. Also they are helpful in monitoring the effect of exogenous toxic. Dysfunctioning of liver is evaluated by ALT, AST, ALP, and LDH. The pathological examinations done in the present study showed effects on kidney and liver. Hence, kidney and liver served as target organs for undoped and doped ZnO nanoparticles. The results of this experimental study indicated that single maximum tolerated dose of doped ZnO NPs don’t show significant effect on the body weight gain and the relative organ’s weight. This is in accordance with previous finding indicating the absence of toxic signs and mortality in rodents exposed to ZnO NPs (Amara et al., 2015).

Blood biochemical indexes further depicted the alterations in the renal and hepatic functions of experimental mice. Severe damage to kidney and liver as well as pathological alterations were indicated in mice exposed to nanoparticles. Therefore, more attention should be paid on the potential toxicity induced by various doped ZnO and pure ZnO nanoparticles exposure. Based on findings in animals, undoped and doped ZnO nanoparticles were dispersed to all of the organs studied, with most concentrated in the liver and kidney. Particle size and concentration of dose is the reason behind pathological alterations (Wang et al., 2006). Literature suggested toxicity of ZnO NPs to human or rodent cells at the concentration above 20 ppm (Brunner et al., 2006) but in our study we have used doped and undoped ZnO nanoparticles range less than 20 nm in size for preclinical study of toxicity potential. Affect of ZnO NPs varies with different cell types and animal systems (Osman et al., 2010; Sharma et al., 2012).

Present studies reported following affect of nanoparticles on animals: weight loss, hypoactivity, altered liver enzymes and altered blood values. Many studies have suggested that toxicity produced by nanoparticles is due to free radical mechanism (Manke et al., 2013; Fu et al., 2014; Fard et al., 2015). Various dopants may increase the acute toxicity of ZnO nanoparticle upto some degree, for which the reason might be related to the synergistic accumulation of dopants and Zinc. Therefore, more attention should be paid on the potential toxicity induced by various routes and concentration of dopant with ZnO nanoparticles exposure to humans.