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RESEARCH ARTICLE

GREEN SYNTHESIS OF IRON AND ZINC OXIDE NANOPARTICLES FROM *Moringaoleifera*POD PEEL EXTRACT AND *IN-VITRO* ANALYSIS OF THEIR ANTIBACTERIAL POTENTIAL

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Abstract

With the enormous applications nanomaterials offer, this study focuses on antibacterial property of nanoparticles. The study involved green synthesis of iron and zinc oxide nanoparticles from *Moringa oleifera*pod peel extract and comparison of the bactericidal efficiency of chemically and green synthesized nanoparticles against both Gram-negative and Gram-positive organisms (*Escherichia coli* and *Staphylococcus aureus*). The synthesized nanoparticles were characterized by UV spectroscopy, SEM-EDS and XRD. *In-vitro* analysis of antibacterial potential of both chemically and green synthesized iron and zinc oxide nanoparticles was assessed. A comparison of the activity of nanoparticles against *E. coli* and *S. aureus* suggested that Gram-positive bacteria are more susceptible to zinc oxide nanoparticles than Gram-negative bacteria. The exact mechanism of the action that justifies this difference in the susceptibility based on the structural differences in cell wall composition is yet to be deciphered. Nevertheless, green synthesis of nanoparticles is emerging as a popular branch of bio-nanotechnology; its popularity is attributed to the lesser energy consumption, biocompatibility and less toxicity of the green synthesized nanoparticles to living systems.

Introduction:-

The reckless use of antibiotics has created havoc in medicine due to the development of antibiotic resistance in pathogenic micro-organisms[6,1]. Micro-organisms have evolved various mechanisms to resist the bactericidal activity of different classes of antibiotics such as secreting enzymes to degrade antibiotics[27], preventing entry of the bactericidal agent in the cell by altering components of the cell wall[9] among others by virtue of mutations in their genome; the frequency of mutated genes is enhanced by their horizontal transfer in the population[1]. Various approaches are been opted to combat this challenge for the health care and pharmaceutical industry. One among them is the use of nanoparticles as antibacterial agents or in conjugation with existing antibiotics as drug delivery systems. Nanoparticles are atomic or molecular entities with one of their dimensions in the range of 1-100nm[26]which renders unique properties to them enabling their dispersed use in various industries. The antimicrobial potential of nanoparticles is a function of their size, shape, surface morphology, charge and zeta potential[38]. Nanoparticle synthesis is achieved by several methods which are characterized into two approaches: top-down[22] and bottom-up[32]. The former approach uses physical methods while the latter involves reduction of precursors into nanoparticles by chemical and biological means[36]. Physical and chemical methods of nanoparticle

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synthesis have several disadvantages in being expensive and are an environment concern as toxic reducing agents are used or by-products are released[32]. Green synthesis is a cleaner and safer approach that focuses on using living systems or their products for nanoparticle formation. Bacteria[29,20], fungi[14,24], algae[31] and plant extracts[28] can be used for green synthesis of nanoparticles. Commonly used nanoparticles or nano-systems can be either organic or inorganic among which metal and metal oxide nanoparticles have been extensively studied for their applications. Two such nanoparticles whose antimicrobial properties have been studied are iron and zinc oxide nanoparticles. The plant extract used for the green synthesis of nanoparticles is *Moringa oleifera* pod peel. Despite the presence of phytochemicals in the pod peel which facilitate synthesis of nanoparticles[34], existing literature provided little evidence about its use in green synthesis.

Materials And Methods:-

Preparation of *M. oleifera* Pod Peel Extract:

Fresh *M. oleifera* pods were collected from the local market in Vasanth Nagar, Bengaluru. The pods were cleaned with distilled water and peeled. The peels were dried in hot air oven at 50℃ for 3-4 days and powdered using electric blender to obtain a fine powder. This powder was stored in an air-tight container[15].

Preparation of aqueous, methanolic and acetone *M. oleifera* pod peel extract:

M. oleifera pod peel powder (5 g) was weighed and dissolved in distilled water, methanol and acetone (50 mL) respectively in different conical flasks. The conical flasks were kept for continuous stirring for 24 hours on a magnetic stirrer at ambient temperature[16]. It was filtered using muslin cloth and centrifuged at 8000 rpm for 15 minutes. The supernatants were collected and stored at 4℃ for 2-3 days[15].

Chemical synthesis of nanoparticles

Chemical synthesis of Fe NPs –

Ferric chloride hexahydrate ($FeCl_3.6H_2O$; 0.01M) and sodium borohydride ($NaBH_4$; 0.002M) was prepared separately in distilled water and was mixed with vigorous stirring on a magnetic stirrer. The addition resulted in the formation of black coloured solution. The mixture was centrifuged, the pellet obtained was washed and dried completely in the hot air oven at 50-60℃ for 2-3 days till fine powder is formed[16,40].

Chemical synthesis of ZnO NPs –

Zinc nitrate hexahydrate ($ZnNO_3.6H_2O$; 0.2M) and potassium hydroxide (KOH; 0.4M) were prepared. KOH was added to $ZnNO_3.6H_2O$ with continuous stirring. The addition of the solutions resulted in the formation of white colloid which was allowed to settle. This precipitate was centrifuged, washed and then subjected to 500℃ for 3 hours[12].

Green synthesis of nanoparticles

Green synthesis of Fe NPs –

M. oleifera aqueous extract and ferrous sulphate heptahydrate ($FeSO_4.7H_2O$; 0.1M) were mixed in the ratio of 2:1 (v/v). This mixture was continuously stirred overnight at room temperature on a magnetic stirrer to obtain a dark brownish-green solution. The solution was centrifuged, washed and dried completely in the hot air oven at 50-60℃ for 2-3 days till a fine powder is formed[39].

Green synthesis of ZnO NPs –

M. oleifera aqueous extract (30mL) was heated gradually till the temperature reaches 60℃; zinc nitrate hexahydrate ($ZnNO_3.6H_2O$; 3 grams) was then added to it with continuous stirring. The solution was kept for overnight stirring with slight heating till the solution started precipitating and a paste like consistency was obtained. The paste was then blazed at 400℃ for 2 hours to obtain fine powder[10].

Characterization of nanoparticles

UV- Visible Spectroscopy –

The synthesized nanoparticles were analysed in the UV-Visible Spectrophotometer (Shimadzu, UV-1700 Pharma Spec). The suspension of NP samples was studied in the absorption range of 200-500 nm for both green and chemically synthesized Fe NPs and ZnO NPs[8,12].

Scanning Electron Microscopy –
The size, shape and surface morphology of the synthesized NPs were analysed using SEM[13]. A thin layer of NP powder was coated on a carbon-coated tape and the film was allowed to dry by putting it under mercury lamp for 5 minutes. The samples were analysed at CeNSE laboratory, IISc, Bengaluru.

Energy Dispersive X-ray Spectroscopy–
The quantitative elemental composition of the nanoparticles synthesized were estimated along with their chemical purity and stoichiometry[13] at CeNSE laboratory, IISc, Bengaluru.

X-Ray Diffraction-
The crystallite structure of NPs was analysed using XRD at CeNSE laboratory, IISc, Bengaluru. The average crystallite size was determined using Scherrer's equation.

$$D = \frac{K\lambda}{\beta \cos\theta}$$

***In vitro* analysis of antibacterial property of chemically and green synthesized NPs**
Well-diffusion method:
The Muller-Hinton agar plates were swabbed with overnight cultures of *E. coli* and *S. aureus* and wells with equal volumes of the samples were incubated overnight at 37℃ in bacterial incubator. Methanol, acetone and distilled water were used as control for methanolic extract, acetone extract and synthesized nanoparticles respectively. The diameter of the zone of inhibition formed was measured[21].

Minimal inhibitory concentration:
The inoculum (*E. coli* and *S. aureus)* was added to test tubes containing broth and increasing concentration (30µL, 50µL, 100µL) ofgreen synthesized ZnO NPs. Suitable controls were maintained for both the bacterial cultures. The test tubes were incubated overnight at 37℃ in shaking incubator. The turbidity of the test tubes was measured using colorimeter at 600nm[3].

Results And Discussion:-

M. oleifera pod peel powder showed considerable decrease in the dry weight after complete drying (72.598%). The *M. oleifera* pod peel aqueous extract prepared had a pale-yellow colourwhile those obtained using methanol and acetone were bright green. The extracts were stored for *in vitro* analysis.

Characterization of the synthesized nanoparticles
UV- Visible Spectroscopic analysis
The absorption spectra for green and chemically synthesized nanoparticles wasread in the range of 200 to 600nm. For both chemically and green synthesized Fe NPs, the absorbance peak was obtained in the range of 290 to 320nm which lies within the characteristic absorption spectra for iron nanoparticles[25]. On the other hand, the green and chemically synthesized zinc oxide nanoparticles showed their absorbance peak in the range of 290 to 450 nm with the absorption peak obtained around 310nm which indicated the presence of ZnO nanoparticles[17,34].

Scanning Electron Microscopy
The SEM analysis of chemically synthesized Fe NPs showed spherical, non-agglomerated nanoparticles with an average diameter of 10-30 nm (Figure 1.A). The green synthesized Fe NPs formed were seen as agglomerated, uniform spherical shaped with an average diameter of 70-90 nm (Figure 3.A). Chemically synthesized ZnO NPs (Figure 2. A) formed showed both oblong and spherical non-agglomeration structures with an average diameter of 90-150nm, and green synthesized ZnO NPs (Figure 4. A), both agglomerated nanoparticles and nanoflakes were formed with average diameter of 250- 400nm which is similar to previous literature[33].

Energy Dispersive X-ray Spectroscopy
EDS analysis of chemically synthesized Fe and ZnO NPs (Figure 1. B and 2. B) showed intense peaks for Fe, Zn and O signifying the presence of their elemental forms in highest concentration. The green synthesized NPs (Figure 3. B and 4. B) also showed high peaks for elemental Fe, Zn and O indicating the formation of nanoparticles along with presence of additional elements like N, S, P, K, Clrooting from the use of *M. oleifera* pod peel extract. Presence of elements like C, O in green synthesized NPs was attributed to the presence of phenols from *M. oleifera* pod peel

extract[37]. Elements like S, N, P showed weak peaks due to the presence of phytocompounds in the *M. oleifera* pod peel extract[23].

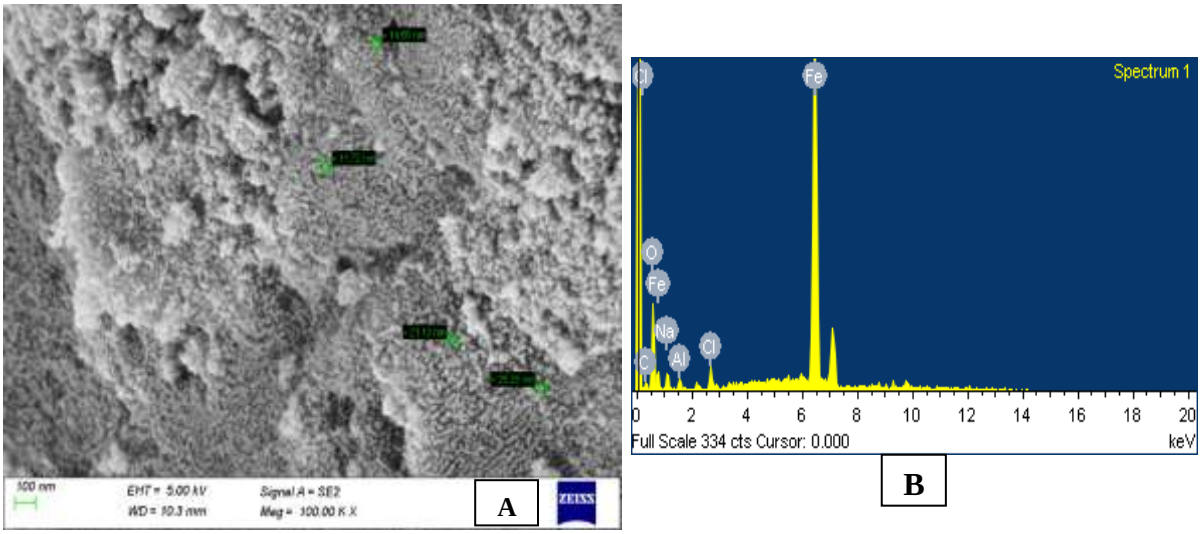

Figure 1:- Chemically synthesized Fe NPs A). SEM image B). EDS spectrum

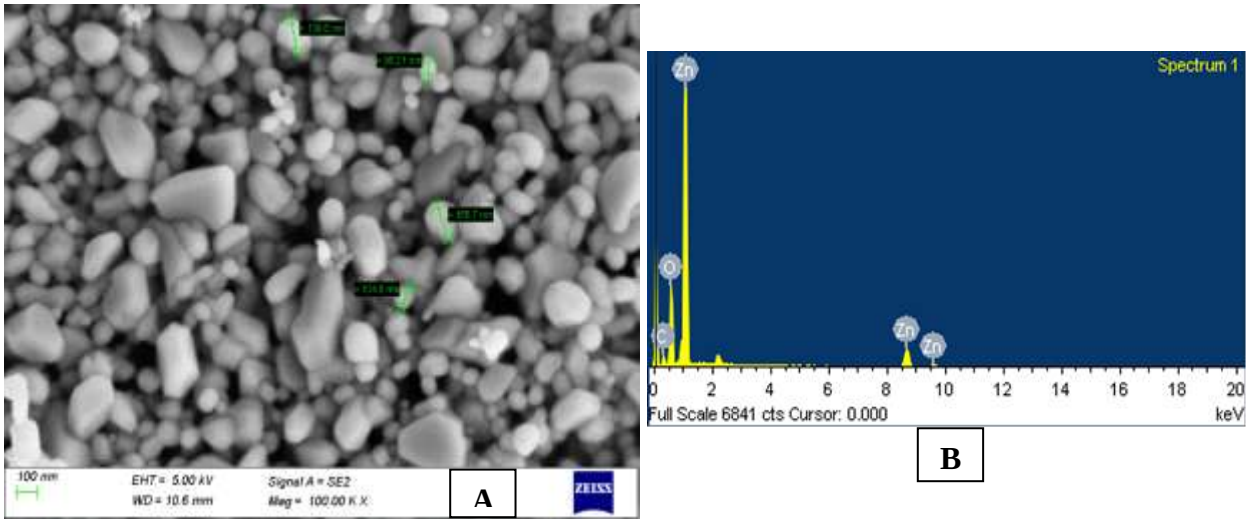

Figure 2:-Chemically synthesized ZnO NPs A). SEM image B). EDS spectrum.

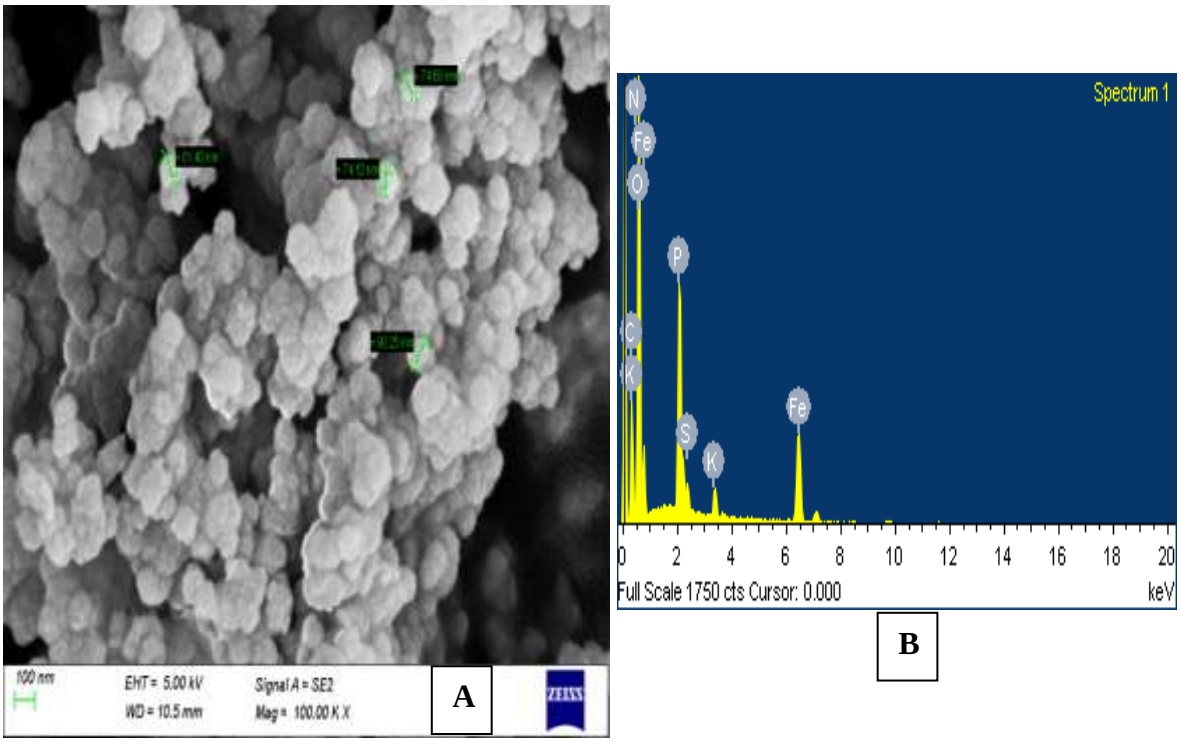

Figure 4:- Green synthesized Fe NPs A). SEM image B). EDS spectrum.

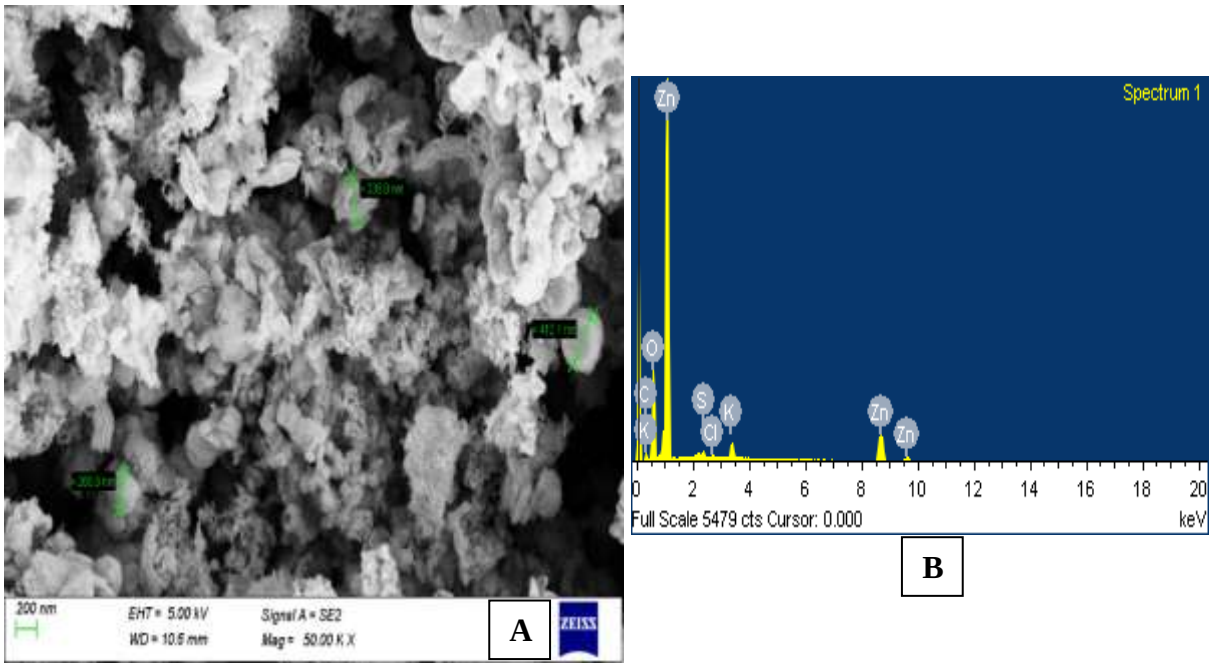

Figure 3:- Green synthesized ZnO NPs A). SEM image B). EDS spectrum.

X-ray Diffraction Analysis

The crystallite size obtained using Scherrer's equation was 7.45nm and 38.93nm for chemically synthesized Fe and ZnO NP and 43.49nm and 17.75nm for green synthesized Fe NPs and ZnO NPs respectively. The XRD data suggests the formation of poly-crystallite structures of the NPs formed which was elucidated by the numerous peaks of various intensities formed by diffraction of X- rays. The presence of peak 311 for chemically and green synthesized Fe NPs(Figure 5. A and C) and peak 101 for chemically and green synthesized ZnO NPs(Figure 5 B and D)confirmed the formation and presence of the respective NPs which is in accordance with previous literature[18,35].

***In-vitro* analysis of antibacterial property of synthesized nanoparticles**

The antibacterial property of *M. oleifera* pod peel extracts (methanol and acetone) and synthesized NPs was studied against Gram-negative *E. coli* and Gram-positive *S. aureus*. *E. coli* showed sensitivity to green synthesized ZnO NPs (100%) forming a zone of inhibition of 16 ± 1 mm and resistance to *M. oleifera* extracts(methanolic, acetone), chemically and green synthesizedFeNPs as well as chemically synthesized ZnO NPs. *S. aureus* showed sensitivity to *M. oleifera* acetone extract (100%) forming a zone of inhibition of 10 ± 0.5 mm, chemically synthesized ZnO NPs (100%) with a zone of inhibition of 10 ± 1 mm, green synthesized ZnO NPs (100%) with zone of inhibition of 25 ±

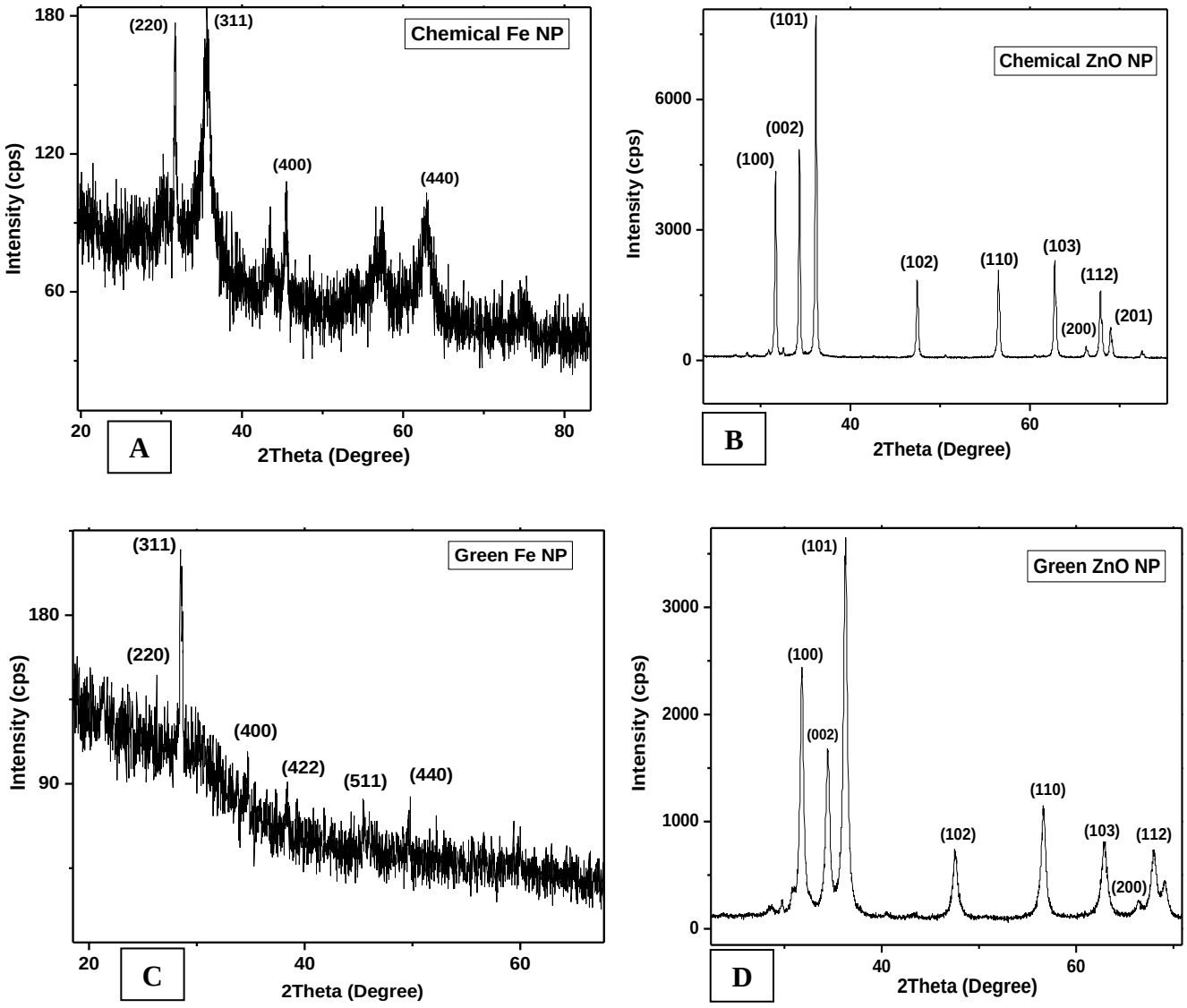

Figure 5: XRD results of chemically synthesized A) Fe NPs B) ZnO NPs and green synthesized C) Fe NPs D) ZnO NPs

1mm (Table 1). It showed resistance to *M. oleifera* methanolic extract and chemically and green synthesized Fe NPs. Green synthesized ZnO NPs inhibited *E. coli*and*S. aureus*, whereas *M. oleifera* acetone pod peel extract, chemically and green synthesized ZnO NPs were able to inhibit *S. aureus*. Since green synthesized ZnO NPs showed sensitivity for both test organisms, the minimal inhibitory concentration was determined using broth dilution method. MIC is the minimum concentration of any biocidal agent required to inhibit the growth of the microbes[5]. In a broth medium, MIC is the concentration of the biocidal agent at which the absorbance of the broth is equal to or less than that of control[30]. The MIC for the test organisms *E. coli* and *S. aureus* was estimated to be 50 µg/µL and 30 µg/µL (Figure 6).

Table 1:- *In vitro* analysis of *M. oleifera* pod peel extracts and synthesized nanoparticles against *E. coli* and *S. aureus*

Extracts/ Nanoparticles	Zone of inhibition (mm)	
	E. coli	***S. aureus***
Methanol extract	Nil	Nil
Acetone extract	Nil	10 ± 0.5
Chemically synthesized Fe NP	Nil	Nil
Green synthesized Fe NP	Nil	Nil
Chemically synthesizedZnO NP	Nil	10 ± 1
GreensynthesizedZnO NP	16 ± 1	25 ± 1

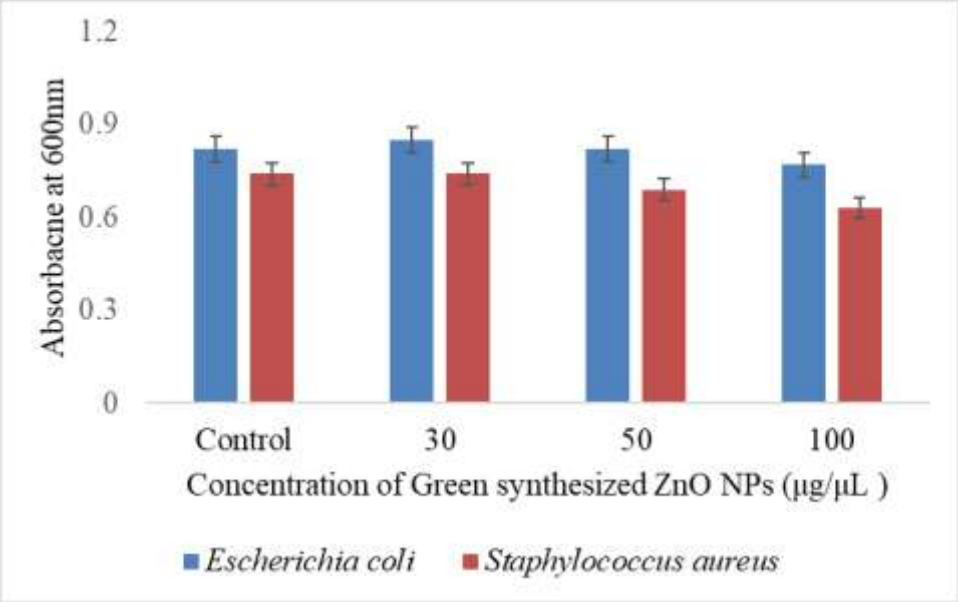

Figure6:- Minimal Inhibitory Concentration estimation of green synthesized ZnO NPs against *E. coli* and *S. aureus*.

The results obtained suggests that green synthesis of NPs was able to inhibit due to the presence of polyphenols and antioxidants that act as capping or reducing agents[10]. The NPs formed showed difference in their bactericidal activity against Gram-negative *E. coli* and Gram-positive *S. aureus*. Previous literature indicate that Gram positive bacteria are more susceptible to ZnO NPs than Gram negative[17,4,2]. However, both Gram-negative and Gram-positive test organisms showed resistance to chemically and green synthesized Fe NPs. This might be caused due to the oxidation of FeNPs and corrosion of its surface[7]due to dissolved oxygen in the NPs suspension. This may have caused negligible bactericidal effect[19]. The bactericidal property is lost as the zero-valent FeNPsis converted to oxides which have low bactericidal properties against both Gram-positive and Gram-negative microbes. A recent work by Ewunkem*et al*, 2021 suggested that some strains of *E. coli* have the potential to develop resistance to a group of FeNPs (magnetite NPs). They are able to do so by the virtue of evolving their metabolic pathways that influence Fe uptake by the bacterial cell[11].

Conclusion:-

Moringa oleifera was utilized for the synthesis of iron and zinc oxide nanoparticles. On assessing the antibacterial action of green synthesized NPs by *in vitro* methods like well diffusionand Minimum Inhibitory Concentration (MIC) assay, against *E. coli* and *S. aureus* and comparing it with the action of their chemically synthesized counterpart, it was found that the green synthesized nanoparticles had better capacity to inhibit the bacteria. Plant extracts involved in green synthesishave incredible antimicrobial properties which is enhanced many folds as it is utilized for synthesis of nanoparticles. This work ventures the antibacterial potential of vegetable waste and their capacity to replace the toxic capping or reducing agents required for nanoparticle synthesis.

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