FACILE GREEN SYNTHESIS OF ZnO AND Ag-DOPED ZnO NANOPARTICLES USING *CROTALARIA LABURNIFOLIA* AQUEOUS EXTRACT FOR ANTIMICROBIAL APPLICATIONS

Antimicrobial activity of phytosynthesized Ag doped and undoped ZnO nanopartcles

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Abstract*:* The current study explains the use of *Crotalaria laburnifolia* aqueous extract in the synthesis of ZnO nanoparticles and Ag doped ZnO nanoparticles using co-precipitation method. Synthesized Ag doped and undoped ZnO NPs were characterized using powder XRD, Dynamic Light Scattering, Scanning Electron Microscope and Energy Dispersive Spectroscopy. The study assessed the antimicrobial potential of Ag doped ZnO and undoped ZnO NPs against human pathogenic bacteria, *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, P. aeruginosa and Salm*onella *typhi* . The results of the study revealed that the Zn-NPs and Ag doped ZnO showed antibacterial efficacy as well as minimum inhibitory concentration against different human pathogenic bacteria such as *B. subtilis, S. aureus, E. coli, P. aeruginosa* and *S. typhi.* The Ag doped ZnO nanoparticles exhibited better antimicrobial activity compared to undoped ZnO and showing activity against all tested microbes except *P aeruginosa* at 5mM concentration. Resazurin based micro-titre assay showed varied results ranging from 3.25% to 100% inhibition of various microorganism. It also showed that efficacy of antimicrobial activity increased with increase Ag concentration (5mM to 8mM). From the results of the study, it could be concluded that *C. laburnifolia* successfully caused reduction of Zn and Ag salts to doped and Ag undoped ZnO nanoparticles. Both doped and undoped ZnO caused favorable antimicrobial activity against the tested microbes with Ag doped variant causing better antimicrobial activity. Hence the ZnO and Ag doped ZnO synthesized using *C laburnifolia* aqueous extract can be further investigated for their toxicity in cells and animals to develop them as potent antimicrobials for biological applications.

***Keywords: Crotolaria laburnifolia*, Antibacterial, green synthesis, doping.**

I. INTRODUCTION

Nanotechnology is the ability to work at the atomic, molecular and super molecular levels (on a scale of 1–100 nm). It opens up a wide array of opportunities in various fields like medicine, pharmaceuticals, electronics and agriculture. The potential uses and benefits of nanotechnology are enormous [Mihail, 2003]. Nanoparticles are atomic or molecular aggregates with at least one dimension between 1 and 100nm that can drastically modify their physico-chemical properties and biological behavior when compared to their bulk counterparts [Jamadagni et al., 2018, Hasnidawani et al, 2016]. Nanomedicine and nanopharmaceutics is getting highly popular because of high tunable properties of nanomaterials that can be used up in targeted drug delivery, cancer treatment, treatment of hypothermia, nano-antibiotics and so on. Among these applications, the nano-antibacterials are getting popular as alternative to antibiotics.

Among different class of nanomaterials, the metal oxide based nanoparticles are gaining popularity as potent antimicrobials. They are important group of engineered nanoparticles, and are widely used in cosmetics and sunscreens, self cleaning coatings and textiles. [Tomasz Puzyn *et al*, 2011].Metal nanoparticles with bactericidal activity can be immobilized and coated on to surfaces, which may find application in various fields, i.e., medical instruments and devices. Metal nanoparticles may be combined with polymers to form composites for better utilization of their antimicrobial activity [Baranwal et al., 2018]. The major concern that arises in the use of metal nanoparticles for their biomedical applications is the questionable toxicity of these nanoparticles. Earlier it was documented that metal oxide nanoparticles (e.g., TiO2, ZnO, and Fe2O3) can enter the human body and exhibit some toxicity, such as inflammatory and cytotoxicity responses and cell membrane leakage [Brunner *et al.*, 2006]. The major reason behind their toxicity not only lies with their size, but also the synthesis method taken up. Most of the metal oxide nanoparticles are synthesized via chemical and physical routes [Khalil et al 2014, LinC and Li YY 2009]. Commonly used methods include chemical vapor synthesis, Laser ablation, solvothermal, thermal decomposition and sol-gel method [Shubha et

al., 2018]. These conventional physical and chemical processes involve expensive chemical and physical attributes that often use toxic materials with potential hazards such as environmental toxicity, cytotoxicity, and carcinogenicity. Procedurally also they involve difficult separation procedure, high pressure and high energy requirement [Shubha et al., 2017]. Therefore, in recent years, use of natural sources in synthesis of metal oxide nanoparticles is highly documented. Among the natural sources the herbal extracts are much explored reducing agents in the synthesis metal oxide nanoparticles. Synthesis using herbal extracts involve single step, does not require any complicated/specialized equipment, fast and cost effective. They are environmentally friendly green alternatives to chemical synthesis methods [Shubha et al., 2019]. Based on these facts, in the current study, *C. laburnifolia* aqueous extract has been used as reducing and capping agent, in the synthesis of ZnO and Ag doped ZnO nanoparticles. The Ag doping is known to enhance the antimicrobial activity of ZnO nanoparticles [Shahid et al 2018, Ahmed K & Jaffri S, 2018]. For this we employed aqueous extract of *Crotalaria lubrunifolia* as reducing and capping agent. *C laburnifolia* is an important medicinal plant used in various ethnomedicinal practices in India found growing in tropics and subtropics. *Crotalaria* is a genus of flowering plants in the legume family fabaceae. It is commonly known as rattlerods. *Crotalaria* possesses many characteristics of a cover crop, being a poor or non host for a large group of pests and pathogens, competitive with weeds without becoming a weed, growing vigorously to provide good ground coverage, performing symbiosis with rhizobium to fix nitrogen [Llamas, 2003]. The undoped and Ag-doped ZnO nanoparticles synthesized using *C laburnifolia* aqueous extract is used in the present study as antimicrobial agent in *invitro* conditions against various pathogenic microorganisms.

II. MATERIALS AND METHODS

The plant material was obtained and authenticated at DOS in Botany, University of Mysore. $ZnSO_4$, NaOH, AgNO3 used in the experiments were obtained from Sigma Aldrich. Culture Media used in bacteriological studies were obtained from HIMEDIA laboratories.

2.1 Preparation of aqueous extract of *C. laburnifolia*

The *C laburnifolia* leaves were separated and washed several times in running water and shade dried for 4-5 days. The dry leaves were powdered finely and sieved through cheese cloth to obtain fine powder. 30 g of this powder was packed in a double pouch made of Whatmann's filter paper #1 and extracted extensively in ultrapure water (18. 2 MΩ (Options ELGA) at its boiling temperature, using Soxhalet apparatus. After 8-9 cycles of extraction, greenish –brown colored aqueous extract was obtained, which was used in synthesis of nanoparticles.

2.2 Synthesis of Ag doped ZnO nanoparticles using co-precipitation method

20ml of fresh aqueous extract of *C. laburnifolia* was maintained at 70 ^{o} C for 1 h. Then 0.1 ml of 3M $ZnSO_4$ was added to above solution and stirred continuously. 8 mL of NaOH was added drop wise to above mixture until *pH* reached 12. At this point, a yellow precipitate of ZnO was obtained. This was considered as source of pure compound. (undoped ZnO NPs), which was dried for 12 h at 60 ^{O}C in hot air oven.

The doped variant was synthesized using same method with minor modifications. 20ml of plant extract was taken and stirred until it reaches 60 to 70 ^{o}c. Further 8ml of NaOH was added and stirring was continued for 10minutes followed by addition of 0.1ml of 3M zinc sulphate salt. Further, 2ml of 0.05 M AgNO3 was added to this and the colour changes to brown. The Precipitate formed was allowed to stand for a day in room temperature, which was further filtered and washed with ultrapure water followed by ethanol. The sample was dried in hot air oven for 12 h at 60^{o}c.

2.3 Characterization

Synthesized ZnO and ZnO-Ag nanostructures were characterized at Centre for Material Science and Technology, University of Mysore. The PXRD studies were performed using Rigaku smart Lab–II, CuKα radiation with step size of 0.0001 deg. External morphology and elemental composition was determined using Hitachi S-3400 N Scanning Electron Microscope with attached EDS. Particle size of synthesized nanoparticles was obtained by DLS using Microtrac Zeta analyser.

2.4 Assessment of Antibacterial activity of pure ZnO and Ag-doped ZnO nanoparticles

The antimicrobial activity assay of pure (ZnO) and doped (ZnO-Ag) nanostructures were determined using standard microbiological assays. The pure cultures for the study were obtained from Microbial Type Cell Culture and Gene banking (MTCC), Chandigarh. The bacteria used in the study were *Escherichia coli* (MTCC 433), *Bacillus subtilis* (MTCC 441), *Staphyloccous aureus*(MTCC 3160) , *Pseudomonas aeruginosa* (MTCC 1934) and *Salmonella typhi* (MTCC 98).

2.4.1 Preliminary antimicrobial activity using disk diffusion method

To assay the preliminary antimicrobial activity of ZnO and Ag doped ZnO nanoparticles, disk diffusion assay was performed in concentration range of 5 and 8 mM [Muzamil et al., 2013]. In the experiment, the required quantity of nanoparticles (ZnO/ZnO-Ag) was dispensed in nano-pure water and sonicated for1 hr to obtain complete dispersion. Filter paper disks that were cut out from Whatman's filter paper # 1 of radius 5 mm were saturated with nanoparticles suspension. 20 µL of respective pure cultures whose concentration was adjusted to 10^6 CFU/mL were spread onto the surface of Muller Hinton agar plates for bacteria. The filter paper disks were saturated with ZnO/ZnO-Ag nanoparticles and were placed on to the surface of agar. Sterile water was used as negative control. Penicillin G (standard anatibiotic) was used in concentration range similar to nanoparticles as positive control. The prepared plates were incubated at 37^{o} C for 24 h and further observed for growth or inhibition.

2.4.2 Determination of antibacterial effectiveness using Resazurin based micro titre assay

The antibacterial effectiveness of ZnO and Ag doped ZnO was determined using Resazurin based microtitre assay at concentrations 5mM and 8 mM using 96 well plates [Elshikh et al., 2016] in triplicates. Experimentally, 12 wells of each row were filled with 0.5 ml sterilized Mueller Hinton broth. Each well received 15 µl of respective microorganisms. Sequentially, wells 2–11 received an additional 0.5 ml of a mixture of culture medium and nanoparticles at 2 concentrations, of 0.512 ml and 0.008 ml. Well 1 served as growth control, well 12 as antibiotic control. Tetracycline Hydrochloride (0.1mg/ml) and Amoxicillin (0.1mg/ml) were used as standard controls. The well plates were incubated for 24h at 37°C. After the incubation period, resazurin (0.015%) was added to all the wells (each well receiving 30 µl) and further incubated for 2-4 hr. The resulting color change was observed, and after 24h MIC was determined by taking OD at 600nm with a Beckman DU-70 UV-Vis Spectrophotometer.

III. RESULTS AND DISCUSSION

3.1 Powder XRD

The PXRD studies were performed using Rigaku smart Lab–II, CuKα radiation with step size of 0.0001 deg. Figure 1 below shows the results of PXRD of pure ZnO without doping with Ag (WOD) and doping with Ag (WD).

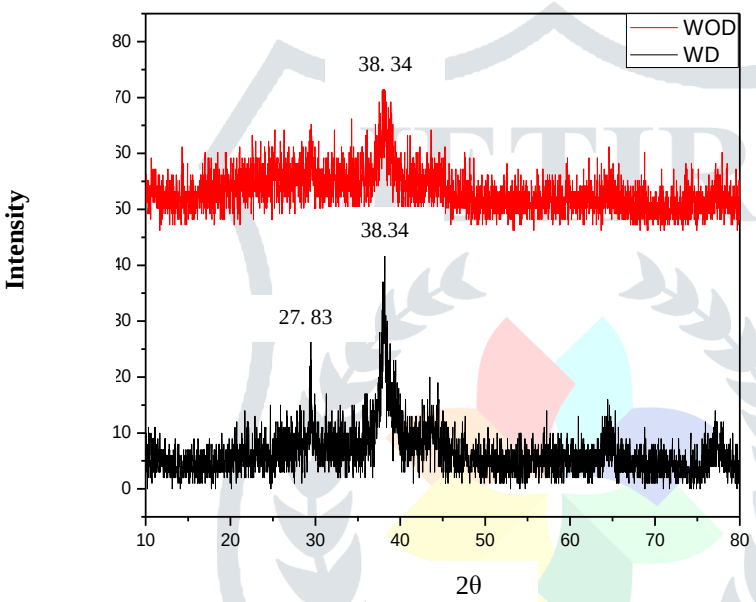
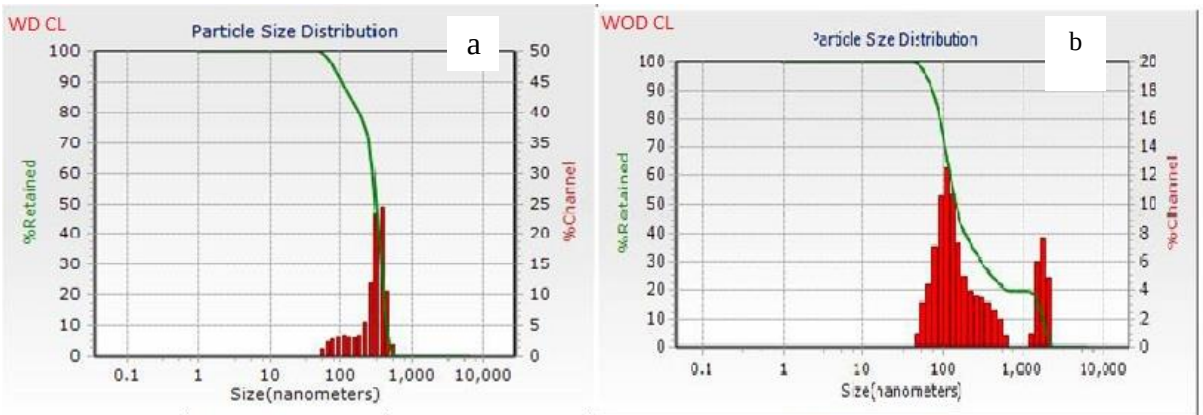


Fig 1-PXRD results of Ag doped and undoped ZnO nanoparticles

The above graph used to identify the d-Spacing value showed distinct diffraction peaks, the peak appeared at 38.34 in pure ZnO, whereas in the case of Ag doped ZnO, displacing values of these peaks to 27.83.and 38.34 respectively. XRD results also suggested that the crystallization of the bio-organic phase occurs on the surface of the silver nanoparticles or vice versa.

3.2 Particle size measurement using Dynamic light scattering



	PEAK SUMMERY			PEAK SUMMERY	
Dia(nm)	Vol%	Width	Dia(nm)	Vol%	Width
92.8	15.2	54.2	125.4	80.9	197.8

Fig 2-Particle size distribution of Ag doped and undoped ZnO nanoparticles

The particle size distribution (DLS) of synthesized pure ZnO and Ag doped ZnO was assayed using Microtrac zeta analyzer. The fig 2 shows the data of particle size measurement. **2A** shows the average particle size of Ag doped ZnO nanoparticles, which is 125.54nm diameter and 92.8nm width, while the undoped pure ZnO nanoparticles **(fig 2B)** showed 197.8 nm width and 125.4 nm diameter(As seen in fig 2b). The results of the DLS measurement clearly shows that doping with silver nanoparticles has noticeably reduced the size of nanoparticles. As size of the nanoparticles play a critical role in their antimicrobial activity, the smaller sized doped nanoparticles will have better membrane permeability, attachment to bacterial cell wall and cell death as studied elsewhere [Jung et al., 2006].

3.3 Scanning Electron Microscopy

For further insight into morphology and size details, the SEM studies were performed using Hitachi N-400 Scanning Electron Microscope. Fig 3 A and 3B shows the SEM images of pure undoped ZnO and Ag doped ZnO nanoparticles.

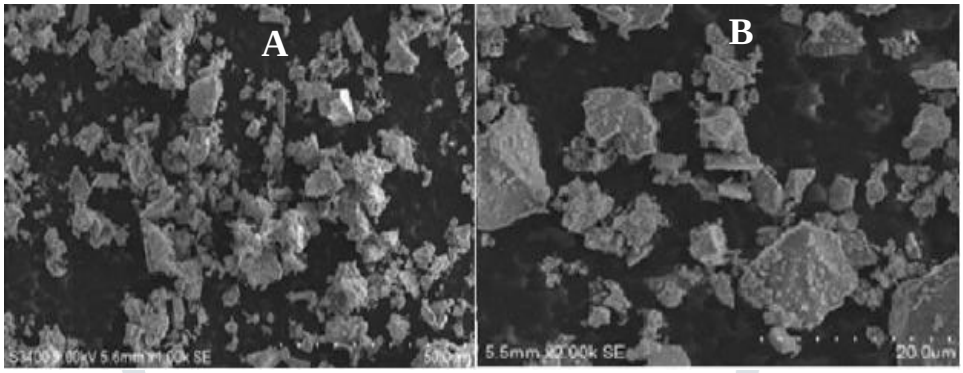


Fig **3A**-SEM images of undoped ZnO nanoparticles
3B-SEM image of Ag doped ZnO nanoparticles

The Fig 3A shows flakes of ZnO nanoparticles that are well agglomerated, while the fig 3B shows deposition of spherical Ag nanoparticles decorated on the flakes of ZnO. The SEM images suggest that incorporation of Ag^{+} in place of Zn^{2+} provoked the decrease in size of nanocrystals as compared to pure ZnO [Reddy et al., 2015].

3.4 Energy Dispersive spectroscopy

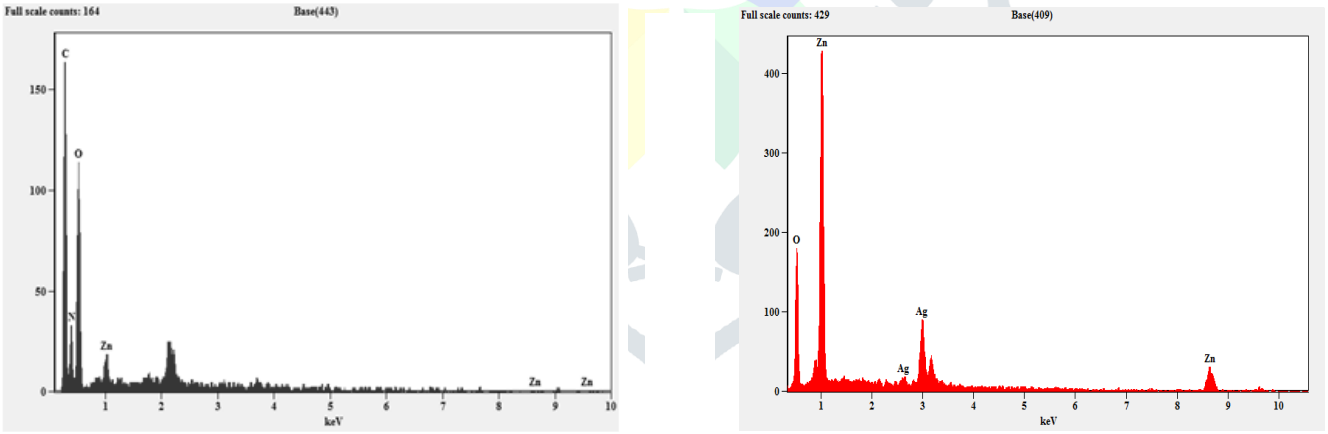


Fig 4A-EDS spectra of pure ZnO nanoparticles
Fig 4B-EDS spectra of Ag doped ZnO nanoparticles

Elemental composition of Ag doped and undoped ZnO nanoparticles was determined using Hitachi S-3400 N SEM with attached EDS. Fig 4A and 4B represents the ED spectra of *C laburnifolia* synthesized ZnO and Ag doped ZnO nanoparticles. Fig 4 A shows Zn as the single major element in the spectra apart from C and O as other major elements, which shows that there is formation of ZnO. Fig 4 B shows successful doping of ZnO with silver (as seen in spectra) and also as confirmed by previous literature [Saravanan et al., 2014].

3.4 Assessment of antimicrobial activity of pure and Ag doped ZnO nanoparticles

3.4.1. Assessment of preliminary antimicrobial activity using disk diffusion method

The agar disk diffusion assay was carried out at 5 mM and 8 mM concentration of pure ZnO and Ag doped ZnO nanoparticles using Muller-hinton agar media. The table I below shows the results of MIC.

Sl No	ZnO-Ag		Pure ZnO		Penicilln G standard	
Concentration of nanoparticles in mM	5mM	8mM	5mM	8mM	5mM	8mM
ZOI (in mm)						
E. coli	7	8	4	6	12	15
S. aureus	2	4	4	6	13	15
B. subtilis	3	4	3	5	8	8
P. aeruginosa	-	*2*	-	-	8	11
S. typhi	5	7	-	4	3	10

Table -1-Minimum inhibitory concentration by Ag doped and undoped ZnO (in mm)

From the table it can be made out that, the Ag doped ZnO nanoparticles showed better antimicrobial activity than the undoped ZnO against all the tested bacteria except *P aeruginosa*. The undoped ZnO did not cause any inhibitory effect against *P aeruginosa*.

Nanosized ZnO is bactericidal and can inhibit the growth of both gram positive and gram negative bacteria [Karunakaran et al., 2011]. According to literature, doping of ZnO with Ag nanoparticles has caused increase in its antimicrobial activity based on the syntergistic activity. The earlier studies have hypothesized that as the amount of silver increases, the resultant bactericidal activity further increases due to more attachment of bacterial cell with subsequent after attachment activity followed by a contribution from zinc oxide with the generation of H_2O_2, hence resulting a synergistic activity. Also, it was mentioned that silver has an important antimicrobial effect, which depends upon superficial contact, where in silver can inhibit enzymatic systems of the respiratory chain, there by altering the DNA synthesis [Talari et al., 2012; Suresh kumar et al., 2017].

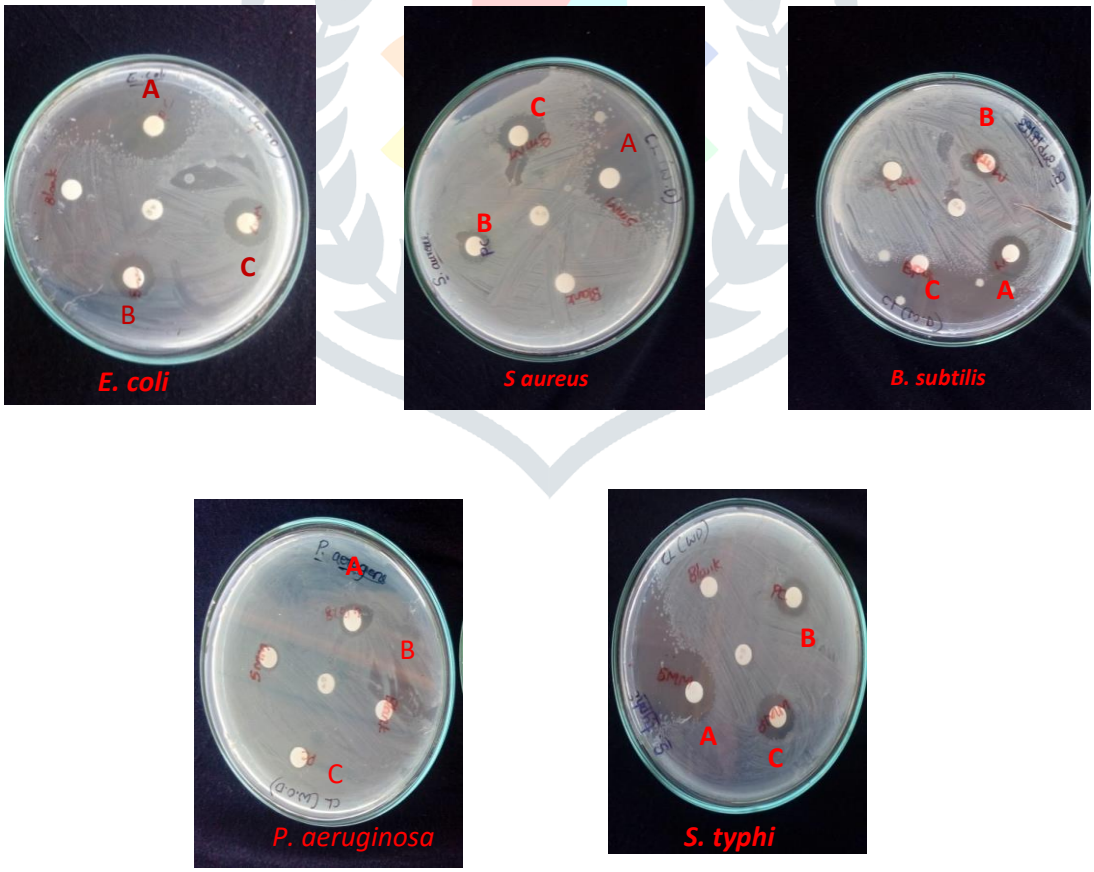


Fig 5-Assesment of Antimicrobial activity of Ag doped ZnO against selected bacteria using disk diffusion assay

3.4.2 Evaluation of antibacterial effectiveness using Resazurin based microtitre assay

The resazurin based microtitre assay was originally described in the year 2000 by Drummond and Waigh. This is modified to achieve a very sensitive measure of antimicrobial activity of natural products, crude extracts. The modified method is simple, very sensitive, rapid, robust and reliable method to assay the antimicrobial activity of natural products even in very small quantity [Sarker et al., 2007]. In the current study, the antimicrobial activity of Ag doped ZnO and undoped ZnO at 5 mM and 8 mM concentrations were assessed using the same assay and the minimum inhibitory concentration was determined. The results of the study showed that the undoped ZnO caused 0.78% (5mM) and 3.25% (8mM) of inhibition of *E coli, whereas, the* Ag doped ZnO caused 3.25% (5mM) and 6.35%(8mM) growth inhibition of *E coli. Similarly, the* undoped ZnO caused 25% growth inhibition of *S aureus* at 5 mM concentration and 50% inhibition at 8 mM concentration and the Ag doped ZnO at 5 mM concentration caused 50% inhibition and 100% in8 mM concentration of Staphylococal growth. Against *B subtilis*, Ag doped ZnO caused 25% inhibition at 5 mM concentration and 40% at 8 mM while the undoped ZnO showed inhibitory effect of 6. 25% (5 mM) and 12.50% at 8 mM concentration. Against *P aeruginosa* undoped ZnO caused no inhibition at 5 mM concentration and 3.25% inhibition at 8 mM concentration while Ag doped ZnO caused 12.5% inhibition at 8 mM concentration. At lower concentration it could not cause any inhibitory effects over *P aeruginosa*. Against *S typhi* the undoped ZnO caused 12.5% inhibition (5 mM) and 25% (8 mM) concentrations. The Ag doped ZnO caused 50% inhibition at 5mM concentration and 100% inhibition at 8 mM concentration. Fig 6A shows the inhibitory effect caused by Ag-undoped ZnO and Fig 6B shows the inhibitory effect caused by inhibitory effect caused by undoped ZnO.

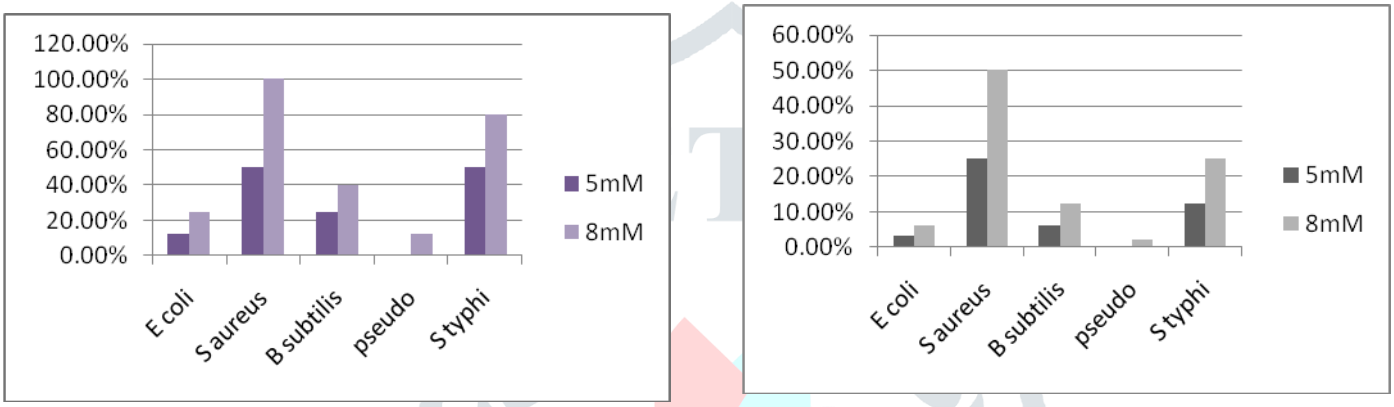


Fig 6A-Inhibitory effect on various bacteria by Ag doped ZnO; 6B- Inhibitory effect caused by undoped ZnO

IV. CONCLUSIONS

In the current study, a facile green synthesis method using *C laburnifolia* leaves aqueous extract was adapted to successfully synthesize the ZnO nanoparticles and Ag doped ZnO nanoparticles. The synthesized nanoparticles were 100 nm (Ag-doped) and 192 nm (Undoped) respectively. Both the nanoparticles caused considerable amount of inhibition of selected gram positive and gram negative bacteria. The Ag-doping has caused remarkable increase in antimicrobial activity of ZnO by two folds. The study has the scope of further extension to in vitro and *invivo* toxicological assays to extend it to clinical trials in future.

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