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Green Synthesis of Zinc Oxide Nanoparticles, Characterization, Antibacterial and Cytotoxicity Against HepG2 Cells Using *Syringodium isoetifolium*

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ABSTRACT: Green nanoparticle synthesis using biological systems, particularly plant extracts is a growing subject in nanotechnology. Leaves extract of *Syringodium isoetifolium* was used to successfully show the environmentally friendly manufacture of zinc oxide nanoparticles (ZnO NPs) and zinc nitrate were used as precursor in this study. The nanoparticles were examined using X-Ray Diffraction (XRD), Energy-Dispersive X-ray analysis (EDX), UV spectroscopy (UV-VIS), Scanning Electron Microscopy (SEM), and Fourier Transform Infrared Spectroscopy (FT-IR). The absorption peak in 223 nm range was discovered via UV–Vis spectroscopy. XRD and FTIR verified the involvement of *Syringodium isoetifolium* bioactive substances in the steadiness of zinc oxide nanoparticles. SEM and EDX investigation indicated an agglomerated asymmetrical, hexagonal morphology and presence of O, Zn, C and K. Elemental mapping study of produced ZnO NPs revealed 60% zinc distribution, 26% oxygen distribution, and 13% carbon distribution. The produced ZnO NPs had a mean particle size of 26 nm was determined using dynamic light scattering (DLS) measurements. Additionally, an *in vitro* assay was used to assess the ZnO NPs' cytotoxic effects on the HepG2 cell line. The ZnO NPs had a strong cytotoxic effect on the HepG2 cancer cell line, as shown by the results of the MTT assay. ZnO NPs exhibited excellent antimicrobial potencies against four different bacterial via pour plate method, and their zone of inhibition values were calculated.
KEYWORDS: Zinc oxide nanoparticles, *Syringodium isoetifolium*, Green synthesis, Characterization, MTT assay, Cytotoxicity potential

1. INTRODUCTION

Nanotechnology has risen to eminence in technological progressions due to their tunable physicochemical characteristics like melting point, electrical and thermal conductivity, wettability, catalytic activity, and light absorption and scattering which result in enhanced performance than bulk equivalents. Nanoparticle synthesis is a potential area of nanobiotechnology research plays vital role to control the shape, size, and composition of nanoparticles, as each of these parameters plays a key role in deciding various applications [1]. Nanotechnology, as a new research area that encompasses a wide range of nanoscale technologies is playing a progressively significant part in the development of innovative methods for producing new products [2]. Herbal medications are becoming increasingly popular among the general public, with these "natural medicines" being seen as milder as and safer than their synthetic equivalents, with less side effects and adverse drug responses. Clinical evidence to support the use of herbal medicines as a treatment for a wide range of ailments. In addition to the foregoing, there are concerns about the deprived or indeterminate bioavailability of active phytochemical ingredients, with herbal medicine's *in vitro* shown potential not necessarily translating to beneficial *in vivo* therapeutic uses. Novel medication delivery technologies have the potential to overcome natural medicine's constraints.

By applying nanostructures and nanophases to diverse branches of study, nanotechnology is proven to bridge the gap between biological and physical sciences [3]. Researchers exhibited inordinate interest in using aqueous plant extract and microorganisms to synthesize metal and metal oxide NPs because they are environmentally friendly, stable, therapeutically adaptive, biocompatible and cost-effective. Natural

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chemicals found in biological systems serve an important and flexible materials are used to make nanoparticles and act as overlaying agents to
keep them stable. Plants have significant benefits over other biological systems according to the research review. Plant extracts produce more stable nanoparticles than synthetic nanoparticles because they are readily available and safe to handle [4]. Furthermore, biological things may serve as a template for nanoscale assembly, synthesis, and organization [5], electrochemical depositions [6], chemical vapor deposition [7], Laser ablation [8], hydrothermal methods [9], sol–gel method [10], Green synthesis [11, 12] and combustion method [13] have all been documented. Ultrasound [14], microwave-assisted combustion [15], two-step mechanochemical-thermal synthesis [16], anodization [17], co-precipitation [18] and electrophoretic deposition [19] have all been used to make ZnO NPs recently. This study's aim was to analyze the eco-friendly ZnO NP's activity against various pathogen strains and assess its cytotoxic effects on the HepG2 cell line using the MTT assay.

2. RESULTS AND DISCUSSION

2.1. UV-Visible Spectroscopy

The production of ZnO NPs from zinc nitrate was studied using UV spectroscopy. The absorption peak is 223nm within the absorption range for ZnO NPs, which is 220–230 nm, according to our findings. Figure 1 shows the UV–visible absorbance of produced ZnO NPs. The greater blue shift absorption of manufactured ZNPs compared to bulk ZnO could be owing to a significant reduction in particle size [22].

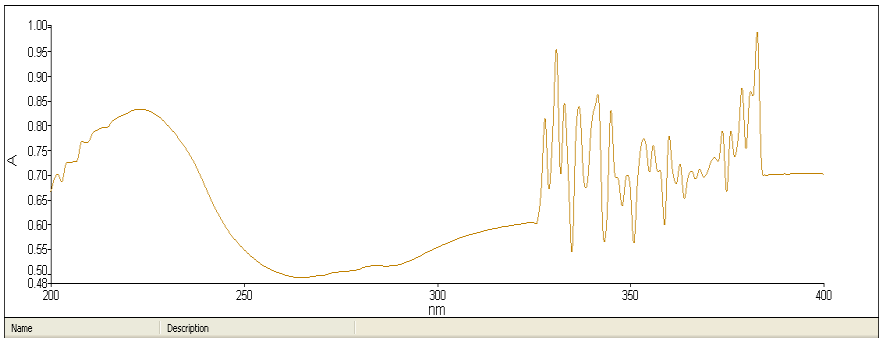

Fig 1: Absorption spectra of ZnO Nanoparticles

2.2. FTIR Analysis

At room temperature, FTIR measurement of the produced ZnO NPs was performed between 4,000 cm^{-1} to 500 cm^{-1}. Spectra (FTIR) reveals *Syringodium isoetifolium* bioactive compounds and their distribution on ZnO NPs surface. Functional groups in the plant extract that are involved in the bonding process with ZnO NPs were identified using FTIR analysis. Fig 2(a) shows the spectra of synthesized ZnO NPs which demonstrated that the NPs have different
absorption bands. As a result, FTIR spectra showed multiple peaks at 3374, 2358, 1561, 1413, 1022, 888, 681, 615, 544 cm^{-1}. The O–H bending of water molecules adsorbed on the material was linked to broad absorption bands around 3,374 cm^{-1}. Peaks observed at 2368 cm^{-1} illustrate the H–O–H vibration of a group of crystallised water molecules [23]. C=C stretching of an aromatic ring and stretching in polyphenol (C=O), C–H bending vibration of an alkane group, stretching of C–N, and bending vibration of C–H, respectively, have peaks at 1561 cm^{-1}, 1413 cm^{-1}, 1022 cm^{-1}, and 888 cm^{-1}[24]. The presence of C-alkyl chloride and ZnO hexagonal phase may be seen in tiny peaks observed at 681 cm^{-1}, 615 cm^{-1}, and 544 cm^{-1}[25].
Fig 2(b) shows the spectra of plant extract which demonstrated that *S. Isoetifolium* have different absorption bands. The band occurred at 1604 cm^{-1} attributes to the presence of C=N stretching, peak at 1413 cm^{-1} corresponding to adenine NH_2 and peak observed at 1198 cm^{-1} was overlapping of protein amide III. Glycogen is represented by the peak at 1021 cm^{-1}, while Sulphur compounds are represented by the peak at 486 cm^{-1}.

Previous research on the phytochemical contents of *S. Isoetifolium* backs up the current findings. Plant extracts comprise phenolic chemicals, flavonoids, saponins, tannins, alkaloids.
The presence of these phytochemicals was justified by their IR bands. Furthermore, the biosynthesized ZnO NP's FTIR spectra revealed a strong and intense band at 544 cm^{-1}, confirming the occurrence of Zn-O vibrations [26].

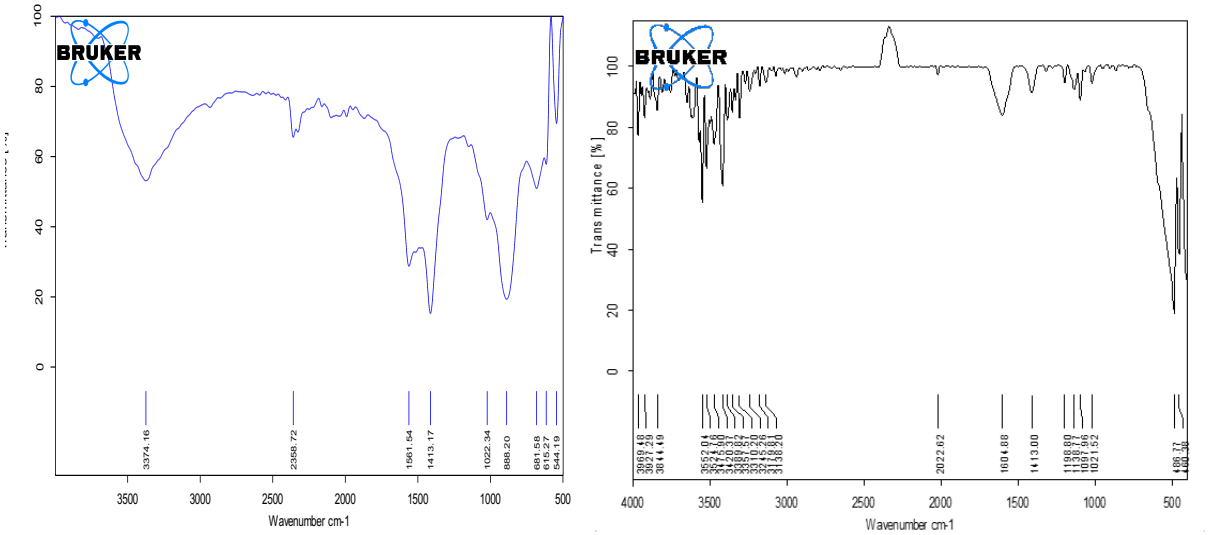

Fig 2: IR Spectra of *Syringodium isoetifolium*

a-Nanoparticle b-Leaf extract

2.3. Dispersive Energy X Ray Spectroscopy

EDX spectra were used to reveal the surface chemical composition of the generated ZnO NPs. In green nanoparticles, distinct Zn signals were observed, as well as one signal each for C, O, and K. The presence of C, O and K signals on generated ZnO NPs confirmed the presence of *S. Isoetifolium* bioactive compounds.
The produced ZnO NPs had a 60 percent zinc distribution, 26 percent oxygen distribution and 13 percent carbon distribution, according to an elemental mapping analysis. The lack of additional elements validates the purity of produced nanoparticles, as shown in Fig 3A. Elemental analysis of ZnO NPs was confirmed by EDX as shown in Fig 3A. The EDX results suggested that the largest quantity of zinc may be used to determine green ZnO NPs production. The presence of carbon in trace amounts suggests that plant phytochemical groups are engaged in the reduction and capping of ZnO NPs that have been synthesised [27].
SEM pictures were investigated in various magnifications to study the surface morphology of the synthesized ZnO NPs [28], shown in Fig 3B. The nanoparticles were found to be in the
agglomerated form. The outcome of surface morphology and its correlation with the synergistic activity of ZnO were observed from the already reported literature [29]. SEM images represents the particles in oval and hexagonal in shapes, which was further ensured by means of XRD.

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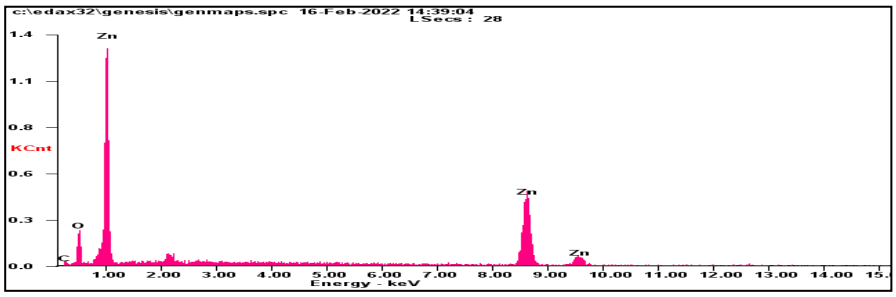

Fig 3A: EDX spectra of ZnO Nanoparticles

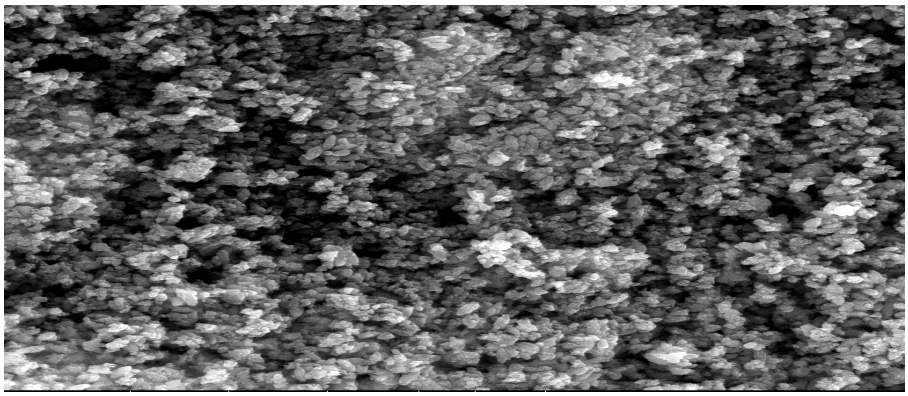

Fig 3B: SEM images of ZnO NPs synthesized using *Syringodium isoetifolium* extract

2.4. Particle Size Analysis

The size of the Synthesized Zinc oxide nanoparticles produced was determined using dynamic light scattering (DLS) measurements. Figure 4A depicts the particle size distribution curve of the synthesized NPs. The prepared ZnO NPs were ultrasonically suspended in water for 10 min. Particle size analyzer spectroscopy was used to examine the prepared sample. Under dynamic light scattering, the produced ZnO NPs had a mean particle size of 26 nm. PXRD are used to verify the crystalline size calculated from PSA.
In addition, the zeta potential (a measure of nanoparticle stability) was -12.7 mV (Fig.4B). The negative value indicated that the nanoparticles were stable. The results are compatible with ZnO NPs generated from *S. isoetifolium* leaf extract. The negative potential value might be attributed to the capping action of biomolecules found in *S. isoetifolium* leaf extract.

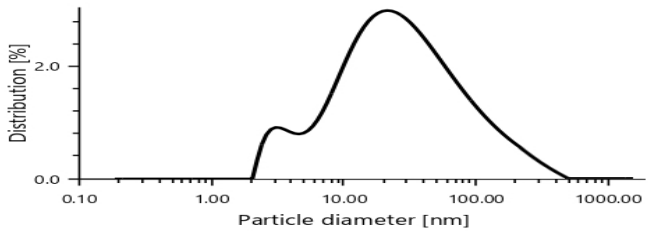

Fig 4A: Particle size analyzer graph of ZnO Nanoparticles

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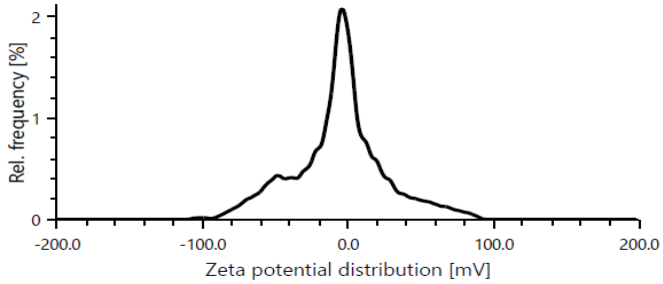

Fig 4B: Zeta potential for ZnO NPs synthesized using leaf extract of *Syringodium isoetifolium*

2.5. X-Ray Diffraction Study

The XRD design of ZnO NPs evidently shows a crystal-like structure (Figure 5). At 2θ values, strong diffraction peaks were seen at 31.87, 34.52, 36.36, 47.62, 56.67, 62.94, 66.44, 67.94, 69.20, 72.58 and 77.05 degrees. These peaks resemble to the diffraction lattice planes (100), (002), (101), (102), (110), (103), (200), (112), (201), (004) and (202). Further it also confirms that ZnO NPs were free of impurities as it did not contain any characteristic XRD peaks other than ZnO [30].
The average particle size of the NPs was calculated using the Scherrer equation.

$$D = \frac{K\lambda}{\beta \text{Cos}\theta}$$

where, D is the particle's crystallite size, K is the Scherrer constant, which is 0.9, ʎ is the diffraction wavelength (l.14 1.54A), the diffraction peak's FWHM (full width at half maximum) is b, and the angle of reflection is q. The average crystallite size of the ZnO NPs was 18 nm, according to the XRD pattern. The data were shown in Table 1.

Table 1: Grain size of zinc oxide nanoparticles

S. No	2θ of the intense peak (deg)	hkl	θ of the intense peak (deg)	FWHM of intense peak (β) radians	Size of the particle (D) nm
1	31.87	100	15.93	0.0061	23.89
2	34.52	002	17.26	0.0045	33.00
3	36.36	101	18.18	0.0068	24.31
4	47.62	102	23.81	0.0088	17.33
5	56.67	110	28.34	0.0081	19.52
6	62.94	103	31.47	0.0095	17.32
7	66.44	200	33.22	0.0116	14.28
8	67.97	112	33.97	0.0251	6.66
9	69.20	201	34.60	0.0106	15.93
10	72.58	004	36.29	0.0088	19.8
11	77.05	202	38.53	0.0139	12.8
				Total	204.84
				Average	**18.62**

The nanoscale range of the generated products is shown by the XRD spectrum line extension of diffraction peaks, and diffraction peaks from other metal species were absent, indicating that the synthesized ZnO NPs are devoid of impurities.

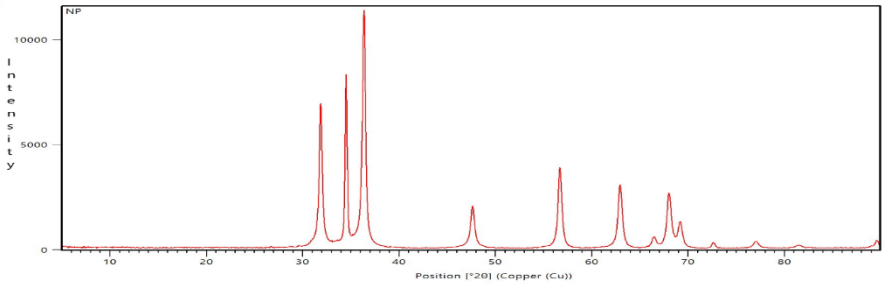

Fig 5: XRD Pattern of ZnO NPs synthesized using leaf extract of *Syringodium isoetifolium*

2.6 MTT Assay

The cytotoxic potential of ZnO nanoparticles was assessed in this study using the HepG2 cell line and the MTT assay. ZnO nanoparticles were compared to the reference medication Cisplatin to assess their impact. The ZnO nanoparticle treatment gradually reduced the viability of HepG2 cells as the compound's concentration increased. On HepG2 cell lines, the ZnO nanoparticles had strong cytotoxic action, with an IC_{50} value of 75.15±0.05 µg/ml. On HepG2, the IC_{50} value for the standard medication was discovered to be 29.05±0.05 µg/ml. It was discovered that the ZnO nanoparticle's performance against HepG2 cell lines was almost identical to that of cisplatin. Additionally, it is selectively toxic to cancer cells while remaining non-toxic to healthy cells. It is clear from our experiment that both ZnO nanoparticles are toxic to the HepG2 liver cancer cell line while having very little effect on healthy cells. As a result, ZnO nanoparticle is a medicine that is safer to use than cisplatin.

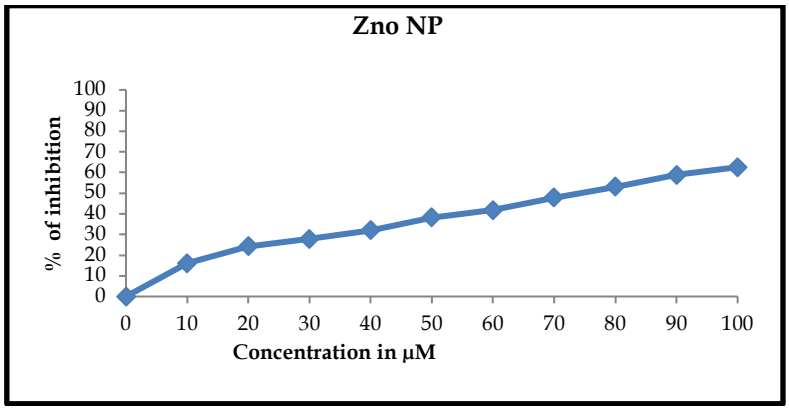

Figure 6: Determination of IC_{50} value of ZnO NPs

2.7. Morphological changes using fluorescence microscopy (Apoptosis)

ZnO nanoparticle promotion of HepG2 cell apoptosis was investigated utilizing fluorescence microscopy apoptosis tests. AO is a crucial colouring agent that can colour both live and dead cells and displays green fluorescence. The red fluorescence of cancer cells is visible in EB. Untreated HepG2 cells fluoresced green and had typical morphologies; in contrast, cells treated with ZnO nanoparticles fluoresced orange-red and had fragmented chromatin under the microscope. It implies that ZnO nanoparticles at IC_{50} concentrations mostly promote apoptosis in the examined cells. The apoptosis rate was calculated at random and displayed in Fig.**7**

2.8. Bactericidal activity of Zinc oxide nanoparticle

Gram positive bacteria like CoNS (Coagulase Negative Staphylococcus), *Streptococcus aureus*, and Gram-Negative bacteria like *Pseudomonas aerug*inosa and *Klebsiella pneumoniae* were found to be resistant to the nanoparticles of See grass with ZnO nanoparticle. For *S. aureus, CONS, P. aeruginosa,* and *K. pneumoniae*,

the measured zones of inhibition for 150 ug were 25 1.82, 24 0.71, 26 3.1, and 20.5 1.41 (mm), respectively. Zones for S. aureus, CoNS, P. aeruginosa, and K. pneumoniae were 16 1.71, 18 1.21, 20 1.52, and 14.5 0.47 (mm) for 100 ug, which showed relatively less inhibitory effect against the bacterial strains. Zones of inhibition were seen at concentrations of 50 ug, and they were reported to be 11.5 0.75, 12 2.41, 13 2.12, and 9 0.71. (mm)respectively.

Table 2: Zone of inhibition of ZnO nanoparticles against different microbial cultures

S.no	Microbial culture	Concentration of Nanoparticles (ug)	Zone of Inhibition (mm)
1	*Streptococcus aureus*	50	11.5 ± 0.75
		100	16 ± 1.71
		150	25 ± 1.82
2	*CoNS*	50	12 ± 2.41
		100	18 ± 1.21
		150	24 ± 0.71
3	*Pseudomonas aeruginosa*	50	13 ± 2.12
		100	20 ± 1.52
		150	26 ± 3.1
4	*Klebsiella pneumoniae*	50	9 ± 0.71
		100	14.5 ± 0.47
		150	20.5 ± 1.41

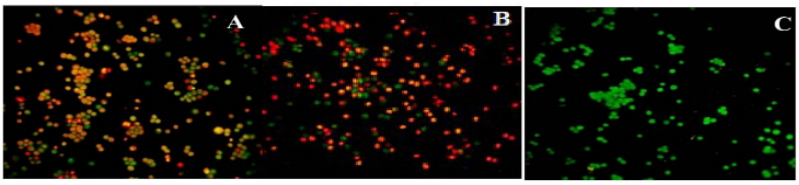

Fig. 7: Dual AO/EB fluorescent staining of HepG2 cells after treatment with control (A), Cisplatin (B) and ZnO nanoparticle (C) for 24 hours

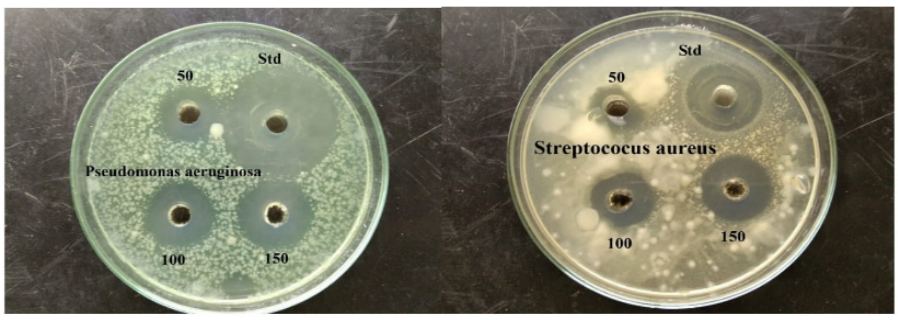

Fig 8: Zone of inhibition of *syringodium isoetifolium*

3. CONCLUSION

This work describes the green synthesis of ZnO NPs utilizing an aqueous extract of *S. Isoetifolium*. UV absorption peaks at 223 nm were seen in the produced ZnO NPs. The crystalline structure was also shown by XRD analysis. EDS with SEM and DLS were used to describe the ZnO NPs, which revealed the presence of

ZnO NPs with an average size of 26 nm. EDX results revealed the presence of zinc, carbon, and oxygen. FT-IR investigations convincingly demonstrated the synthesis of ZnO NPs. The zeta potential of generated ZnO NPs was -12.7mV in the absence of any electrolyte. The results show that the precursors had a significant impact on the surface form and structure of ZnO NPs. Green produced ZnO NPs shown significant antibacterial action against bacterial pathogens. As a result, it is anticipated that the environmentally friendly and highly effective zinc oxide nanoparticles created using *S. isoetifolium* leaf extract would have more widespread applications to avoid bacterial contamination. Our findings suggest that *Syringodium isoetifolium* can be used to make ZnO NPs in a simple, rapid, and ecologically friendly manner.

4. MATERIALS & METHODS

4.1. Materials

Zinc nitrate and Sodium hydroxide were used. All the chemicals used were analytical reagent grade from Merck (Mumbai, India) and the solutions were made using deionized water. *Syringodium isoetifolium* leaves were collected with the help of coastal peoples from Devipattanam, Tamilnadu.

4.2. Extract Preparation

Syringodium isoetifolium (leaves), often known as noodle seagrass were collected from Devipattanam, Ramanathapuram district. The extraction was carried out with slight changes based on the previously reported method [20]. The leaves were cleaned, dried, and powdered with the help of mortar. Then 20g of the coarse powder of Syringodium isoetifolium was combined with 200 ml water and heated at around 80°C for a period of 30 minutes while shaking constantly. The filtrate was kept at 4°C after being filtered with Whatman no 1 filter paper. The filtered liquid was then centrifuged at 3000 rpm for 10 min to eliminate any remaining debris & contaminants before being stored for future use. The plant was authenticated by Dr M. U. Sharief, Scientist E & Head, Botanical Survey of India, Coimbatore, India.

4.3. Green Synthesis of ZnO Nanoparticles

For many years, traditional methods were used, but studies have shown that green methods are more successful for the generation of NPs because due to their less chances of failure, inexpensive nature, and ease of characterization [21].

To make ZnO NPs, 25 ml of 0.05M zinc nitrate solution was mixed with 4ml leaf extract of Syringodium *isoetifolium*. Magnetic stirrer was used to agitate the liquid for a period of 2 hours at room temperature. Then, drop by drop, NaOH (0.02 M) was added until the pH reached 12. For an additional hour, the mixture was stirred until it produced a white solid product with a little yellow hue. The white powder obtained was calcined at 400°C for 6 hours in a muffle furnace.

4.4. Characterization of ZnO Nanoparticles

The following approaches were used to characterize the produced ZnO NPs.

4.4.1. UV Spectroscopy

UV spectrophotometer was used to record the spectra between 200 and 400 nm (Perkin Elmer). For UV analysis, sample volume of 1 ml was extracted by using ethanol and sonicated at 4000 rpm for 15 minutes.

4.4.2. Fourier Transform Infrared Spectroscopy (FTIR)

Using attenuated total reflectance (ATR)-FTIR, the functional group of ZnO NPs was established. Nanoparticle ATR-FTIR spectra were acquired using Bruker FTIR spectrophotometer with ATR sampling cells (Bruker Optics. Germany) between 4000-400 cm^{-1} spectra were measured. Each sample was scanned 24 times and the spectrum was captured using the OPUS 6.4 programme.

4.4.3. Scanning Electron Microscopy (SEM)

SEM was used to study the nanoparticle's surface morphology. A drop of nanosuspension was placed on an aluminum stub to prepare the sample. SEM was used to settle the particles. The sample was coated by using a 30 nm layer of gold sputter coater for 30 seconds and observed with EDX under the JOEL JSM-IT200. EDX was employed to govern the purity and elemental content of the generated ZnO NPs.

4.4.4. Particle Size Analyzer

The average size and size distribution of nanoparticles were measured using Dynamic Laser Scattering (DLS) technology (Zeta sizer Ver. 6.20, Malvern Instruments). The samples were diluted to the required quantities with water and sonicated for 2 minutes to create a homogeneous dispersion of NPs. After that, the samples were placed in a quartz cuvette. The hydrodynamic diameter of the NP was measured at 25°C using a He-Ne laser with a scattering angle of 90°.

4.4.5. Zeta Potential (ZP)

To estimate the surface charge, zeta potential measurements were accomplished in purified water with the conductivity set to 0.0504 mS/cm. In the original dispersion medium, the zeta potential was measured (water). Zeta Potential (ZP) study was used to measure particle stability.

4.4.6. X ray Diffraction Spectroscopy (XRD)

The crystallinity of prepared ZnO NPs was determined by using Rigaku ZSX Primus II. The experiment was carried out utilizing an X-ray diffractometer with a nickel filter and a copper anode as the radiation source. Samples were flattened to a smooth surface by mounting it on aluminum stages. The measurement was made across a 10° to 90° angular range, 2-theta (2θ).

4.4.7. Cytotoxicity by MTT assay

The MTT assay is an easy and simple colorimetric method used to measure viability of cells that works by measuring cell metabolic activity. Briefly, the method is based on the transform of MTT into formazan by the mitochondrial succinate dehydrogenase. The determination of mitochondrial dehydrogenase activity is related to the presence of viable cells. The anticancer effect of Zinc oxide nanoparticle was recognized by using MTT assay. The human hepato cancer cells were placed in a 96-well culture plate (1.0 x 10^5cells/ml) using appropriate media having 10% FBS. 100 µl of cell suspension was added in each well. Then add 100 µl of ZnO in each well and incubated for 24hr at 37°C in a 5% CO_2 incubator. After incubation, added 100µl of MTT (2mg/ml) in PBS to each well and incubated at 37°C for 4 hours in a 5% CO_2 incubator. The quantity of formazan dye produced directly connected to the number of viable cells in the culture and is calculated at 590 nm using a spectroscopic method. The effects of the ZnO nanoparticles as an anticancer agent by using the following formula.

Percentage cell viability = Intensity of treated cells/Intensity of control cells X100

4.4.8. Apoptosis study

Acridine orange-ethidium bromide (AO-EB) staining

Apoptosis, a type of programmed cell death, is an active process. There are many methods have been developed that detect changes in cell morphology and surface markers associated with apoptosis. AO/EB colouring is used to see the nuclear changes in cell level. Here, AO-EB fluorescent colouring was employed to access cell apoptosis in HepG2 cells upon treatment with ZnO nanoparticle. The cells were placed in 94-well plates at a density of 4000 cells/well and incubated at 37 °C for 24 hours. Zinc oxide nanoparticle (IC_{50} µM) were incubated with seeded cells. After 24 hours, the 10 µL of colouring solution containing AO and EB (each 100 µg/mL) was added to each well (500 µL). Immediately, the cells were visualized using a fluorescence microscope (Olympus, CKX-53, Japan).

4.4.9 Antibacterial assay

Four bacterial strains were utilized to test the antibacterial effectiveness of the ZnO nanoparticles. Bacterial Strains and Culture Collection The bacteria were Klebsiella pneumoniae (ATCC13883) and Staphylococcus aureus (ATCC 12600), and they were collected from the Bolineni KIMS hospital's American type culture collection in Rajamahendravaram, Andhra Pradesh.

Before the experiment begins, all the strains are subcultured in freshly made nutrient agar media. Each culture receives 5000 CFU inoculation, and nutrient plates are made using the pour plate method. Borers are used to create holes in solidified agar while keeping an equal spacing between each one. Inoculated into previously labelled wells are various quantities of sea grass Zinc oxide nanoparticles at 37 degrees. The Kirby-Bauer Disk Diffusion technique is used to measure the zone of inhibition.

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Conflict of interest statement: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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