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Research Article

Cytotoxic Effects of Biosynthesized Zinc Nanoparticles on Normal Fibroblast Cells and Their Antimicrobial Effect on Pathogenic Strains

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ABSTRACT

Nanoparticles, particularly zinc nanoparticles (ZnNPs), have garnered substantial interest in biomedical research for their potential applications in therapeutics and antimicrobial therapy. In this study, we investigated the cytotoxic effects of biosynthesized zinc nanoparticles on normal fibroblast cells and evaluated their antimicrobial efficacy against pathogenic strains. The biosynthesis of zinc nanoparticles was achieved through an eco-friendly and cost-effective approach utilizing biological agents. The cytotoxicity of biosynthesized zinc nanoparticles was assessed on normal fibroblast cells, serving as a model for evaluating biocompatibility. Our results indicate that biosynthesized zinc nanoparticles exhibited minimal cytotoxicity towards normal fibroblast cells, suggesting their potential safety for biomedical applications. Furthermore, the antimicrobial activity of biosynthesized zinc nanoparticles was evaluated against pathogenic strains, including antibiotic-resistant bacteria. The antimicrobial efficacy of zinc nanoparticles demonstrated promising results, highlighting their potential as alternative antimicrobial agents. Overall, this study provides valuable insights into the cytotoxic effects of biosynthesized zinc nanoparticles on normal fibroblast cells and underscores their antimicrobial potential against pathogenic strains, offering opportunities for the development of nanoparticle-based therapeutics and antimicrobial agents in medicine and healthcare.

1. INTRODUCTION

In recent years, the emergence of nanotechnology has revolutionized various fields including medicine and microbiology. Nanoparticles, due to their unique physical and chemical properties, have garnered significant attention in biomedical research for their potential applications in drug delivery, imaging, and antimicrobial therapy. Among these nanoparticles, zinc nanoparticles (ZnNPs) have gained prominence owing to their biocompatibility, low toxicity, and antimicrobial properties [1].

In the context of biomedical research, understanding the cytotoxic effects of nanoparticles on normal cells is crucial to ascertain their safety profile for potential therapeutic use. Fibroblast cells play a pivotal role in tissue repair and maintenance, making them an important model for assessing the biocompatibility of nanoparticles [2]. Thus, investigating the cytotoxic effects of biosynthesized zinc nanoparticles on normal fibroblast cells serves as a fundamental step in evaluating their potential biomedical applications.

Moreover, the antimicrobial properties of zinc nanoparticles have drawn attention as a promising strategy to combat microbial infections [3]. Pathogenic strains of bacteria pose a significant threat to public health due to their increasing resistance to conventional antibiotics. Hence, exploring the antimicrobial efficacy of biosynthesized zinc nanoparticles against these pathogens is imperative for developing alternative antimicrobial agents.

In this study, we aim to investigate the cytotoxic effects of biosynthesized zinc nanoparticles on normal fibroblast cells and evaluate their antimicrobial activity against pathogenic strains. The biosynthesis of zinc nanoparticles offers an eco-friendly and cost-effective approach, utilizing natural resources or biological agents such as plant extracts, fungi, or bacteria. By utilizing biosynthesized zinc nanoparticles, we aim to explore their potential advantages over conventionally synthesized nanoparticles, including enhanced biocompatibility and reduced environmental impact.

Understanding the cytotoxic effects of biosynthesized zinc nanoparticles on normal fibroblast cells will provide valuable insights into their safety profile for biomedical applications. Additionally, assessing their antimicrobial efficacy against pathogenic strains will contribute to the development of novel antimicrobial agents to combat microbial infections.

In summary, this study addresses the dual objectives of assessing the cytotoxic effects of biosynthesized zinc nanoparticles on normal fibroblast cells and evaluating their antimicrobial activity against pathogenic strains. The findings from this investigation hold significant implications for the development of nanoparticle-based therapeutics and antimicrobial agents with potential applications in medicine and healthcare.

In present study, zinc nanoparticles were biosynthesized using green chemistry route and characterized using UV-Vis spectroscopy and Scanning Electron Microscopy. These biosynthesized zinc nanoparticles were tested for antimicrobial activity and bio toxicity assay.

2. MATERIALS and METHODS

2.1. Herbal Plants

Procurement of herbal plants from authentic sources was an extremely important factor in this research. Hence, all herbal plants under evaluation were collected from the botanical garden of Navsari Agriculture University (N.A.U.), Navsari. Botanical identification and authentication was carried out by the authorities of the Herbarium of N.A.U. Plants used for the study are *Mallotus phillipensis*, *Kalanchoe pinnata*, *Syzygium cumini*, *Citrus sinensis*, *Aegle marmelos* and *Flacourtia indica*.

2.2. Bacterial Strains

All bacterial cultures were procured from N.C.I.M., Pune, India. This study included Gram positive bacterial strains, Gram negative bacterial strains and also fungal strains. Bacterial and fungal cultures used in the study are *Staphylococcus aureus* (NCIM 2079), *Escherichia coli* (NCIM 2065), *Pseudomonas aeruginosa* (NCIM 2863), *Salmonella typhi* (NCIM 2501), *Klebsiella pneumonia* (NCIM 2883), *Aspergillus niger* (NCIM 1004) and *Candida albicans* (NCIM 3102).

2.3. Chemicals and Media

DMSO and Methanol were purchased from Finar. Zinc nitrate was procured form Loba Chemie. Nutrient agar, Nutrient broth, Muller Hilton agar, Rose Bengal agar, RPMI 1640/ D-MEM growth medium supplemented with 10% foetal calf, MTT solution were purchased from Hi-Media Laboratories Pvt Ltd (Mumbai, India).

2.4. Synthesis of ZnNPs

The present work was carried out at Department of Microbiology, B. P. Baria Science Institute, Navsari, Gujarat, India. All the plants after their identification were thoroughly washed with water and dried under shade for about ten days. The dried plant samples were ground well into fine powder in a mixture grinder. 10 gm leaves powder of *Flacourtia indica*, *Syzygium cumini*, *Citrus sinensis*, *Mallotus phillipensis*, *Kalanchoe pinnata* and *Aegle Marmelos* were each mixed with 100 ml organic solvent (methanol) separately in each flask. The mixture was incubated at room temperature for 48-72 hours on rotary shaker. Mixture was filtered using Whatmann Filter Paper No.1 and evaporated in the hot air oven at room temperature to yield the powder form of pure extract. Stock solutions of crude extracts were prepared by mixing organic solvent with appropriate amount of dried extracts to obtain a final concentration of 100 mg/ml. The solvent used was 20% DMSO. The filtrates were collected and stored at 4 ºC for conducting further experimental work related to biosynthesis of nanoparticles.50mL of each leaf extract was taken and boiled to 60-80 ˚C. 5 grams of zinc nitrate was added to the solution as the temperatures reached 60 ˚C. This mixture is then boiled until deep, yellow-colored precipitates are formed and flask was kept overnight at room temperature [4].

2.5. Characterization of ZnNPs

2.5.1. UV Visible Spectral Analysis

Although, color change is an indicator of nanoparticle formation, it cannot be the sole, reliable parameter indicating formation of ZnNPs, hence the shift in the absorption spectra for ZnNPs as observed by UV-Spectrophotometer (Equip-Tronics, EQ-824). The shift in the absorbance spectra indicated the formation of nanoparticles. Distilled water taken as blank.

2.5.2. Scanning Electron Microscopy (SEM)

The SEM analysis was performed at Sardar Vallabhbhai National Institute of Technology (SVNIT), Surat, Gujarat, India. Scanning Electron Microscopy (SEM) was done to examine the average particle size and morphology of the ZnNPs. A drop of aqueous solution containing the ZnNPs was placed on the SEM's

sample stage. Once vacuum is attained in the sample chamber, the operator then proceeds to align the electron gun in the system to the proper location. The electron gun shoots out a beam of high energy electrons, which travels through a combination of lenses and apertures and eventually hits the sample. As the electron gun continues to shoot electrons at a precise position on the sample, secondary electrons will bounce off the sample. These secondary electrons are identified by the detector. The signal found from the secondary electrons is amplified and sent to the monitor, creating a 3D image.

2.6. Antimicrobial activity by agar diffusion method

Antimicrobial activity were performed at B.P.Baria Science Institute, Navsari, Gujarat, India. Antimicrobial activity of zinc nanoparticles was tested individually against test organisms by Agar Well Diffusion method. Pure cultures were subcultured into sterile nutrient broth and incubated at 37 ºC for 24-48 hours. Each test organism was spread uniformly onto the individual plates using spread plate technique. Wells of 8 mm diameter were made on pre-incubated nutrient agar plates using cup borer. Using sterile micropipette tips, 100 µL of each biosynthesized nanoparticle was pipetted into the above mentioned well in all the plates. After incubation, the diameters of zone of inhibition were measured.

2.7. Bio toxicity Assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was conducted using a modified method of **Labieniec *et al.* (2003)** and **Lapshina *et al.* (2005) [5,6].** Normal fibroblast cell were used in this assay, it was obtained from extra part of product of conception material at Shree Krushna Gene Lab and Research Center, Rajkot. The normal fibroblast cells were cultivated in RPMI 1640/D-MEM growth medium supplemented with 10% foetal calf. The cells were grown in incubator at 37oC and 5% CO_2.

Confluent cells (70–80%) in Corning flasks were detached using a trypsin solution followed by removing the trypsin. The cells were re-suspended in D-MEM growth medium and counted using a hemacytometer. Cell densities were adjusted to 1x105 cells/mL. The suspension of cells (1000 µL) was seeded into a pre-labeled micro centrifuge tubes. Three set for replica for each concentration of compound used were made. The tubes were incubated at 37°C and 5% CO_2 for 24 hours.

After seeding the cells for 24 hours, the tubes were taken out from the incubator and the cells were exposed with D-MEM growth medium containing test compound and incubated for a further 24 hours at 37°C and 5% CO_2. Each compound was tested in ten different concentrations for the normal fibroblast cells. There after 100 µL of MTT solution (5 mg/ml final concentration) was added to the wells and incubated for 1 hour at 37°C and 5% CO_2. At the end of the treatments, the medium was removed prior to the addition of DMSO (200 µL) into each well. The 24-wells plate was allowed to stand for 10 minutes followed by shaking for 15 seconds. The colour was measured at wavelength 562 nm. The absorbance obtained was used to determine the IC_{50} values. The cytotoxic nature of ZnNPs was calculated using the following formulas [7].

$$\% \text{ Cell viability} = (\text{Absorbance of test} \div \text{Absorbance of control}) \times 100$$

$$\text{Cytotoxicity} = 100 - \% \text{ cell viability}$$

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3. RESULTS

3.1. Phytochemical Screening

The results of phytochemical tests indicated presence of secondary metabolites in leaf extracts of *Flacourtia indica (Burm. f.) Merr., Syzygium cumini L., Citrus sinensis, Mallotus phillipensis, Kalanchoe pinnata* and *Aegle marmelos* as listed in Table-1. Preliminary phytochemical screening has indicated the presence of alkaloids, tannins, saponins, flavonoids, proteins, steroids and terpenoids in almost all the plant extracts.

Tablo 1. Qualitative phytochemical analysis of methanolic extract of *Flacourtia indica* (Burm. f.) Merr., *Syzygium cumini, Citrus sinensis, Mallotus phillipensis, Kalanchoe pinnata* and *Aegle marmelos*

Sr.N	Phyto-constituents	Results					
		Flacourtia indica (Burm. f.) Merr.	***Syzygium cumini***	***Citrus sinensis***	***Mallotus phillipensis***	***Kalanchoe pinnata***	***Aegle marmelos***
1	Alkaloids	+	-	+	+	+	+
2	Tannins	+	+	-	+	+	+
3	Saponins	+	+	-	-	+	+
4	Anthraquinones	-	-	-	-	-	-
5	Anthocyanides	+	-	-	-	-	-
6	Phenolic flavonoids	+	+	-	+	+	+
7	Flavonoids	+	+	-	+	+	+
8	Carbohydrates	-	-	-	+	-	-
9	Proteins	+	+	+	+	+	+
10	Steroids	+	+	-	+++	-	-
11	Terpenoids	+	++	-	+	+	+
12	Cardiac glycosides	-	-	-	-	-	-
13	Phlobatannins	-	-	-	-	+	+

3.2. Synthesis of ZnNPs

Various herbal plants have been used for the green synthesis of zinc nanoparticles. Synthesis of ZnNPs is most commonly done by reduction of the zinc nitrate. The possible mechanism involved in the formation of zinc nanoparticles is dependent on the reaction of the zinc ions present in the solution with the polyphenols such as the tannins, glycosides, and flavonoids that are present in the plant extract forming the complexation [8]. Light brown to dark brown colour indicates the formation of zinc nanoparticles in the reaction vessels (Figure 1). Different color change observed in the reaction vessel is due to an effect called Localized Surface Plasmon Resonance.

Figure 1. Biosynthesis of Zinc Nanoparticles. (A) 1mM $ZnNO_3$, Biosynthesis of Zinc nanoparticles, using B) *Kalanchoe pinnata,* C) *Mallotus phillipensis,* D) *Citrus sinensis,* E) *Aegle marmelos, F) Flacourtia indica* and G) *Syzygium cumini* respectively.

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3.3. Characterization of ZnNPs

3.3.1. UV-Vis Analysis

Figure 2 represents the UV absorption spectra of zinc nanoparticles biosynthesized using 6 different herbal plants. The absorption peak of the all prepared zinc nanoparticles was found in between 350-410 nm of UV-Vis range.UV-Vis analysis infers that these biosynthesized nanoparticles represent LSPR bands in the visible region. It is related to excitation of electrons present in the metal nanoparticles after their unique interaction with electromagnetic field of light. These excited electrons in plasmonic nanoparticles oscillate collectively to only specific wavelength of light. Therefore, they exhibit selective photon absorption, which can easily be monitored using UV-Vis spectrophotometer [9].

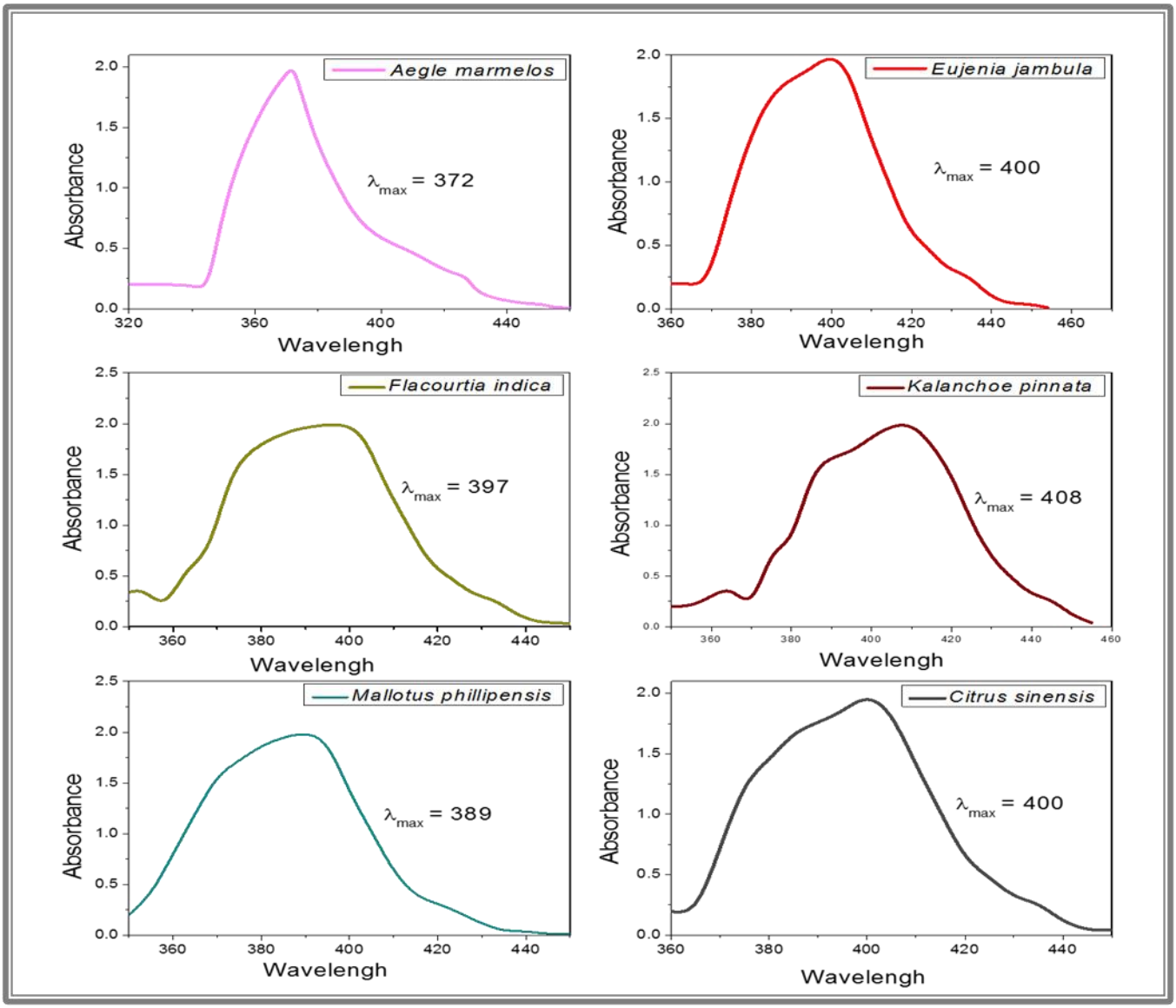

Figure 2. UV-Vis Spectra of zinc nanoparticles prepared using different herbal extracts: *Citrus sinensis*, *Aegle marmelos*, *Mallotus phillipensis*, *Kalanchoe pinnata*, *Flacourtia indica* and *Syzygium cumini*.

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The use of different herbal plants in the biosynthesis process can lead to variations in the size, shape, and surface chemistry of the ZnNPs. These factors, in turn, affect the LSPR and the corresponding absorption peak. Plant extracts typically contain various bio molecules like flavonoids, terpenoids, and alkaloids, influencing their optical properties. The unique interaction between the metal nanoparticles and the phytochemicals present in the plant extracts may result in slight variations in the UV-Vis absorption spectra, even though all ZnNPs exhibit LSPR bands within the visible range.

3.3.2. SEM Analysis

The nanoparticles shape and size was characterized by high-resolution scanning electron microscopy. SEM images were seen in 2 µm magnification range, which clearly demonstrated the presence of almost all spherical shaped nanoparticles with average particle size ranging from 65-99 nm (**Figure.3 A-F**).Out of the total six nanoparticles synthesized, zinc nanoparticles synthesized from *Flacourtia indica* has smallest size nanoparticle (~65-72 nm) as compared to others.

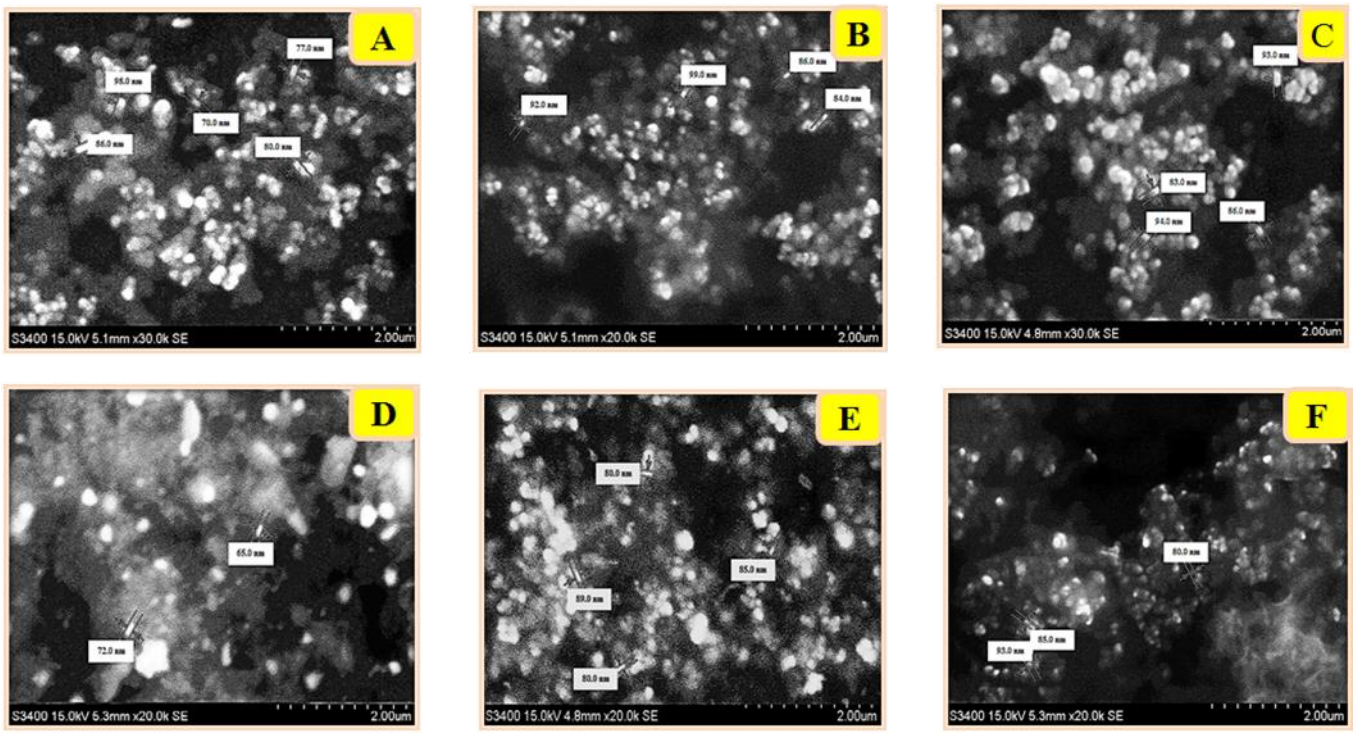

Figure 3. SEM images of zinc nanoparticles prepared using different herbal extracts**,** (A) *Aegle marmelos*, (B) *Citrus sinensis*, (C) *Syzygium cumini*, (D) *Flacourtia indica*, (E) *Mallotus phillipensis*, (F) *Kalanchoe pinnata*.

3.4. Antimicrobial Potency

The biosynthesized zinc nanoparticles from 6 different herbal plants were noticed to be active against fungi than bacteria (Figure 4), thereby exhibiting strong activity and wide antimicrobial action. From the graphical representation (Figure 5), it can be clearly seen that in case of antifungal activity, zinc nanoparticles synthesized from all the six plants showed highest activity against *Aspergillus niger*as well as against *Candida albicans*. Moderate activity was recorded for antibacterial activity against all bacterial

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strains. Antifungal activity is likely derived through electrostatic attraction between the negatively charged cell membranes of microorganisms and the positively charged nanoparticles [10]. Inhibition zones vary according to types of pathogens involved, synthesis methods applied and concentrations of nanoparticles and size of biosynthesized metal nanoparticles used.

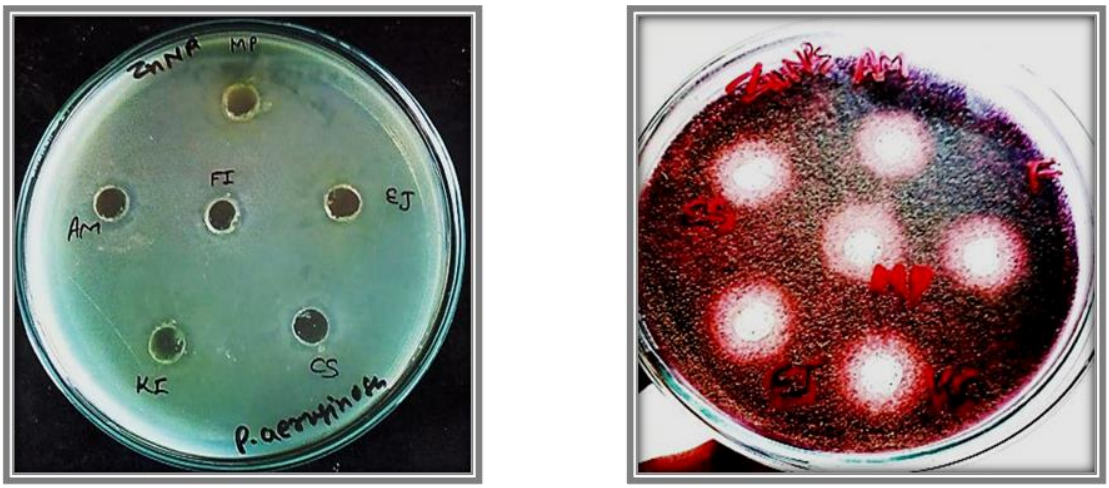

Figure 4. Antimicrobial activity of zinc nanoparticles

In case of *Escherichia coli* and *Klebsiella pneumoniae* lower standard deviation indicates that the zone sizes are relatively consistent across the different plant extracts. Whereas in case of *Aspergillus niger*, *Candida albicans* higher standard deviation suggests more variability in the zone sizes, possibly due to the differing effectiveness of the plant extracts against these strains.

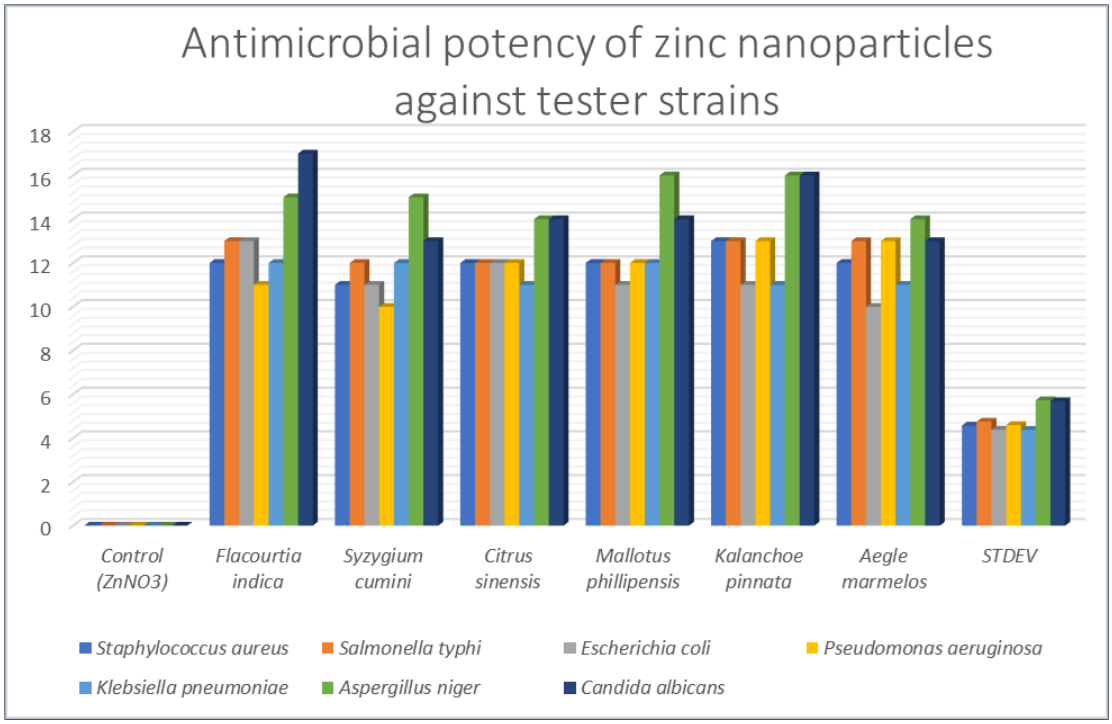

Figure 5. Antimicrobial potency of zinc nanoparticles against tester strains

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Tablo 2. Antimicrobial activity of biosynthesized zinc nanoparticles against pathogenic strains with Standard Deviation

Microbial strains / Plant extracts	*Staphylococcus aureus*	*Salmonella typhi*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Aspergillus niger*	*Candida albicans*
Control (ZnNO3)	0	0	0	0	0	0	0
Flacourtia indica	12	13	13	11	12	15	17
Syzygium cumini	11	12	11	10	12	15	13
Citrus sinensis	12	12	12	12	11	14	14
Mallotus phillipensis	12	12	11	12	12	16	14
Kalanchoe pinnata	13	13	11	13	11	16	16
Aegle marmelos	12	13	10	13	11	14	13
STDEV	4.5721726	4.75094	4.386125	4.598136	4.375255	5.72796	5.682052

The p-value obtained from the ANOVA test for the given data **(Table.2**) is approximately 0.828. Since the p-value is much greater than the common significance level of 0.05, there is no statistically significant difference in the inhibition zones among the different plant extracts for the various microbial strains. This suggests that the plant extracts do not differ significantly in their effects on the microbial strains tested.

3.5. Bio toxicity Assay

MTT assay was performed on normal cells. Here in this study as mentioned earlier, normal fibroblast cellswere chosen. The small size and the relatively large surface area of nanoparticles resulted in increased toxicity when compared to particles in micrometer size. Therefore, the present study was carried out with objective to evaluate the toxic potential of biosynthesized zinc nanoparticles on exposure to normal fibroblast cells [11]. The cytotoxicity of zinc nanoparticles was initially investigated by measuring the cellular activity. The results of the MTT viability assay showed that zinc nanoparticles have significant toxic effect as per used different concentrations (0.125 µL to 100 µL) on normal fibroblast cells.

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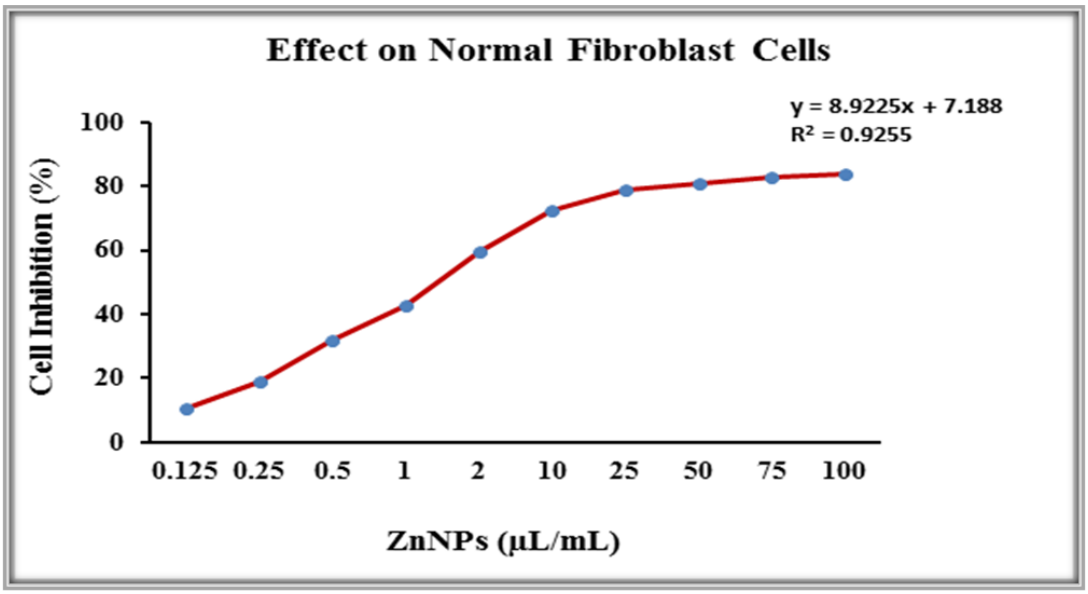

Figure 6. Effect of zinc nanoparticles on cell viability

However, the IC_{50} values were calculated from a regression curve (Figure.6).These values represent the effective concentration of copper nanoparticles that decreases the amount of viable cells to 50% after 24 hours. Values of IC_{50} obtained by MTT assay resulted to be higher for normal fibroblast cells (4.79 µL/mL). In the present study, exposure of zinc nanoparticles caused concentration dependent cytotoxicity as revealed by cell viability assay.

4. DISCUSSION

The green synthesis of zinc nanoparticles (ZnNPs) using plant extracts is an eco-friendly and sustainable approach that has garnered significant attention in recent years. The method utilizes the rich phytochemical content of plants, particularly polyphenols like tannins, glycosides, and flavonoids, which act as both reducing and stabilizing agents during nanoparticle synthesis. The mechanism underlying the formation of ZnNPs is largely attributed to the reduction of zinc ions (Zn^{2+}) in the presence of these bioactive compounds, resulting in the formation of zinc nanoparticles.

The process of ZnNP formation begins with the reduction of zinc nitrate, a common precursor, in the presence of plant extracts. According to Solabomi *et al.* (2019), the interaction between zinc ions and polyphenols is critical, leading to the complexation that ultimately results in the formation of nanoparticles [8]. Polyphenols play a dual role; they not only reduce Zn^{2+} to Zn^{0} but also stabilize the newly formed nanoparticles, preventing their aggregation. This mechanism has been supported by several other studies. Sharmila *et al.* (2019) reported the successful synthesis of ZnNPs using *Tecoma castanifolia* leaf extract, highlighting the role of quercetin, a potent flavonoid, in reducing Zn^{2+} and forming stable nanoparticles [12].

The color change observed during the synthesis, from light brown to dark brown, is an important visual indicator of nanoparticle formation. This color change is associated with a phenomenon known as Localized Surface Plasmon Resonance (LSPR), which occurs due to the collective oscillation of electrons on the surface of the metal nanoparticles when excited by light at specific wavelengths. Other studies have also

observed similar UV-Vis absorption patterns, corroborating the findings related to LSPR. Supriya and kumari (2019), synthesized AgNPs using Aloe vera extract and observed an absorption peak at around 430 nm, consistent with the LSPR effect [13]. This observation was similar to the results obtained using other plant extracts, reinforcing the role of LSPR as a fundamental property of metallic nanoparticles.

The smaller size of ZnNPs synthesized using *Flacourtia indica* (~65-72 nm) can be attributed to the specific phytochemicals present in the extract, such as polyphenols, flavonoids, and tannins. These compounds not only reduce zinc ions but also act as capping agents, stabilizing the nanoparticles and limiting their growth, which results in smaller sizes. A recent study by Kumar et al. (2022) demonstrated that ZnNPs synthesized using *Azadirachta indica* (Neem) had sizes ranging from 75-85 nm, with the phytochemical composition of the extract being a key factor in determining the nanoparticle size [14]. For instance, smaller ZnNPs have been shown to possess enhanced antibacterial properties due to their ability to interact more effectively with microbial cells.

Moreover, in vitro antibacterial and antifungal activities of ZnO nanoparticles have been widely reported [15,16]. Green synthesized ZnO NP using *Beta vulgaris, Cinnamomum tamala, Parthenium hysterophoru*, orange fruit extract showed strong inhibition against *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Candida albicans, Aspergillus niger, Botrytis cinerea, Penicillium expansum* etc. [17, 16, 18, 19, 20]. Similarly, our study highlighted the antibacterial and antifungal potential of green synthesized ZnNPs against different bacterial strains i.e., *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Klebsiella pneumonia* and fungal strains *Aspergillus niger* and *Candida albicans* (Table: 2)

The cytotoxic effects observed in your study can be attributed to the small size of the nanoparticles, which increases their surface area relative to volume, thereby enhancing their interaction with cellular components. This increased surface area allows for greater reactivity, which can lead to higher levels of oxidative stress, membrane damage, and ultimately, cell death. A study by Chen *et al*. (2019) supports these findings, showing Size-dependent cytotoxicity study of ZnO nanoparticles in HepG2 cells [21]. Zinc nanoparticles (ZnNPs) hold significant potential in cancer therapy due to their unique physicochemical properties, including small size, large surface area, and ability to generate reactive oxygen species (ROS). However, further research needs to optimize their design, improve targeting efficiency, and ensure safety in clinical applications, ZnNPs could become a valuable tool in the fight against cancer.

4. CONCLUSIONS

Total six different herbal plants were used to biosynthesize zinc nanoparticles. All of the zinc nanoparticles biosynthesized during the current study showed potent biological activities against all microbial tester strains. Out of the total six nanoparticles synthesized, zinc nanoparticles synthesized from *Flacourtia indica* was chosen for bio-toxicity assay due smallest size nanoparticle (~65-72 nm) as compared to others. Zinc nanoparticle has significant toxic effect as per used different concentrations on normal fibroblast cells. Therefore, our results concluded that sensitivity towards nanoparticle exposure is depends on the concentration of nanoparticles.

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The Declaration of Conflict of Interest/ Common Interest
No conflict of interest or common interest has been declared by the author.

Author's Contribution
The authors contributed equally to the study

The Declaration of Ethics Committee Approval
This study does not require ethics committee permission or any special permission.

Declaration of research and publication ethics
The authors of the paper declare that we followed the scientific, ethical and citation rules of Environmental Toxicology and Ecology in all processes of the paper and that we did not make any falsification of the data collected. Furthermore, we declare that ETOXEC and its Editorial Board are not responsible for any ethical violations that may have occurred and that this study has not been evaluated in any other academic publication environment than ETOXEC.

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