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ORIGINAL ARTICLE

Biosynthesis of FeO Nanoparticles and Study of Antifungal Activity from DPJ

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ABSTRACT
Nanoparticles are important for various fields, including biomedical, antimicrobial, antioxidant, phytotoxicity, cytotoxicity, and anticancer applications. Nanoparticles nowadays are the most studied and interesting subject among researchers. Nanoparticles are synthesized from physical and chemical methods, but researchers are moving towards new ways, such as eco-friendly and cost-effective methods. Therefore, they find out biologically synthesized nanoparticles utilizing plants, microorganisms like bacteria, fungi, and also algae. This biological phenomenon of nanoparticle synthesis has gained a lot more attention due to its uniqueness and advantages. Plants become a trustworthy candidate to synthesize nanoparticles because of the phytochemicals. These phytochemicals provide stability and act as a reducing agent for nanoparticle synthesis. In this work, we mainly focus on the plant, Medicago sativa L., which is known for its medicinal properties, and it exhibits secondary metabolites and the phytochemicals. Fresh leaves of Medicago sativa were used for preparation of deproteinized juice (DPJ). DPJ reacting with ferric chloride resulted into Iron oxide (FeO) nanoparticles synthesize. Then, the synthesized nanoparticles were confirmed by using characterization techniques such as FESEM, UV-Vis spectra, EDS, FTIR, and XRD. Finally, the applicability of Iron oxide nanoparticles was assessed through antifungal activity utilizing a fungal strain. All the results confirmed that DPJ is capable of synthesizing Iron oxide nanoparticles.
KEYWORDS: *Green Synthesis, Deproteinized juice (DPJ), Iron oxide nanoparticles, Antifungal activity, FESEM, EDS.*

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INTRODUCTION

Deproteinized juice (DPJ) is rich in water-soluble nutrients from leaves. DPJ provides essential components such as Carbohydrates, Amino acids, and Vitamins, making it a valuable resource for various applications in agriculture and biotechnology (1). Additionally, its potential in enhancing microbial growth and supporting sustainable agricultural practices highlights the importance of DPJ in modern farming techniques. Furthermore, the versatility of DPJ allows it to be utilized not only as a growth medium for microorganisms but also as a source of nutrients for animal feed and organic fertilizers. The multifaceted applications of DPJ underscore its significance in promoting both crop yield and soil health, thereby contributing to sustainable agricultural practices (2,3). The diverse uses of DPJ in agriculture, particularly its role in enhancing soil health and crop productivity, make it a crucial component in sustainable farming systems. The ongoing research into DPJ's applications further emphasizes its value in mitigating environmental impacts while improving agricultural productivity. The juice or leaf extract obtained during the process of green crop fractionation (GCF) is commonly utilized to produce leaf protein concentrate (LPC). This involves heating the extract, which leads to the coagulation of proteins and the formation of a curd known as LPC. Subsequently, the LPC is isolated through filtration from the residual juice, referred to as deproteinized juice (DPJ). The DPJ serves as a by-product of the GCF system and is generated in substantial quantities. This brown, aqueous liquid is also identified as "whey" or "liquor" (2)(4). Deproteinized leaf juice (DPJ) is generated as a by-product during the extraction of leaf protein concentrate (LPC) from thermally processed plant material. Inappropriate disposal of DPJ poses a

potential risk to the environment due to bio-pollution (5). Given the economic inputs associated with its production, the valorization of DPJ through strategic utilization is essential for enhancing its sustainability and value. The continued exploration of DPJ's benefits may lead to innovative practices that further align agricultural productivity with environmental sustainability. The ongoing advancements in the utilization of DPJ reflect its potential to revolutionize the nanotechnology area. DPJ could be used for the synthesis of nanoparticles because DPJ has all the potential to fabricate nanoparticles due to its rich organic content and ability to stabilize various compounds. This capability positions DPJ as a promising candidate for developing eco-friendly nanomaterials. Nanoparticles are small in size, ranging from 1 to 100 nm. Nanotechnology is a rapidly evolving field that enables the manipulation of matter at the nanoscale, with significant implications for various industries and applications (6,7). Nanotechnology has seen significant growth recently, especially in fields like medicine, chemistry, and biotechnology, offering a new possibility in drug delivery, gene delivery, and biosensing. Nanosized particles exhibit high reactivity due to their large surface-to-volume ratio. Synthesis methods for nanoparticles include physical, chemical, and biological approaches, with green synthesis gaining popularity due to its environmentally friendly processes and cost-effectiveness potential for sustainable production of nanomaterials (8–10). Green synthesis methods reduce harmful by-products and enhance the efficiency of nanoparticle production, aligning with the principles of green chemistry. Green synthesis involves natural materials like plant extracts, reducing the need for hazardous substances and minimizing environmental impact. This approach not only promotes sustainability but also enhances the safety of nanoparticles for applications in antimicrobial, drug delivery, and medicine.

Phytochemicals present in the plant extract serve as both reducing and stabilizing agents, with essential compounds like Terpenoids, Flavonoids, Phenolic compounds, and more contributing to the reduction process (11). The composition of the leaf extract significantly impacts nanoparticle synthesis, with factors like pH, temperature, contact time, metal salt concentration, and phytochemical profile influencing synthesis, stabilization, and nanoparticle quantity (12). Metal ions are stabilized post-reduction in three phases: activation (involving metal ion reduction and nucleation), growth (ensuring nanoparticle stability), and termination (determining nanoparticle shape). Various metals like copper, silver, gold, zinc, Iron, and others form their oxides under the influence of phytochemicals, enabling metal ion growth and stabilization. Ultimately, the formation of oxygen links metal ions, shaping the nanoparticles distinctly. Iron nanoparticles are also synthesized from the green synthesis approach. Iron nanoparticles have all the properties such as magnetic, high surface area, electrical conductivity, thermal, and non-toxic. Iron nanoparticles synthesized from several plants, such as *Phoenix dactylifera*, *Moringa,* Tea extract, and *Azadiracta indica.* These plants contain medicinal properties; along with this, these plants have active biomolecules like phenols, Flavonoids, Alkaloids, Sterols, Carotenoids, which help in formulation. Plants are used as reducing and capping agents to synthesize nanoparticles.

Iron nanoparticles were synthesized using the extracts of tropical plants. The synthesized FeNPs capped using *Terminalia Billerica* extracts exhibited the highest antioxidant potential. The thermal conductivity of Iron nanoparticles is reported to be higher (13). Durga Devi review on recent studies of Iron nanoparticles isolated through plant-mediated green synthesis. She also argued on the catalytic and biomedical application of Iron nanoparticles (14). In another study, iron nanoparticles were synthesized using a green approach and their potential was investigated by removing nitrate from surface and ground water, and investigating antibacterial activity against *Escherichia coli*. Sreerangaraj 2019 reported the first-time formulation of iron nanoparticles from *R. tuberosa* leaf extract. Iron nanoparticles illustrate strong photocatalytic activity and antimicrobial activity (15). Nowadays, the green approach is exceptionally favorable for the non-toxicity and eco-friendly nature of iron oxide nanoparticles. This study reports that Iron oxide nanoparticles were isolated from *Carica papaya* (Papaya) (16). In another study, *Echhornia crassipes* was used for the synthesis of Iron oxide nanoparticles and to report its antibacterial activity (17). The biogenic fabrication of iron oxide nanorods (FeO-NRs) utilizing FeCl3 and encapsulated with *Moringa oleifera* (MO) has been established in the present study. The synthesized FeO NRs stand out as effective, efficient, and exceptionally promising candidates for antibacterial applications, owing to their affordability, non-toxic characteristics, and simple synthesis methods in therapeutic biomedical settings.

The present work aims to biosynthesis of Iron oxide nanoparticles using the Deproteinized juice of *Medicago sativa* L. A Characterization study was conducted, including UV-vis spectra, scanning electron microscope, EDS, X-ray crystallography (XRD), and FTIR. Investigating its antifungal activity against *Candida tropicalis* and *Candida albicans.*

MATERIA AND METHODS

Medicago sativa L. was collected from the farm field of Vadgaon Shinde, Lohegaon, Pune. The chemicals used include Sabouraud Dextrose Agar (SAB). Petri plates, Microtiter plate, Beaker, Glass rod, Cork borer, Cotton swab, Lamp, Wire loop, Laminar air flow, and Incubator.

Test Organism: Fungi such as *Candida tropicalis* (Berkhout)*, Candida albicans* (Berkhout)*.* Compared with antibiotics, Amphotericin B.

Preparation of DPJ

Fresh leaves of *Medicago sativa* were used for the preparation of DPJ (5). Leaves are washed two to three times with distilled water to avoid contamination and dust. Then the leaves were macerated by using a mortar and pestle. A homogenised liquid mixture was synthesized. This mixture filtered through cotton clothes, and the green colour juice was isolated. The juice released during filtration was employed for heating for 45 min at 90°C. After that, heating was stopped and the solution was cooled down. After the cooling filter, the solution is filtered through Whatman filter paper. Preparation of Leaf Protein Concentration by heat coagulation, and the brown colour juice was released after precipitation. This brown colour juice was called Deproteinised juice (18)(19).

Biosynthesis of Iron Oxide nanoparticles

In a 2:3 ratio, 30 mL of DPJ and 20ml of 0.1M Ferric chloride ($FeCl_3$) are mixed. The mixture was heated under continuous stirring for 40-60 min using magnetic stirring at 1000rpm. 1M NaOH was added dropwise to maintain a pH of 8.0, which resulted in black colour precipitate formation. The reaction solution was centrifuged at 3500 rpm for 10 minutes and washed repeatedly with distilled water to remove impurities and oven dried at 90°C for 8 hours. The obtained dried powder was mashed using a mortar and pestle. Made ready for further characterizations (20).

Well diffusion method

Fungal strains were grown in nutrient broth at 37°c for 24h. This activity was carried out using the well diffusion method, following previous studies (21)(22). Sabouraud Dextrose Agar (SAB) plates were prepared. A sterile cotton swab was dipped into each inoculum and swab streaked over the entire surface of the respective SAB plates, and then, with the help of a cork borer, wells were made in the plates. Different conc. (20µl, 40µl, 60µl) FeO nanoparticles were loaded into the wells, and the plates were incubated at 37°C for 24h. Amphotericin B (1mg/ml) was used as a control. After incubation, the zone of inhibition was measured.

Preparation of Inoculum

A loopful of culture was inoculated in broth (SAB), Sabouraud Dextrose agar broth (for fungus), and grown for 24 hours. The culture OD was adjusted to 0.5 McFarland standard to get 1 × 108 CFU/ml.

The Minimum Inhibitory Concentration (MIC) of the samples against the test organism was determined by using the Resazurin microtiter plate assay (REMA). This assay was performed using flat-bottom 96-well clear microtiter plates. The wells in the first column of the plate were filled with 100 µl of 1X SAB broth (for fungus) with standard antibiotic Amphotericin B (5mg/ml), while the second column was filled with 100 µl 1X media and 100 µl of sterile distilled water.

From columns 3 to 8, the 1st rows were filled with 100 µl 2X media and 100 µl of the sample. Then, in the 2nd row onwards, each well was filled with 1X media. An identical two-fold serial dilution was made from the 1st row to the 6th row. Lastly, in all the wells, 10 µl of culture was added and mixed thoroughly. The cultured microplate was sealed with the lid and incubated at room temperature for 24 hrs. The MIC of the sample was detected following the addition of 10µL of 2mg/mL Resazurin in all the wells and incubated at 37°C for 30 min. Microbial growth was determined by observing the change in colour of Resazurin in the microplate wells. MIC is defined as the lowest sample concentration showing no colour change and exhibiting complete inhibition of microbial growth (21).

RESULTS AND DISCUSSION

FESEM AND Energy Dispersive X-ray Spectroscopy

FESEM images were taken at different magnifications to examine the shape and size of the nanoparticles synthesised, as shown in Figure 1. The surface morphology confirms the formation of FeO nanoparticles in their agglomerated form. The nanoparticle size was found to be between 9nm and a maximum of 24nm. The shape of FeO NPs at the nanoscale level shows a spherical shape.

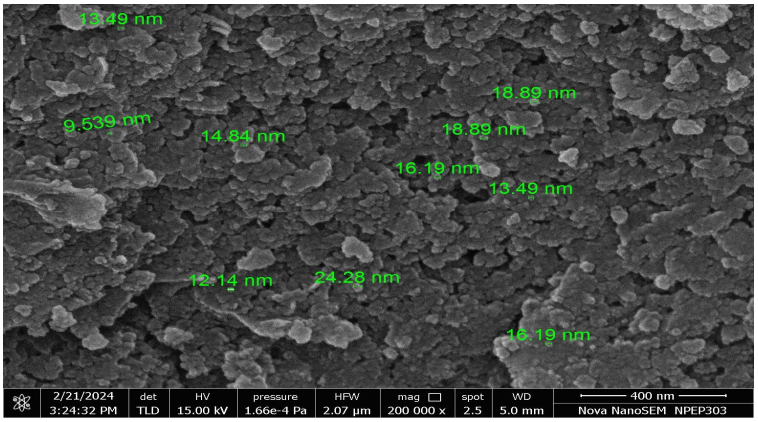

Figure 1: FESEM of Iron oxide

EDS confirms peaks of C, Zn, along with Fe and O, as shown in Figure 2. C signals are attributed mainly to the polyphenol groups and other C-containing molecules present in the DPJ. The atomic percentages were obtained by EDS quantification: 48.74% O, 19.95% Zn, 31.16% C, 0.14% Fe. These values are helpful in the atomic content on the surface and near-surface region of the FeO NPs (20).

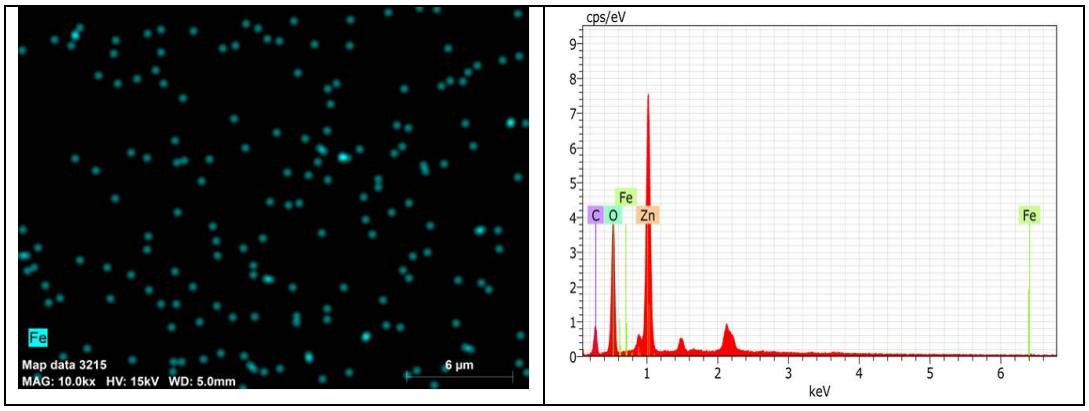

Figure 2: EDS result of Iron oxide nanoparticles

UV-VIS Spectrophotometer

The synthesis of FeNPs was confirmed by UV-Visible analysis. The wavelength range is set at 200-800nm. Peaks were observed at 312nm. Different absorbance peaks were observed for FeNPs synthesised from different salt concentrations, as shown in Figure 3.

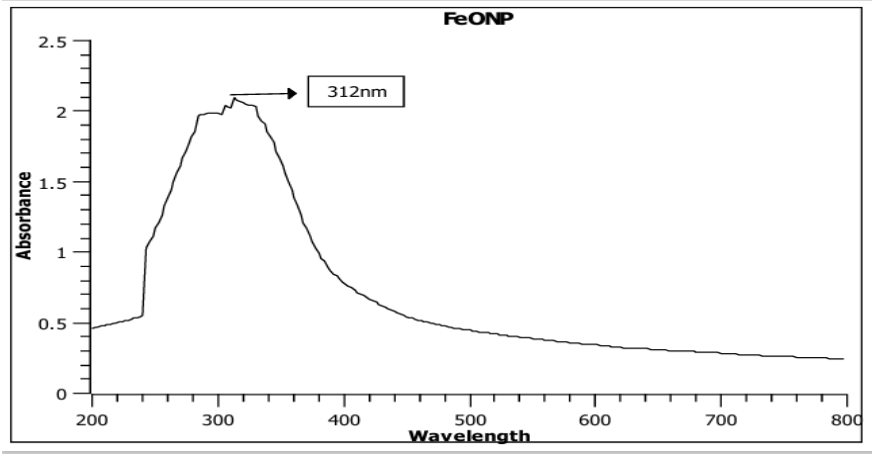

Figure 3: UV-Visible spectra of Iron oxide nanoparticles

Fourier Transform infra-red (FTIR)

FTIR spectroscopy was used to identify the presence of functional groups responsible for the synthesis of FeNPs. Figure 4 represents the absorbance bands of FeNPs in the range of the wave region between 500

and 4000 cm^{-1}. Characteristic peaks were observed at 1589cm^{-1}, 3301cm^{-1}. 1589 cm^{-1} corresponds to C=O stretching and 3301cm^{-1} vibration stretching assigned to O-H stretching carboxylic group.

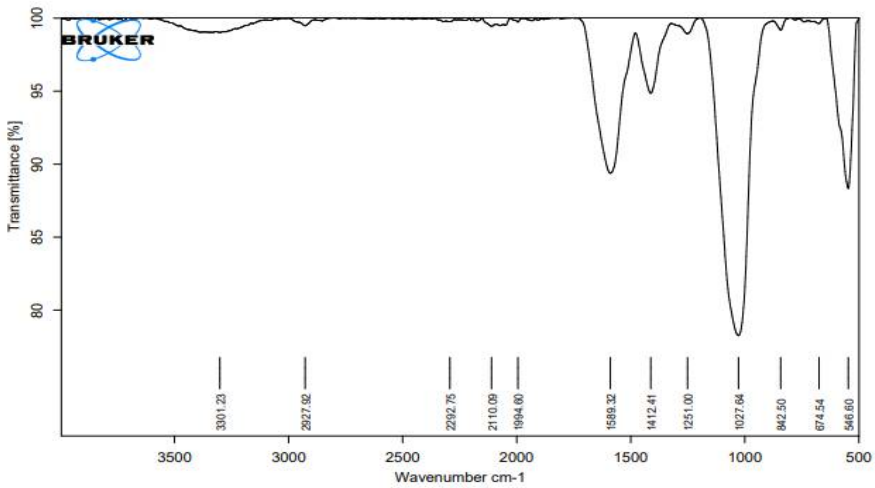

Figure 4: FTIR result of Iron oxide nanoparticles

X-ray diffraction crystallography (XRD)

The XRD patterns of the FeO NPs lack distinct diffraction peaks; they exhibit small intensity peaks, which resulted in the FeNPs being amorphous. Figure 5. The result of XRD is compared with the previous study of nanoscale zerovalent iron nanoparticles synthesized using tea polyphenols; the second sample shows small intensity peaks. The author interprets that small intensity peaks were amorphous in nature. (Wang, 2013),

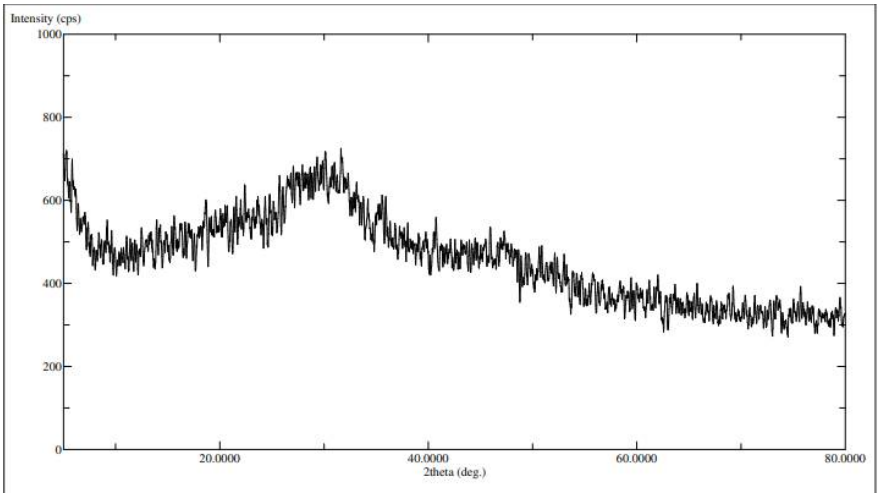

Figure 5: XRD result of Iron oxide nanoparticles

Antifungal result

The antifungal activity against *Candida albicans* exhibits zones ranging from 17.24 mm to 17.92 mm (Figure 6). The Minimum Inhibitory Concentration was 0.83 mg/ml. The result of the antifungal activity of iron oxide nanoparticles agrees with previous results. The fungi strain *Candida tropicalis* exhibits antifungal activity ranging from 17.94 mm to 18.28 mm, and the Minimum Inhibitory Concentration was 0.166 mg/ml. This result also agrees with previous studies conducted on iron oxide nanoparticles. The result of iron oxide nanoparticles proved that iron oxide nanoparticles will be utilized in the future in the biomedicine field or clinical sciences. More research in this direction opens opportunities for iron nanoparticles to become a better alternative for drug resistance.

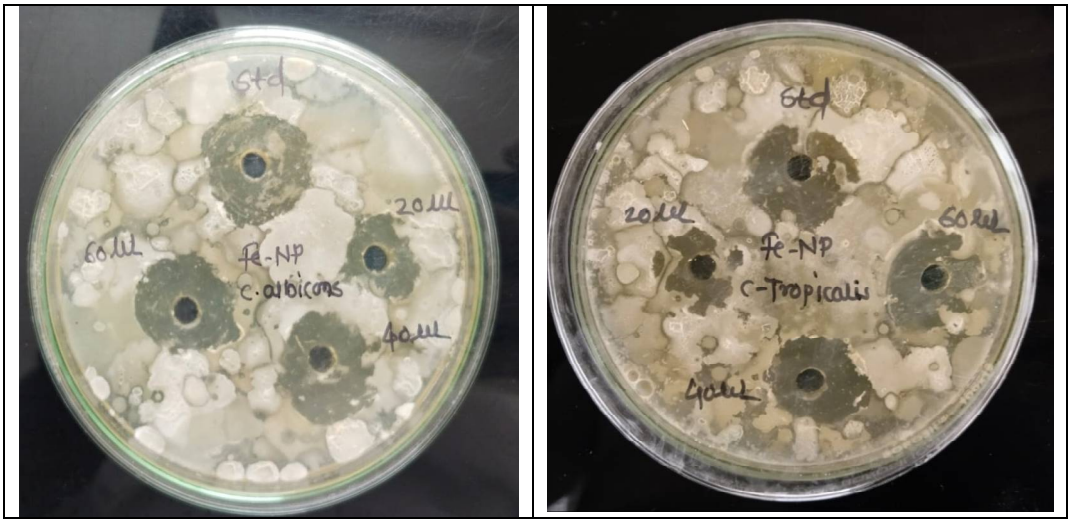

Figure 6: Antifungal activity of *Candida albicans* and *Candida tropicalis*

CONCLUSION

This research work concluded that Iron oxide nanoparticles can be synthesized from deproteinized juice (DPJ) of *Medicago sativa*. All the characterization techniques confirmed the formation of iron oxide nanoparticles from DPJ. Fabricated nanoparticles are exhibiting inhibition against fungal strains, i.e., *Candida albicans* and *Candida tropicalis.* Therefore, Iron oxide nanoparticles are capable of inhibiting fungi. In the future, it might be an alternative to antifungal drugs. This approach of iron nanoparticle synthesis was eco-friendly and cost-effective. The use of DPJ means avoiding bio-pollution and best use of by-product for nanoparticle synthesis, which could open a new point of view of research. Antifungal activity made this Iron oxide nanoparticle more valuable and productive; in the future, more studies will open new fields of biomedical and clinical sciences.

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