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Natural Synthesis of Nanoparticles using Flower Extract of Hymenocallis littoralis (Jacq) Salisb and Evaluation of its Antimicrobial activity

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Abstract: ***The development of biologically synthesised nanoparticles using plant extracts plays an important role in the field of nanotechnology as it is eco-friendly and does not involve any harmful chemicals. The present study deals with green synthesis and characterization of Copper oxide and Iron oxide nanoparticles using Hymenocallis littoralis floral extract. To our knowledge, this is the first report where this flower extract was found to be a potential source for the green synthesis of copper oxide and iron oxide nanoparticles. Aqueous floral extract of fresh flowers of Hymenocallis littoralis were utilised for the synthesis of nanoparticles. The aqueous extract acts as both reducing and capping agent. The synthesised oxide nanoparticles of copper (CuONPs) and iron (IONPs) were confirmed by the change of colour after addition of floral extract into the precursors*** $CuSO_4.5H_2O$ ***and*** $FeCl_3.6H_2O$ ***salt solutions separately. The synthesised nanoparticles were characterised by UV-Visible Spectroscopy and Scanning Electron Microscopy(SEM). The UV-VIS spectroscopy showed the absorption peaks at 300nm and 314nm for CuONPs and IONPs respectively. The SEM analysis confirmed the presence of CuONPs and IONPs nanoparticles and the average size of some selected particles ranged from 100nm-200nm. It was observed that the aqueous floral extract can reduce copper and iron ions into their oxide nanoparticles within a day. Following the synthesis and characterization, the antimicrobial activity of the nanoparticles was evaluated. Thus, the method followed has the potential for simple, safe and stable biosynthesis of these nanoparticles.***

Keywords: Natural synthesis, Hymenocallis littoralis, nanotechnology, Copper oxide nanoparticles, Iron oxide nanoparticles

I. INTRODUCTION

The term "Nanotechnology" has been coined by "Norio Taniguchi", a researcher at University of Tokyo, Japan in 1974 [1]. It may be defined as the manipulation of particle with one of its size dimension smaller than 100nm and having specific properties which can be used in particular applications [2]. Nanoparticles are of great interest because they act as bridge between the atomic/molecular structure [3] to the material in bulk as they exhibit completely new or improved properties based on specific characteristics such as size, shape, distribution, ionic strength, capping agent and morphology [4], [5].

The interface of nano-sciences and biology has created a bright area of research for bio mediated synthesis of multifunctional nanoparticles [6], [7]. While the bioinspired synthesis of silver and gold nanoparticles has been popular, the plant-mediated green method has now also been extended to various metal oxide nanoparticles [8], [9]. Metal oxide nanoparticles are widely studied because of their unique magnetic, optical, biological and electronic properties, of whose properties differ from those of normal metal oxide particles [10].

Copper has been recognised as a hygienic material since the beginning of civilisation and, during the last two centuries, much of the historical evidence has been amply supported by scientific research to show that copper possesses antimicrobial qualities, which means it may inhibit the growth of harmful microorganisms (bacteria, algae, fungi, and viruses). Today, copper is utilised as an antibacterial and antifouling agent, an algaecide, a fungicide, a nematocide, and a water purifier [11]. A more cost-effective and better alternative method for creating copper and copper oxide nanoparticles (Cu and CuONPs) has been reported by the green synthesis methods. Both of these NPs have been applied as dietary additives, lubricant supplements, chemical sensors, coating materials in addition to large number of biotechnological and pharmaceuticals applications [12].

Iron has been known since the ancient times. Some of the earliest traces of iron use as a material dates back to Egypt around 3500 BC. At this time, iron beads which were meteorite-derived were discovered. Because of its celestial associations, meteoric iron was a highly valued substance.

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However, at that time, the only naturally occurring source of iron was meteoric iron, of which were mainly used by the ancient civilisation for weaponry purposes.

Among various types nanomaterials, iron oxide nanoparticles (IONPs) have excellent catalytic and reductive properties to be used for waste-water treatment and it has the advantage of the ease of separation as compared to the other nanomaterials requiring highly extensive centrifugation for separation [13]. IONPs are usually used for a wide range of applications from removal of heavy metals, dyes, antibiotics from water sources to the biomedical field like site-specific drug delivery and damaging tumour cell [14]. Again, iron-based nanoparticles is found to be effective against various pathogenic bacterial strains and fungi as they can effectively produce highly reactive oxygen species (ROS) [15].

The nanoparticles can be synthesised by many procedures, and are categorised mainly under physical and chemical techniques. The drawback of the physical technique is that the resultant nanoparticles have defective surface formation, low production rate, high cost of manufacturing and large energy requirement [16], [17]. The chemical method for synthesis of nanoparticles involves usage of toxic chemicals, concentrated reducing agents, high level of radiation [18] and formation of hazardous by-products and contamination from precursor chemicals [19] which is an alarming threat in every aspect of flora, fauna and human health. The synthesis provided in this paper is eco-friendly, non-hazardous and non-toxic in nature as it involves the use of medicinal plant.

Due to various inherent benefits not present in the chemical or physical processes of synthesis, the interface of medicinal plants and nanoparticles has become a bright area of research [20]. Plant-mediated biosynthesis of nanoparticles is a one step, economical, safe and free of any waste generation. Due to this, medicinal plants mediated biosynthesis of nanoparticles has gained much popularity [21]. A variety of phytochemicals found in medicinal plants, including phenolics, flavonoids, alkaloids, and others, are thought to be responsible for the efficient chelation and stability of bioinspired nanoparticles [22].

The present contribution reports a one step, complete green biosynthesis of CuONPs & IONPs using the aqueous floral extracts of Hymenocallis littorals (Beach Spider Lily) without any further addition of acid or base. Beach Spider lily belonging to Amaryllidaceae family is a well-known plant species for its medicinal properties. It originates from South and Central America. But it is cultivated as a garden ornamental plant and naturalised in tropical Africa, Asia and the Pacific islands. In the Philippines the bulbs of H. littoralis are used as a vulnerary (of use in the healing of wounds). However, they are regarded as being too toxic to be eaten in Thailand. Hymenocallis leaves are applied externally to bruising and swelling in traditional Chinese medicine. In Central America, the bulbs of the Hymenocallis plant are frequently used in traditional medicine, most often in the form of an ingested decoction to cure asthma and as a poultice on boils. The floral extract can occasionally be found in cough decoctions as well. Thus, H. littoralis is a fascinating medicinal plant, which deserves more recognition in South-East Asia. Although it is a source of compounds with anticancer and antiviral (including anti-HIV) properties, bulb extracts are also said to have positive interior effects on asthma, cough and exterior effects on boils, wounds, swellings, and bruises [23].

II. MATERIALS AND METHODS

A. Chemicals

Analytical grade copper sulphate pentahydrate ($CuSO_4.5H_2O$) and ferric chloride hexahydrate ($FeCl_3.6H_2O$) chemicals were used in this study without any further purification.

B. Collection of Flowers

Flowers of Hymenocallis littoralis were collected from Yuvaraja's College campus, University of Mysore, Mysuru.

C. Preparation of floral extract

Weigh 20g of fresh flowers and wash them thoroughly in distilled water for a few minutes. The washed flowers are dried, chopped into fine pieces and boiled in 100ml of distilled water for 15minutes in a 500ml borosil beaker. The extract obtained was filtered through muslin cloth and then through Whatmann no: 1 filter paper (pore size 25µm) and used immediately for the biosynthesis of copper oxide and iron oxide nanoparticles.

D. Green Synthesis of CuONPs

For the synthesis of CuONPs, 5ml of fresh flower extract was added to 25ml of 1% aqueous copper sulphate in 250ml borosil conical flask separately at room temperature. The colour of the extracts changes from pale yellow to green in colour indicating the formation of CuONPs. The synthesis is carried out in shade and left inside a BOD incubator for 24 hours for the reactions to be completed.

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E. Green Synthesis of IONPs

$FeCl_3.6H_2O$ was used as the precursor for the synthesis of the iron oxide nanoparticles. The synthesis of Iron oxide nanoparticles was done by adding 0.01 M Ferric Chloride and the fresh flower extract in 1:1 proportion separately in clean sterilised flasks. The solution resulting from the addition of flower extracts and 0.01 M Ferric Chloride had an immediate colour change and was blackish brown in colour. This solution is then left in shade, inside a BOD incubator for 24 hours for the reactions to get completed.

F. Extraction of Nanoparticle Samples

To collect the nanoparticle samples, the reaction mixture was centrifuged two times at 4000 rpm for 10 min and washed twice with distilled water and alcohol respectively. The obtained greenish brown coloured sample of copper oxide and blackish brown coloured sample of iron oxide particles from using fresh floral extracts were then dehydrated with the help of vacuum dryers, or even they can be shade dried at room temperature if a dehydrator is not available. The dehydrated product obtained is dry grounded to fine powder.

G. Characterization Techniques

All ultraviolet-visible (UV-vis) spectra were recorded on the Beckman Coulter DU 730 UV-vis spectrophotometer. The absorption spectra of the prepared NPs were recorded by taking the aqueous dispersion of the NPs and scanned in the range of 200–900 nm, operated at resolution of 1nm. Distilled water was taken to adjust the baseline. Scanning electron microscopy (SEM) was used study the morphological features of synthesised nanoparticles. SEM images were recorded using Carl Zeiss Germany, Model : EVO MA 15 SEM instrument of the IOE Research Facility at University of Mysore, India.

H. Antibacterial Assay

Escherichia coli and Bacillus subtilis species were collected from Dept. of Microbiology, Yuvaraja's college, University of Mysore. The sub-cultures were maintained using nutrients agar media for further use. The zone of inhibition was calculated using the agar plate well diffusion method. E. coli and Bacillus subtilis were cultured in nutrient broth, kept at 37 ^{0}C for the duration of a night. The experiment made use of this overnight culture of bacteria in nutrient broth. For each bacteria in this procedure, a sterilised nutrient agar plate was used. Using a UV-Visible Spectrophotometer, the optical density (OD) of every bacterial culture was adjusted to 0.1. (Spectrumlab 1200RS, Japan).

To counteract the impact of assay reagents, the spectrophotometer was initially set to auto zero using a blank. Then, using a sterile cotton swab, these two bacterial pathogens were applied to selected agar plates.

These plates were then given time to dry. A sterile cork borer of approximately 8.0 mm diameter was then used to dig four wells into each agar plate. Subsequently, 30 µl and 70 µl of NPs stock solutions prepared (1mg/ml), the suspension of streptomycin antibiotic (0.1ml of 25 µg/ml) and distilled water was poured into individual wells of each inoculated plate. DMSO was used to prepare the stock solutions. The antibiotic was taken as the positive control and the distilled water was used as the negative control. The plates were left in place for 1 hour to allow for complete diffusion, then incubated for 24 hours at 37 ^{0}C and measured the diameter of inhibitory zones in millimetres.

I. Antifungal Assay

The fungal species *Aspergillus niger* and *Fusarium oxysporum* were collected from the Dept. of Microbiolgy, Yuvaraja's college, University of Mysore. The sub-cultures were maintained using potato dextrose agar media for further use. The antifungal activity of the NPs was determined by agar well diffusion method. The fungal inoculums prepared were used to test the antifungal potential of selected NPs. Potato dextrose agar medium was prepared and 20ml was poured into each of the 90 mm Petri plates. After that, a laminar airflow chamber was used to allow the culture plates to solidify. 0.5ml of fungal inoculum was inoculated into the petriplates using the spread plate method.

Then three wells were made on the agar plate using a 8 mm standard cork borer. Different amounts of NPs (30 and 70 µl of 0.30 mg/ml NPs) and distilled water (negative control) were added to respective wells. DMSO was used to prepare the stock solutions of synthesised nanoparticles. Standard sterile discs presoaked in Bavistin (1ml of 25 µg/ml) was used as standard (positive control).

The effect of NPs against the fungal pathogens was evaluated and compared with the standard used during the study. The plates were then sealed and incubated at room temperature for 2 days. Finally, the antifungal activity was calculated by measuring the zone of inhibition diameter via standard scale.

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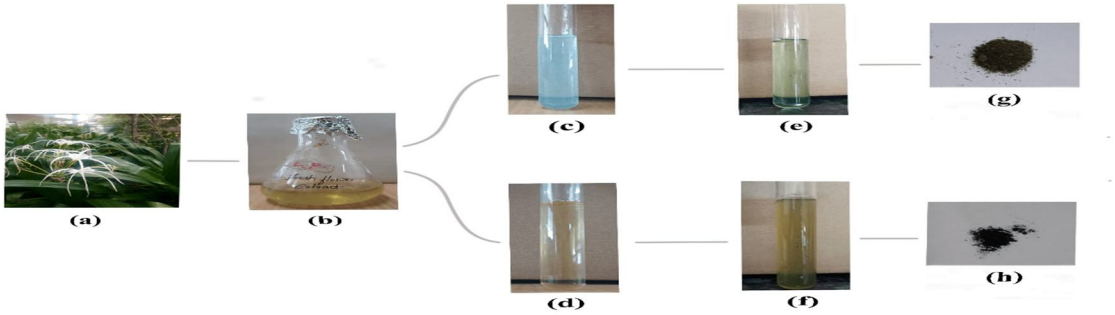


Fig. 1: Various steps involved in the preparation of NPs sample (a) image of the flowers, (b) floral extract (c) 1% $CuSO_4$, (d) 0.01 M $FeCl_3.6H_2O$, (e) formation of CuONPs (f) formation of IONPs, (g) finely crushed CuONPs, (h) finely crushed IONPs

III. RESULTS AND DISCUSSION

A. Synthesis of CuONPs

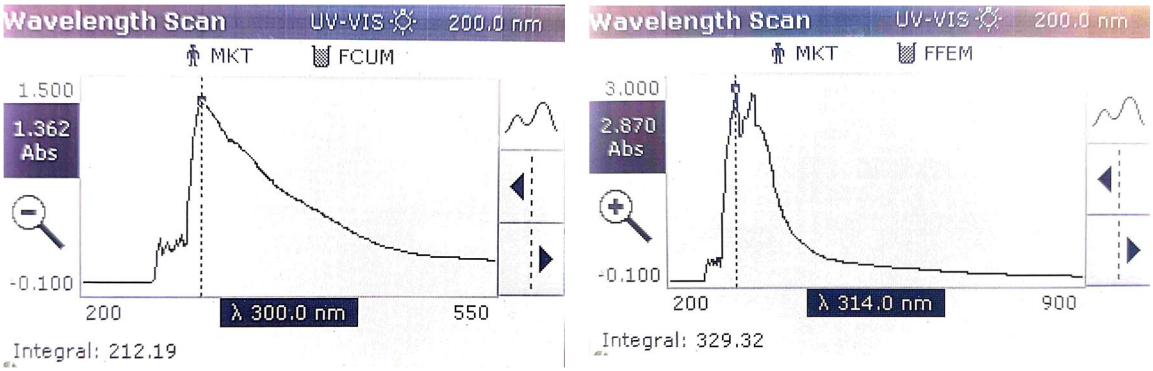
The CuONPs were synthesised by using copper sulphate as a precursor and fresh floral extracts as reducing and capping agent. The change in colour of the solution from blue to light greenish brown visually indicates the formation of copper oxide NPs. The CuONPs were washed with distilled water followed by ethanol to remove any unwanted particles. Thereafter, CuONPs was dried, ground and later subjected to various characterization methods.

B. Synthesis of IONPs

The various phytochemicals present in the fresh floral extracts equally act as reducing and stabilising agents for the synthesis of IONPs. The formation of black colour precipitates occurred due to the interaction between these phytochemicals and metal ions ensuring the formation of iron oxide nanoparticles. After mixing of iron salt solution with floral extracts at definite reaction conditions, it is not be able to reduce Fe^{3+} to Fe^0 rather, the phytochemicals react with the iron ions to give iron oxide NPs [24].

C. UV – Vis Spectroscopy

UV-Vis analysis is one among the foremost important characterization methods to review nanoparticles. The surface plasmon resonances (SPR) of synthesized oxide nanoparticles have been studied by UV-Vis Spectrophotometer. The absorption of visible radiations because of the excitation of SPR, imparts various colours to nanoparticles. The colour of the solution is also expected to change as the size of the nanoparticles changes. Therefore, the production of nanoparticles is quite sensitive to the UV-Vis absorption spectrum. The two nanoparticle samples were subjected to UV-Vis study. Fig. 2 shows the UV-Vis spectrum of the samples. The highest peak bands of copper oxide nanoparticles was observed around 300 nm and that of iron oxide nanoparticles was around 314 nm. From the various literature studied pure Cu NPs show peaks near 590nm and the peaks for CuONPs are around in the range of 250 nm. As the observed UV result for the synthesised copper particles are nearer to the later, it is assumed that the synthesisied particles predominantly consists of CuONPs. The same goes in the case of IONPs as various literature survey indicates the peak observations of IONPs are in the range between 250-350 nm.

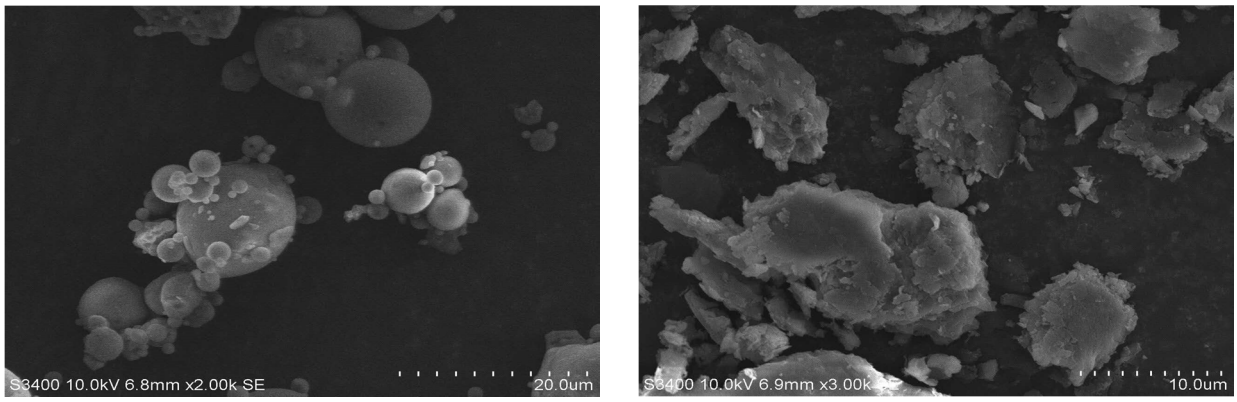


a b

Fig. 2: UV-Visible spectrum of (a) CuONPs, (b) IONPs

D. SEM (Scanning Electron Microscopy)

Scanning Electron Microscopy provided further insight into the morphology and size details of the synthesized nanoparticles. The typical SEM image shows that the product mainly showed the presence of particles like copper oxide and iron oxide nanoclusters with panoramic view and some of the selected particles measured size ranged between 120-200 nm. The nanoparticles were measured from the SEM image with the help of Image J software. Average size of the selected CuONPs and IONPs were found to be 146nm and 180nm respectively. Some of the nanoparticles were almost spherical and some were of distorted shaped, which are shown in the SEM images. SEM showed that the nanoparticles are agglomerated in some amount due to sticky nature of the plant extract. The SEM micrographs taken at low resolution is depicted in the inset of Fig. 3. Small nuclear particles are self-aggregated and orient themselves to form larger particles.



a b

Fig. 3: SEM images of (a) CuONPs, (b) IONPs

E. Antibacterial Activity Of Nanoparticles

The antibacterial activity of green synthesised nanoparticle suspensions of different concentrations was done against a gram negative and gram positive bacteria such as E.Coli and Bacillus subtilis. The antibacterial agent's (NPs) capacity to disrupt the bacterial cells was examined using the well diffusion method. Table 1 & 2 displays the antibacterial activity of NPs tested against gram-negative and gram–positive bacteria.

Table1: Antibacterial activity of copper oxide nanoparticles.

	Inhibition zone (mm)			
	+ve control	-ve control	30µl	70µl
Escherichia coli	23	0	15	16
Bacillus subtilis	24	0	16	18

Table2: Antibacterial activity of iron oxide nanoparticles.

	Inhibition zone (mm)			
	+ve control	-ve control	30µl	70µl
Escherichia coli	23	0	12	14
Bacillus subtilis	24	0	14	16

The as possessed antibacterial properties of nanoparticles is because of its nanoscale size allowing it to accumulate or deposit on the surface of studied bacterial strains which is reported by other researchers [25]-[27]. Apart from the NPs, the plant extracts may additionally possess antibacterial activity because of the presence of phytochemical components [14]. However, there are a variety of hypotheses available to clarify the precise mechanism of NPs against the bacterial strains.

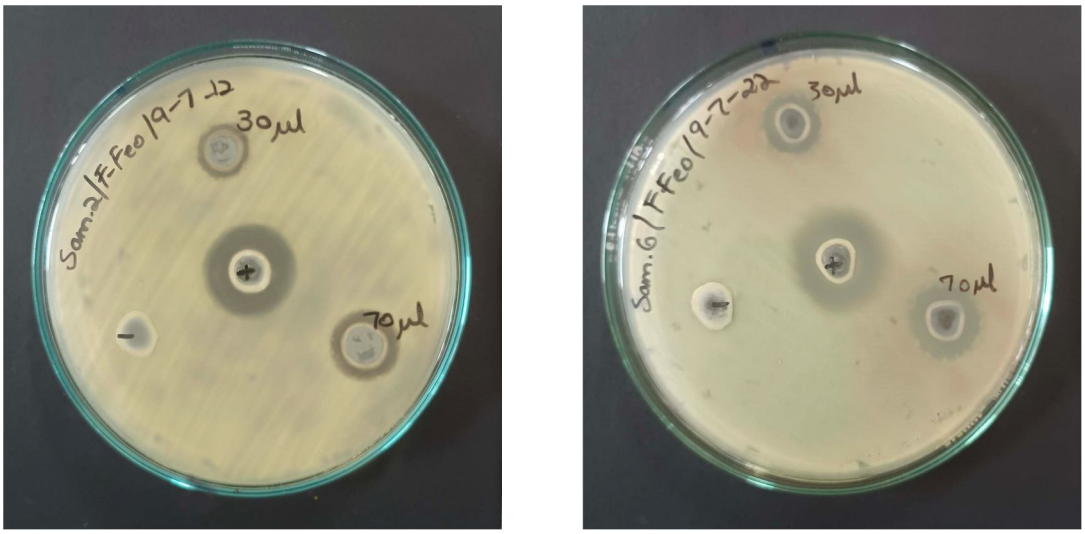
One of the proposed mechanism involves the association of copper with oxygen and its reaction with sulfhydryl (-S-H) groups on the cell wall to form R-S-S-R bonds, thereby blocking respiration and causing cell death. The formation of reactive oxygen species (ROS), oxidative stress brought on by ROS, and the interaction of ions released by nanoparticles with thiol groups (-SH) of the bacterial cell; all contributed to the iron oxide nanoparticles' antibacterial properties [28]. This alters the structure of the microorganisms and prevents DNA replication and protein synthesis [29].

It is clearly hinted by the presence of an inhibitory zone that the membrane rupture may be a factor of the biocidal action of nanoparticles. The degree of suppression is dependent on both the initial bacterial concentration and the nanoparticle concentration. The smaller size of the particles may cause them to adhere more closely to the surface of the bacterial cells, breaking the membrane and allowing internal components to leak out, killing the bacterial cells.



a b

Fig. 4: The antibacterial effect of CuONPs on pathogens (a) E. coli & (b) B. subtilis



a b

Fig. 5: The antibacterial effect of IONPs on pathogens (a) E. coli & (b) B. subtilis

F. Antifungal activity of nanoparticles

The fungi A. niger and F. oxysporum were used as role model fungi to test the antifungal activities of iron oxide NPs. The well diffusion method was used to test the ability of the antifungal agent (NPs) against the fungal cells. The antifungal activity studied against the fungal species at different concentrations of samples are shown in **Table 3** & **4**.

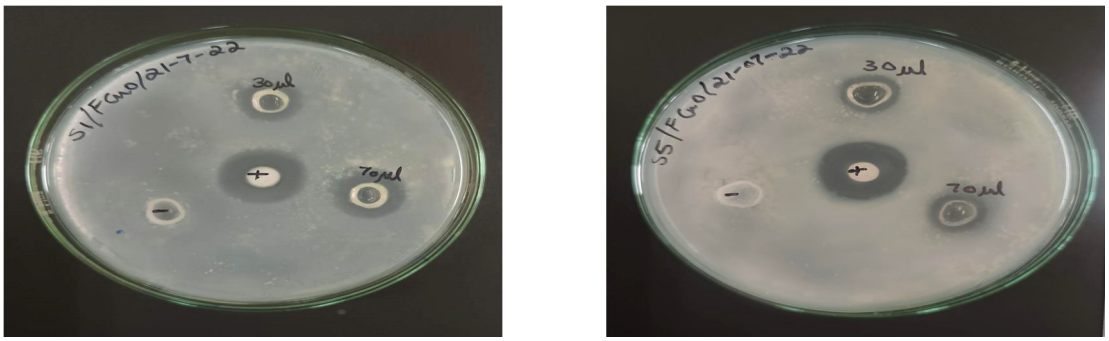
Table3: Antifungal activities of copper oxide nanoparticles.

	Inhibition zone (mm)			
[illegible]	+ve control	-ve control	30µl	70µl
Fusarium oxysporum	21	0	14	16
Aspergillus niger	22	0	13	13

Tabl 4: Antifungal activities of iron oxide nanoparticles.

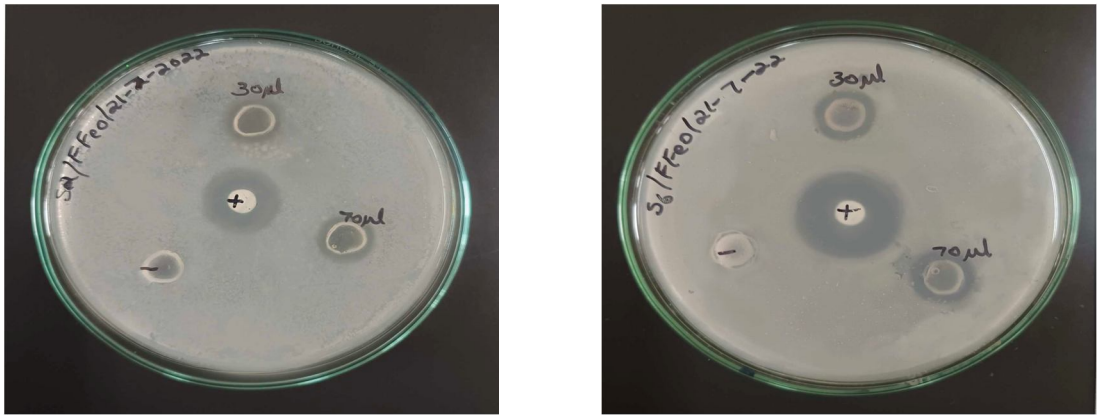
	Inhibition zone (mm)			
[illegible]	+ve control	-ve control	30µl	70µl
Fusarium oxysporum	23	0	13	14
Aspergillus niger	24	0	15	15

Because NPs have a high surface-to-volume ratio, they can strongly adhere to fungal cell surfaces. Furthermore, due to its small size, it can effectively penetrate the cell wall and cause damage to it. Inactivation of the fungi by iron oxide NPs involves the direct interaction of NPs with cell surfaces, thereby affecting the permeability of membranes by inducing oxidative stress in them. This results in cell growth inhibition and eventually cell death [30]. The potential for membrane damage brought on by direct or electrostatic contact between iron oxide nanoparticles and cell surfaces, cellular internalisation of NPs, and the generation of active oxygen species such H_2O_2 in cells as a result of metal oxides have been reported in various literature [31]. Moreover, iron oxide NPs synthesised using Hymenocallis littoralis showed significant antifungal activities against both F.oxysporum and A.niger species in this study.



a b

Fig. 6: The antifungal effect of CuONPs on pathogens (a) Fusarium oxysporum & (b) Aspergillus niger



a b

Fig. 7: The antifungal effect of IONPs on pathogens (a) Fusarium oxysporum & (b) Aspergillus niger

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IV. CONCLUSION

Making eco-friendly and biologically advantageous nanomaterials is now possible thanks to the environmentally friendly synthesis of nanoparticles using plant extracts. Copper oxide and iron oxide nanoparticles were produced using floral extracts from Hymenocallis littoralis. Moreover, UV-visible spectroscopic analysis was used to track the reduction of copper ions into CuONPs and iron ions into IONPs. SEM technology was used to investigate the particle size and shape.

The tailored nanoparticles were tested for their effect as antimicrobial agents against bacteria such as Escherichia coli, Bacillus subtilis and fungi such as Fusarium oxysporum and Aspergillus niger. The greater surface interaction between the synthesized nanoparticles and these microorganisms is the factor responsible for their antimicrobial activities. It is possible to do additional study to examine and contrast the effects of CuONPs and IONPs with those medications typically used to treat these microbial infections, as well as to explore their uses for other environmental applications.

V. ACKNOWLEDGMENT

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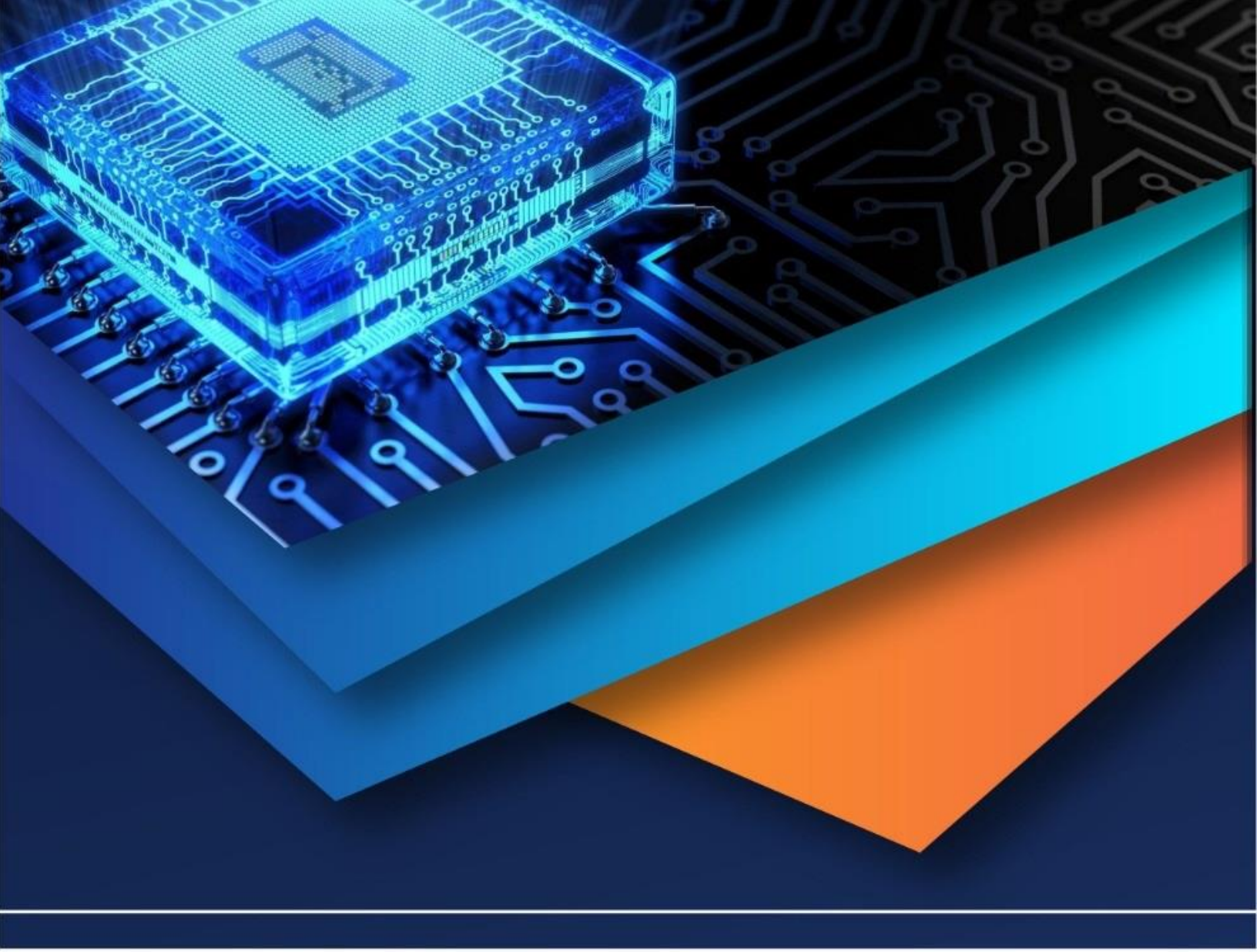
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