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"ANTIMICROBIAL ACTIVITY OF GREEN SYNTHESISED ZINC OXIDE NANOPARTICLE USING *HIBISCUS SUBDARIFFA, FICUS RELIGIOSA AND CATHARANTHUS ROSEUS*"

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Abstract:

Nanoparticles can be synthesized by using microbes and by plant extracts. Under the favourable conditions, size and surface of nanoparticles can be easily altered to achieve both passive and active transportation of drug Nanotechnology is recognized as the study of particle with a minimum of one dimension in nanometers. Synthesis of nanoparticles can be done through three methods like physical, chemical, and green synthesis methods. Green synthesis can be done using microorganisms, enzymes, or plant extracts. This project work is based and focused on the green synthesis method due to its non-toxic and eco-friendly process. In this project, Zinc (II) ion solution is reduced into ZnO nanoparticles using plants like *Hibiscus subdariffa, Ficus religiosa* and *Catharanthus roseus*. The significant antibacterial activity of ZnO NPs was observed against *Staphylococcus aureus* MTCC 9760 (S. aureus), *Streptococcus pyogenes* MTCC 1926 (S. pyogenes), *Bacillus cereus* MTCC 430 (B. cereus), *Pseudomonas aeruginosa* MTCC 424 (P. aeruginosa), *Proteus mirabilis* MTCC 3310 (P. mirabilis) and *Escherichia coli* MTCC 40 (E. coli). The synthesized ZnO NPs have shown antibacterial efficacy against both Gram-positive and Gram-negative pathogens. Overall, the results elucidated a rapid, cost effective, environmentally friendly and convenient method for ZnO NPs synthesis, which could be used as a potential antimicrobial agent against drug resistant microbes. Minimum inhibitory concentrations (MICs) test is performed to determine the susceptibility of organisms to the nanoparticles.

1. Introduction

Zinc oxide nanoparticles are antibacterial and inhibit the growth of microorganisms by permeating into the cell membrane. The oxidative stress damages lipids, carbohydrates, proteins, and DNA (Kelly SA, 1998). Lipid peroxidation is obviously the most crucial that leads to alteration in cell membrane which eventually disrupt vital cellular functions (Siddiqi, K. S., ur Rahman et al., 2018).

Two mechanisms of action have been proposed for the toxicity of zinc oxide nanoparticles, namely (1) generation of ROS and (2) induction of apoptosis. Metal oxide nanoparticles induce ROS production and put the cells under oxidative stress causing damage to cellular components, i.e., lipids, proteins, and DNA (Xia T, 2006). Zinc oxide nanoparticles, therefore, induce toxicity through apoptosis. They are relatively more toxic to cancer cells than normal cells, although they cannot distinguish between them.

2. Materials and Methods

2.1. Synthesis of ZnO Nanoparticles from *Catharanthus roseus*

2.1.1. Preparation of Plant Extracts:

Catharanthus roseus leaves powder prepared from the dried grounded leaves was used for NP synthesis. 6g of dried powder was mixed in 50 ml of distilled water and incubated at room temperature for 24 h. The extract was filtered and centrifuged for 30 min at 4000 rpm. The supernatant was used for ZnO NPs biosynthesis and stored at 4 ^{o} C for further use. [Apoorva Bangroo et al, 2021].

2.1.2. Green synthesis of zinc oxide nanoparticles

Biosynthesis of ZnO NPs was performed by mixing the 50 ml of aqueous solution of 0.01M zinc acetate dihydrate with 1 ml C. roseus leaf extract followed by constant stirring till the formation of the white suspension. The pH was adjusted to 12.0 using 2M and keep on stirring until the ZnO NPs precipitate were completely dissolved. The spectra exhibited an absorption band with a resolution of 1.0 nm between 350 and 500 nm, this indicated the formation of ZnO NPs.

2.2. Synthesis of ZnO Nanoparticles from *Ficus religiosa*

2.2.1.Plant extracts preparation

Ficus religiosa leaves were collected from Hyderabad region. They were thoroughly washed thrice with deionized water and dried in oven for 48 hours at 40–50°C. Then, they were crushed into fine powder using grinder. An intense green coloured powder of *Ficus religiosa* was obtained which was used in the present study. 20 g of finely powdered *Ficus religiosa* leaves were boiled in 100 ml water for 10 min and filtered to obtain *Ficus religiosa* leaves extract. [Arvind Singh K. Heer et al, 2017].

2.2.2. Green synthesis of zinc oxide nanoparticles

For the ZnO nanoparticles synthesis, 50 ml of Ficus religiosa leaf extract was boiled to 60- 80°C using magnetic stirrer and heater. Then 0.02 moles of Zinc Nitrate were added to the leaf extract of Ficus religiosa plant when temperature reaches 60°C. Then boil the solution till it is reduced to deep yellow paste. This paste is dried in oven at temperature 100- 130°C for 40-45 mins. This obtained yellow powder was then collected. The material was mashed in a mortar pestle so as to get a fine nature of particles for characterization.

2.3. Synthesis of ZnO Nanoparticles from *Hibiscus subdariffa*

2.3.1. Plant extracts preparation

5 grams of *Hibiscus sabdariffa* leaves were washed thoroughly with plenty of distilled water and both surface of leaves was sterilized using alcohol by gently rubbing. These leaves were heated for 30 min in 100 ml of distilled water at 50 °C. Then the extract was filtrated with Whatman filter paper no 1 and further filtered using vacuum filter with pore size of 0.2 µm. The final filtrate was stored in cool dry place for further use. [Niranjan Bala, 2015].

2.3.2. Green synthesis of zinc oxide nanoparticles

20 ml of the plant extract was heated at 50 °C for 10 min and 50ml of 91 mM of zinc acetate solution (1 gm of zinc acetate was dissolved in 50 ml of distilled water) was added drop wise to it under stirring. The reaction mixture became yellowish and cream coloured precipitate of zinc hydroxide was formed. The reaction mixture was left for 30 min for complete reduction to zinc hydroxide. Then the precipitate was collected by centrifugation at 16000 rpm for 10 min at 4 °C. The precipitate was vacuum dried at 30 °C and the sample was stored for further studies.

2.4. Phytochemical Analysis

Preliminary qualitative screening for phytochemicals, of the leaf extracts was carried out with the following methods.

Test for Alkaloids (Mayer's Test): 2 ml of leaf extract was treated with 2 drops of Mayer's reagent. Presence of white creamy precipitate indicates the positive test.

Test for Coumarins: 2 ml of leaf extract was treated with 3 ml of 10% NaOH. The formation of yellow colour indicates the presence of Coumarins.

Test for Terpenoids (Salkowski's test): 2 ml of leaf extract was treated with 2 ml of acetic anhydride. Few drops of concentrated sulphuric acid was then added to this solution and observed the formation of blue, green rings that indicates the presence of terpenoids.

Test for Quinones: 1 ml of leaf extract was added to the 2 ml of dilute NaOH. Formation of blue green or red coloration confirms the presence of quinones.

Test for Tannins (Braymer's test): 2 ml of leaf extract was allowed to react with 10% alcoholic ferric chloride solution. Formation of blue or greenish colour of the solution was observed. This was the indication of the presence of the tannins.

Test for Flavonoids (Alkaline reagent test): 2 ml of leaf extract was treated with few drops of IN sodium hydroxide solution and observed the formation of intense yellow colour. This yellow colour becomes colourless on addition of dilute hydrochloric acid, indicating the presence of flavonoids.

Test for Phenolic Compounds (Ferric chloride test): Few drops of the leaf extract were treated with 5% aqueous ferric chloride. Formation of deep blue or black colour indicates the presences of phenolic compounds.

Test for Anthocyanin: 2 ml of leaf extract was treated with 2 ml of 2N hydrochloric acid and ammonia was added to it. The appearance of pink-red colour turning blue-violet was observed. This indicates the presence of anthocyanin.

2.5. Characterization of NPs

UV-Vis spectroscopy: The synthesized ZnO nanoparticles were characterised by Perkin-Elmer UV-Vis spectrophotometer at ordinate mode of A, slit width of 1nm, and scan speed of 240nm/win. Number of cycle was set to 1 per 1 second and lamp change value was set at 326 nm for the determination of the Plasmon resonance property of ZnO nanoparticles, reduction of metal ion and formation of nano particles.

2.6. Antimicrobial activity of ZnO NPs

The antimicrobial potential of the ZnO NPs synthesized from the leaf extracts was tested using the agar well diffusion method. The human pathogenic microorganisms used in this study included six bacterial strains (*Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus*). The bacterial cultures were grown in nutrient broth at 37 ◦C for 24 h, and then the suspensions were spread on Muller Hinton agar plates. The wells were cut and 100 µg L−1 of the synthesized ZnO NPs were loaded in the wells. The plates were then incubated at 37 ◦C for 24 h. Thereafter, the plates exhibited the formation of clear inhibition zones around the wells, which indicate the occurrence of antimicrobial activity. The inhibition zones were then calculated by measuring their diameter around the wells.

3. Results and Discussion

3.1. Synthesis of ZnO Nanoparticles from *Catharanthus roseus*

Fig: 3.1.1

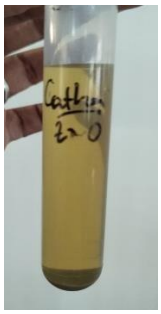


Fig: 3.1.2

Fig: 3.1.3

3.2. Synthesis Of Zno Nanoparticles From *Ficus religiosa*

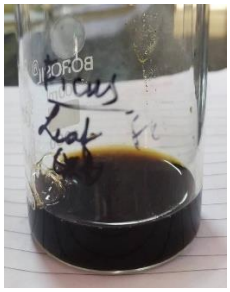


Fig: 3.2.1

Fig: 3.2.2

Fig: 3.2.3

3.3.Synthesis of ZnO Nanoparticles from *Hibiscus subdariffa*

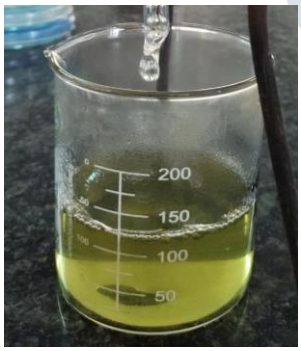


Fig: 3.3.1

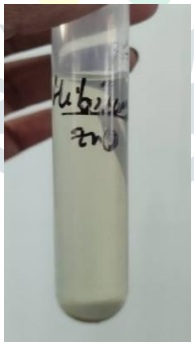
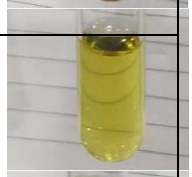
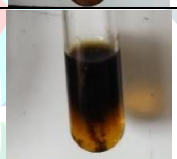
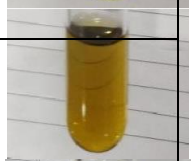


Fig: 3.3.2

Fig: 3.3.3

Fig: 3.1.1: *Catharanthus roseus* leaf extract; Fig: 3.1.2: *Catharanthus roseus* zinc oxide nanoparticles; Fig: 3.1.3: *Catharanthus roseus* dry zinc oxide nanoparticles;
Fig: 3.2.1: *Ficus religiosa* leaf extract;Fig: 3.2.2: *Ficus religiosa* zinc oxide nanoparticles; Fig: 3.2.3: *Ficus religiosa* dry zinc oxide nanoparticles
Fig: 3.3.1: *Hibiscus subdariffa* leaf extract; Fig: 3.3.2: *Hibiscus subdariffa* zinc oxide nanoparticles ; Fig: 3.3.3: *Hibiscus subdariffa* dry zinc oxide nanoparticles

3.4.Phytochemical Analysis

S. No	Plant Constituents	Catharanthus roseus	Ficus religiosa	Hibiscus subdariffa
1	Alkaloids			
2	Terpenoids			
3	Quinones			
4	Tannins			
5	Flavonoids			
6	Phenols			
7	Anthocyanin			
8	Coumarins			

3.4.1. Table showing phytochemical results of leaf extracts

Plant Constituents	Catharanthus roseus	Ficus religiosa	Hibiscus subdariffa
Alkaloids	+	-	-
Terpenoids	+	+	-
Quinones	-	-	-
Tannins	+	+	+
Flavonoids	+	-	+
Phenols	+	+	+
Anthocyanin	-	+	-
Coumarins	+	-	+

The important phytochemicals responsible for the intermediate reduction of Zinc ion into Zinc oxide

nanoparticles in Catharanthus roseus, Ficus religiosa and Hibiscus subdariffa leaf extracts are flavonoids, tannins, terpenoids.

Therefore, the Zinc oxide nanoparticles synthesised using these leaf extracts were then characterised for further analysis.

3.5.Characterisation of ZnO Nanoparticles:

UV-Vis Spectrophotometer analysis:

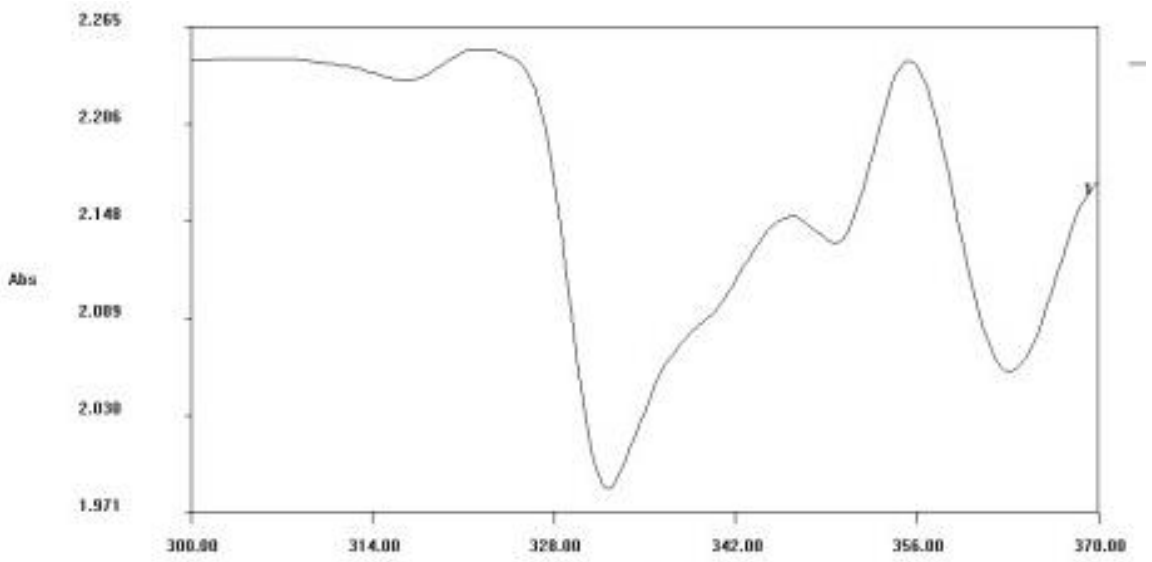


Fig: 3.5.1. UV-Vis analysis of Ficus religiosa ZnO NPs

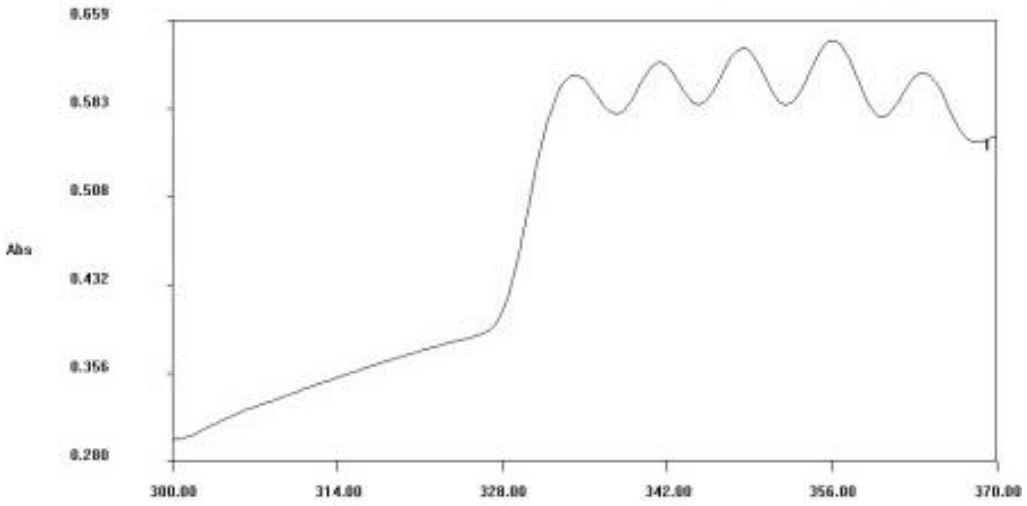


Fig: 3.5.2. UV-Vis analysis of Catharanthus roseus ZnO NPs

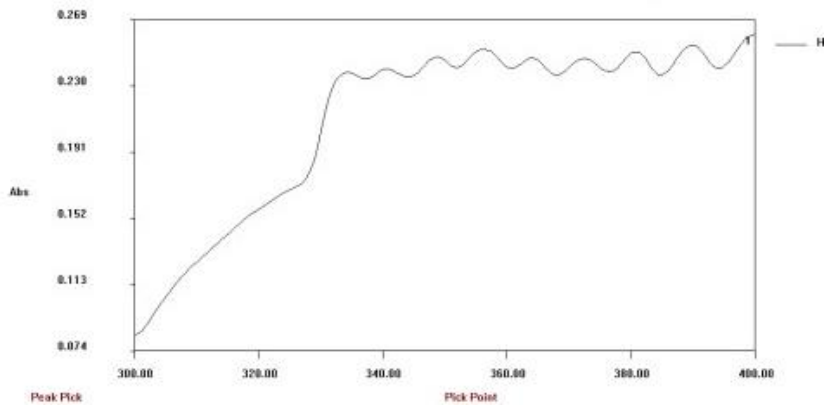


Fig: 3.5.3. UV-Vis analysis of *Hibiscus subdariffa* ZnO NPs

Discussion:

Reduction of zinc ions into zinc nanoparticles during exposure to plant extracts was observedas a result of the colour change. The colour change is due to the Surface Plasmon Resonance phenomenon. The metal nanoparticles have free electrons, which give the SPR absorption band, due to the combined vibration of electrons of metal nanoparticles in resonance with light wave. The sharp bands of zinc nanoparticles were observed around **355.6nm** in case of Ficus Religiosa whereas the bands for Catharanthus roseus were observed around **355.6nm** and thebands for Hibiscus subdariffa were observed around **334.4nm**. From different literatures it was found that the zinc nanoparticles show SPR peak ataround **420 nm.** From our studies we found the SPR peak for Ficus Religiosa at **355.6nm** whereas for Catharanthus roseus it was at **355.6nm** and the bands for Hibiscus subdariffa were observed around **334.4nm**. So, we confirmed that zinc oxide nanoparticles synthesised using leaf extracts have more potential to reduce Zn ions into ZnO nanoparticles than chemically synthesised nanoparticles, which lead us for further research on synthesis of zinc oxide nanoparticles from leaf extracts. The intensity of absorption peak increases with increasing time period. This characteristic colour variation is due to the excitation of the SPR in the metal nanoparticles.

Calculation of Band Gap Energy:

UV Vis Spectroscopy absorption peak means the Electrons are absorbing the Energy at some specific wavelength. Electrons are absorbing Energy means the Electrons are going to excited state from its ground state. Electrons are going to excited state from its ground state means thematerial is having band gap, thus which can be determine by absorption wavelength.

Energy Equation of Quantum Mechanics:

Energy (E)= Planks Constant (h) * Speed of Light (C) / Wavelength (^)

Where, Energy (E) = Band gap, Planks constant (h) = 6.626x10^-34 Joules sec, Velocity of Light (C) = 2.99x10^8 meter/sec and Wavelength (X) = Absorption peak value. Also 1eV = 1.6x10^-19 Joules (Conversion factor)

By this formula band gap can be calculated easily, from UV Vis spectroscopy absorption peak.

3.5. 2: Table showing Band Gap Energies of the Wavelengths observed during UV – Vis analysis

Sample	Band Wavelengths	Band Gap Energy
Ficus religiosa ZnO NPs	355.6nm	3.49 eV
Catharanthus roseus ZnO NPs	355.6nm	3.49 eV
Hibiscus subdariffa ZnO NPs	334.4nm	3.71 eV

Further antimicrobial activity was conducted using 6 bacterial strains (*Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacillus subtilis and Staphylococcus aureus)* to check the activity of zinc oxide nanoparticles synthesised using leaf extracts.

3.6.Antimicrobial Activity of ZnO NPs

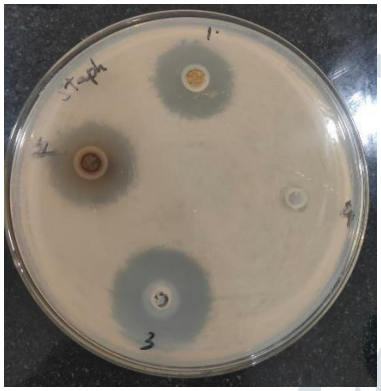


Fig: 3.6.1

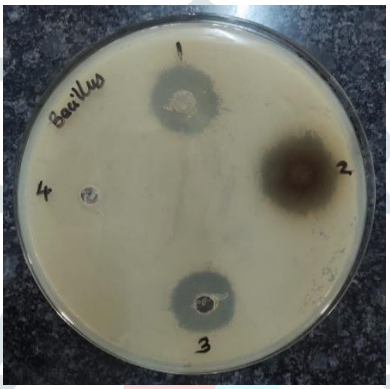


Fig: 3.6.2

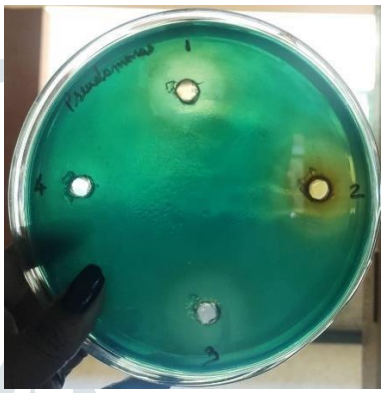


Fig: 3.6.3

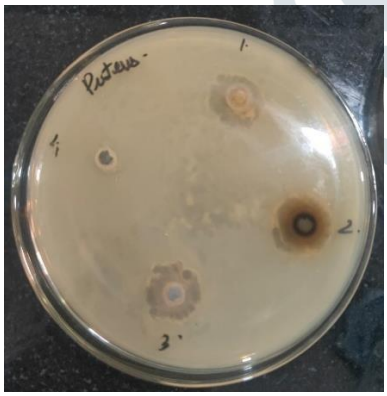


Fig: 3.6.4

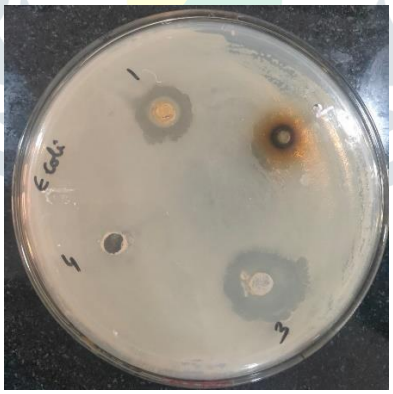


Fig: 3.6.5

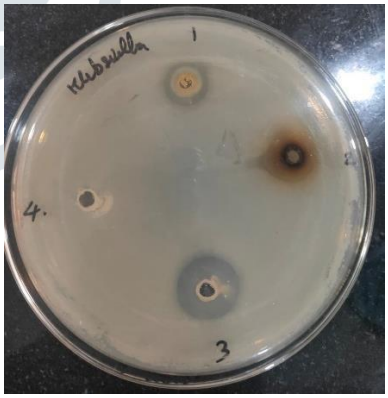


Fig: 3.6.6

Antibacterial activity of ZnO nanoparticles against

Fig: 3.6.1: *Staphylococcus aureus* strain

Fig: 3.6.2: *Bacillus subtilis* strain

Fig: 3.6.3: *Pseudomonas aeruginosa* strain

Fig: 3.6.4: *Proteus mirabilis* strain

Fig: 3.6.5: *Escherichia coli* strain

Fig: 3.6.6: *Klebsiella pneumoniae* strain

Sample 1: zinc oxide nanoparticles -Catharanthus roseus

2: Ficus religiosa - zinc oxide nanoparticles

3: *Hibiscus subdariffa* - zinc oxide nanoparticles dissolved in 4: Control (DMSO)

Table 3.6.1: zone of inhibition of ZnO NPs against Microorganisms

	ZoI of Catharanthu sroseus zinc oxide NP	ZoI of Ficus religiosa zinc oxide NP	ZoI of Hibiscus subdariffa zinc oxide NP	Control (DMSO)
Staphylococcus aureus	**23mm**	**24mm**	**22mm**	**-**
Bacillus subtilis	**23mm**	**28mm**	**23mm**	**-**
Pseudomonas aeruginosa	**20mm**	**21mm**	**21mm**	**-**
Proteusmirabilis	**13mm**	**10mm**	**15mm**	**-**
Escherichiacoli	**10mm**	**15mm**	**20mm**	**-**
Klebsiella pneumoniae	**12mm**	**13mm**	**20mm**	**-**

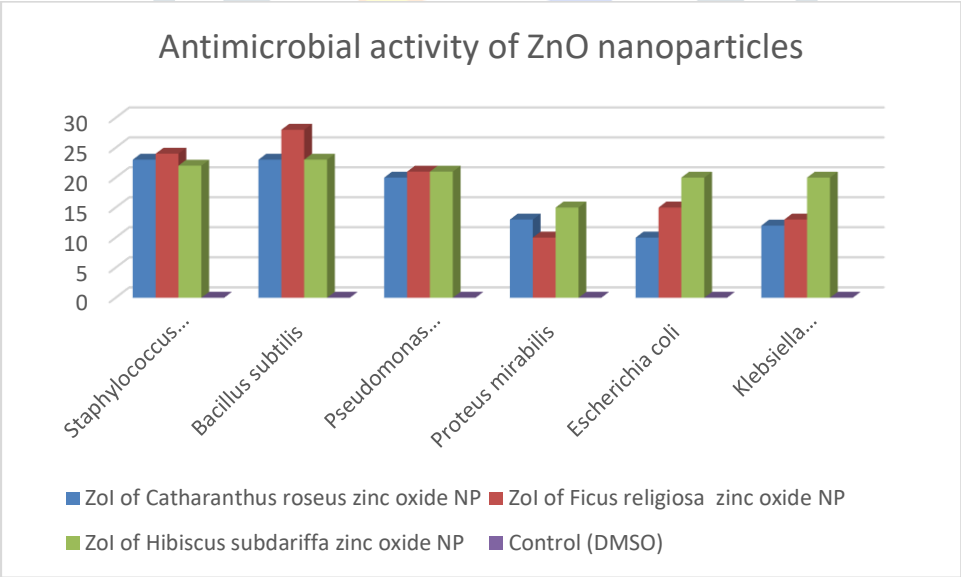


Fig: 3.6.7: zone of inhibition of ZnO Nps against Microorganisms

Discussion:

Figures 3.6.1 to 3.6.6 show that the bacterial strain agar plates with ZnO nanoparticles createdclear zones of inhibition. Each sample showed their unique activity with different lengths of zones of inhibition. It is clear that ZnO NPs synthesised using leaf extracts were very effective against all the cultures.

4. Conclusion

- ZnO nanoparticles have been successfully synthesized using leaf extracts of *Catharanthus roseus*, *Ficus religiosa*, *Hibiscus subdariffa* at room temperature.
- It has been found that flavonoids, tannins, terpenoids are the important phytochemicals responsible for the intermediate reduction of Zinc ion into Zinc oxide nanoparticles inthe leaf extracts.
- These nanoparticles are then characterized using UV Vis Spectroscopy
- The UV-Vis spectroscopic study shows the Plasmon resonance property, confirmed the reduction of metal ion and formation of nanoparticle with plasma resonance peaks between the range of 340nm – 356nm. The ZnO nanoparticles comprises distinguishing colour in colloidal solution due to its miniature dimension. The bands of zinc nanoparticles proves the zinc metal reduces the leaf extract extract more efficiently.
- It has been found that all ZnO nanoparticles were effective antimicrobial agents.

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