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In Vitro Antibacterial Activity of Iron Oxide Nanoparticles Synthesised using Hydrocotyle Umbellata L. Against Oral Pathogens

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Hydrocotyle umbellata L. is a plant known for its therapeutic properties, traditionally used to treat various ailments. The advent of nanotechnology has introduced novel methods for creating nanoparticles with unique properties, including enhanced antimicrobial activity. Iron oxide nanoparticles have been shown to possess significant antimicrobial potential, making them promising candidates for combating antibiotic-resistant bacteria. To synthesise iron oxide nanoparticles using Hydrocotyle umbellata L. and evaluate their antibacterial activity against oral pathogens under in vitro conditions. Preparation of nanoparticle Solution was done by using Hydrocotyle umbellata L.plant extract. The agar well diffusion method was employed using Mueller Hinton agar plates inoculated with Streptococcus mutans, Lactobacillus sp Staphylococcus aureus, and Candida albicans.The antimicrobial activity was measured by the diameter of the inhibition zones in the wells. Time-Kill Kinetic Analysis was done to evaluate the antimicrobial properties of the nanoparticles against the oral pathogens. The synthesised iron oxide nanoparticles exhibited significant antibacterial activity, as indicated by the inhibition zones against all tested pathogens. The highest concentration (100 µg/mL) produced the largest inhibition zones, averaging around 9 mm. The time-kill curve assay demonstrated a concentration-dependent reduction in bacterial counts, with the 100 µg/mL concentration showing the most substantial decrease. Iron oxide nanoparticles synthesized using Hydrocotyle umbellata L. show potent antibacterial activity against various oral pathogens. The green synthesis method employed is both eco-friendly and cost-effective, providing a promising alternative to conventional antibiotics in the treatment of bacterial infections.

Keywords: Antimicrobial activity, Bacterial infections, Biological method, In vitro, Oral pathogens.

1. Introduction

The variety Hydrocotyle (Araliaceae) is a semi aquatic plant of tropical regions. Hydrocotyle umbellata L is local to the American landmass and is customarily involved by networks in Argentina, Brazil and Cuba in the treatment of dermatological conditions (Florentino et al., 2013). The plant has high importance in phytotherapy and in the Ayurvedic medication (Indian) due to its anxiolytic and memory energizer impacts as it contains phytochemical substances like triterpenes, saponins, flavonoids, and polyacetylenes (Oliveira et al., 2017). Nanotechnology is a part of innovative modern science that deals with particles within range of 1-100 nm.The synthesis of nanoparticles is an arising area of innovation and investigation in the field of material science for their interesting size and shape and have dependent elements that are unique in relation to the normal mass design (Begum et al., 2023). For overcoming antibiotic resistance in bacteria the use of metal oxide nanoparticles is one of the promising ways.

Iron oxide nanoparticles have broad applications in the biomedical field. Many studies have shown us the antimicrobial potential of iron oxide nanoparticles (Behera et al., 2012). 10% to 30% patients admitted to hospitals get bacterial infection in India, up to 70% of organisms responsible for infections are resistant to at least one antibiotic (Sathyanarayanan et al., 2013). Bacterial infection and invasion is a serious issue and increasing concern in day to day life and it causes significant damage to several sectors of medicine and food packaging (Díez-Pascual & A. M. 2018). Flavonoids which are present in hydrocotyle umbellata L. are better antimicrobial agents against a broad range of pathogenic microbes. With an increase in infections caused by antibiotic resistance bacteria, flavonoids are better substitutes for antibiotics (Xie et al., 2015). Bacteria can be divided into gram-positive and gram -negative based on their cell wall structure.

According to studies Gram-positive bacteria are more resistant to nanoparticle mechanisms of action due to the differing cell walls (Slavin et al., 2017). Nanoparticles may or may not exhibit size-related properties that differ significantly from those observed in fine particles or bulk materials and individual molecules are usually not referred to as nanoparticles (Rahman et al., 2011). Iron oxide [Fe_2O_3] nanoparticles are capable of depolarizing the microbial membrane to generate oxidative stress that disturbs cellular homeostasis which causes inhibition of growth of the microbe by inducing the production of reactive oxygen species (Caldeirão et al., 2021). This study's motive is to synthesise in a cost effective and eco-friendly way and evaluate the antibacterial activity of iron oxide nanoparticles synthesised using Hydrocotyle umbellata L. against oral pathogens under in vitro conditions.

2. Materials and methods

The preparation of Hydrocotyle umbellata L mediated iron oxide nanoparticles was done in two stages. In the first stage, the preparation of the plant extract was done and in the second, the preparation of the nanoparticle solution.

2.1. Preparation of hydrocotyle umbellata l. plant Extract:

1g of plant dry powder was mixed to 100 ml of water to prepare the plant extract. This solution was later kept in a heating mantle for boiling the solution for 15 to 20 minutes at 50 to 60°C.

The solution was then filtered using a muslin cloth or Whatman no.1 Filter paper. The filtrate was stored.

2.2.Preparation of iron oxide nanoparticle solution:

0.486g of iron chloride was mixed with 50 ml distilled water and then 50 ml plant extract also added in the iron chloride solution for the preparation of the nanoparticle solution. The nanoparticle solution was then placed in an Orbital shaker for the following 2 days. The solution was assessed periodically for any colour changes, followed by UV reading to verify the synthesis of nanoparticles in various different time intervals. The synthesised nanoparticle solution was centrifuged for 10 minutes at 8000 rotations per minute. The pellet formed is collected for the following biomedical research and supernatant is discarded..

2.3. Antimicrobial activity

To evaluate the antimicrobial activity of the green synthesised iron oxide nanoparticles Agar well diffusion technique was used.Mueller Hinton agar plates were prepared and sterilised using an autoclave at 121ºC for 15- 20 minutes, the medium was poured onto the surface of sterile Petri plates and allowed to cool to room temperature. The bacterial suspension (Streptococcus mutans, Lactobacillus sp, Staphylococcus aureus, Candida albicans) was spread evenly onto the agar plates using sterile cotton swabs. Wells of 9 mm diameter were created in the agar plates using a sterile polystyrene tip. The wells were then filled with various concentrations (25, 50, 100 µg/mL) of Fe_2O_3 nanoparticles. An antibiotic (e.g., Bacteria-Amoxyrite, Fungi-Fluconazole) was used as a standard. The plates were incubated at 37°C for 24 hours and 2 days for fungal cultures. The antimicrobial activity was evaluated by measuring the diameter of the inhibition zone surrounding the wells. The zone of inhibition was calculated by measuring the diameter in millimetres (mm) of the zone of inhibition using a ruler. This method was followed by (Rajeshkumar et al., 2019).

2.4. Time kill curve assay:

This technique was followed by (Tharani & Rajeshkumar 2023), A time-kill curve assay was conducted to assess the bactericidal properties and concentration-dependent relationship between Hydrocotyle umbellata L mediated iron oxide nanoparticles and the net growth rate of Lactobacillus sp, C.albicans, S.aureus and S.mutans over regular intervals of time. The assay involved culturing the four wound pathogens in Mueller Hinton Broth supplemented with varying concentrations of iron oxide nanoparticles (25, 50 and 100 µg/ml), followed by time-kill curve analysis. An antibiotic (e.g., Bacteria-Amoxyrite, Fungi-Fluconazole) was used as a standard. After a pre-incubation period of four hours in a medium devoid of any antimicrobial agents, growth curves were carried out before the test to ensure that all pathogens had reached a stable early-to-mid log phase. An inoculum consisting of 0.5 McFarland of each pathogen was created in sterile phosphate-buffered saline. This inoculum was collected from cultures that had been cultivated on Mueller Hinton agar plates at 37 °C for 18–20 h. After that, 30 µL of the inoculum was diluted in 15 mL of antimicrobial-free Mueller Hinton Broth medium that had been pre-heated to 37 °C, and 90 µL of the resultant mixture was distributed evenly over each well of a 96-well ELISA plate. To each well containing 90 µL of pre-incubated wound pathogens, 10 µL of Hydrocotyle umbellata lmediated iron oxide nanoparticles at five various concentrations was added, along with the untreated control.

3. Results and Discussion

3.1. Visual Observation

The green synthesis of iron oxide nanoparticles using H. umbellata, the iron chloride solution was mixed with the plant extract. The plant extract have phytochemical compounds, it has reduce the metal ions. Iron chloride was converted into iron ion and the color change reaction was observed in visually. The pale brown color turned into darkish black shade, this color was confirmed the presence of iron oxide nanoparticles.

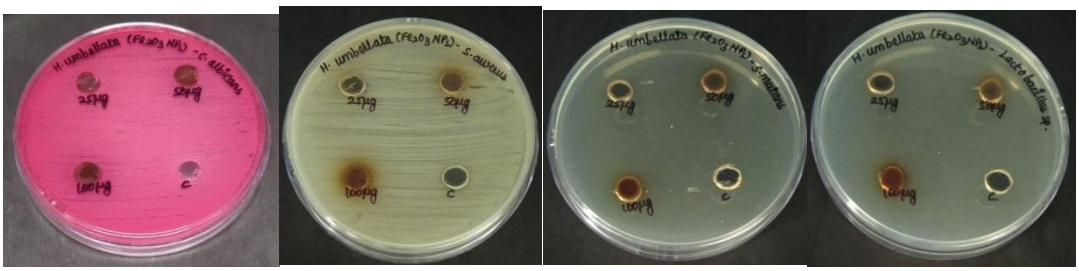
Figure 1: Preparation of iron oxide nanoparticles, A) Weighing of plant powder, B) H. umbellata solution, C) Boiling of H. umbellata, D) H. umbellataaqueous extract, E) Reaction mixture, and F) green synthesis of Iron oxide nanoparticles.

A B C D

E F

3.2. Antimicrobial activity

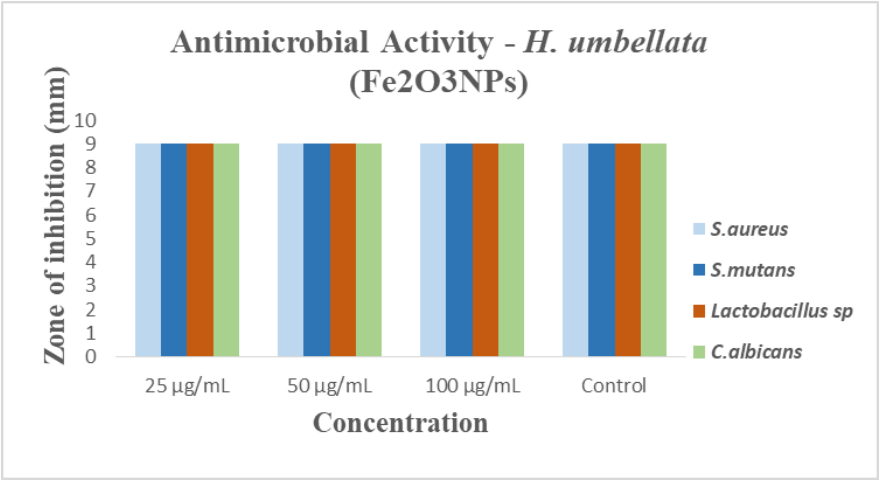
Figure 2: The plate images were representing the antimicrobial activity of iron oxide nanoparticles against oral pathogens A) C. albicans, B) S. aureus, C) S. mutans, and Lactobacillus sp.



A B C D

The antimicrobial activity of H. umbellata (Fe2O3NPs) against four different oral pathogens: S. aureus, S. mutans, Lactobacillus sp., and C. albicans(Figure 2). Antimicrobial activity is measured by the zone of inhibition in millimeters (mm), which is a clear area around the substance where bacteria do not grow, indicating the effectiveness of the antimicrobial agent.

Figure 3: Antimicrobial activity of iron oxide nanoparticles synthesised using Hydrocotyle umbellata L against oral pathogens



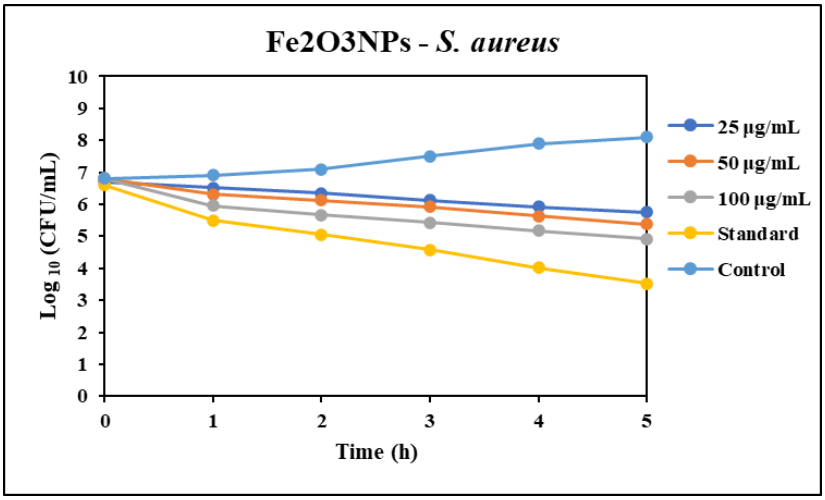
For S. aureus the highest concentration (100 µg/mL) shows the largest zone of inhibition, which is just below 10 mm,for S. mutans with the largest zone of inhibition at the highest concentration, which is slightly above 9 mm. Lactobacillus sp. shows a similar trend, with the zone of inhibition increasing with the concentration of the substance., which is just below 9 mm. C. albicans shows a different pattern, with the zone of inhibition being the largest at the intermediate concentration (50 µg/mL), which is just above 9 mm, and slightly smaller at the highest concentration. The control group does not show any zone of inhibition, which is expected as it serves as a baseline to compare the effects of the substance at different concentrations.Overall, the graph suggests that H. umbellata (Fe_2O_3) has antimicrobial

properties against the tested microorganisms, with generally increased effectiveness at higher concentrations (Figure 3) respectively.

3.3. Time kill curve assay

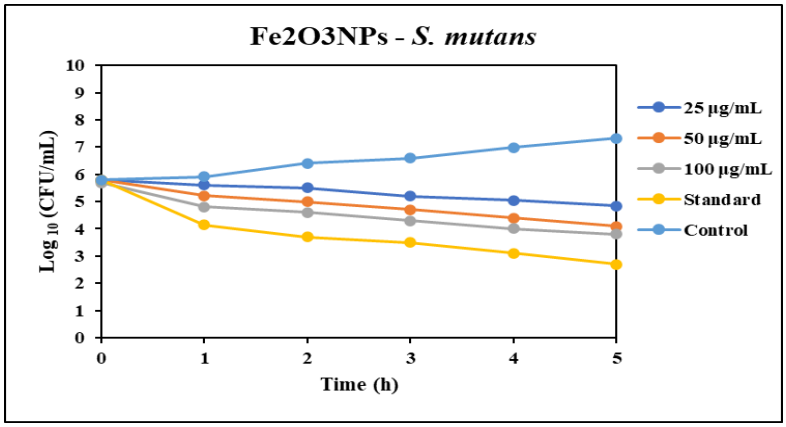
The time-kill curve assay of bio-synthesized Fe_2O_3NPs against the four wound pathogens Lactobacillus sp, C.albicans, S.aureus and S.mutans, has shown concentration-dependent antimicrobial effects when compared to the control group [Figure 4, 5, 6, & 7].

Figure 4: Analysis of Time kill curve assay of Fe_2O_3NPs against S. aureus



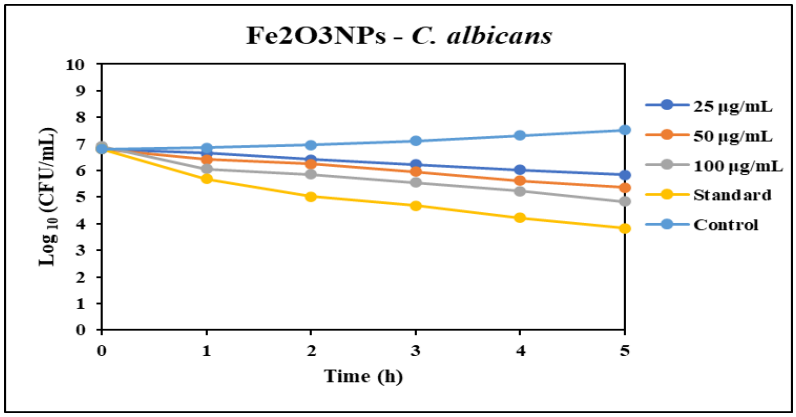
At all concentrations of Fe_2O_3 (25µg/ml, 50µg/ml, and 100µg/ml), there was a noticeable reduction in S. aureus counts compared to the control group over the entire assay duration. In figure 4 Fe_2O_3 exhibit a dose-dependent antibacterial activity against S. aureus. While the standard antibacterial agent consistently showed the most substantial inhibition of bacterial growth, Fe_2O_3 NPs at 100 µg/mL achieved a comparable reduction in CFU/mL. These results suggest that Fe_2O_3 NPs could be a potential alternative or adjunct to traditional antibacterial agents in the treatment of infections caused by S. aureus.

Figure 5: Analysis of Time kill curve assay of Fe_2O_3NPs against S. mutans



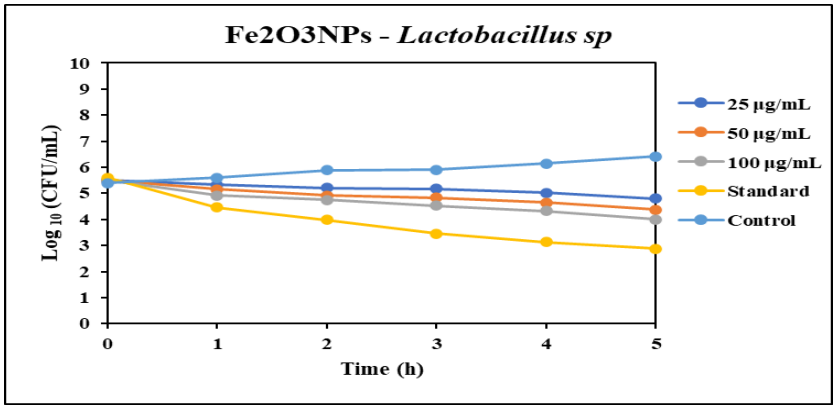
In figure 5, S. mutans the control group's CFU/mL count remains relatively constant throughout the 5-hour period, indicating stable bacterial growth without any treatment.The standard antimicrobial treatment group shows a decrease in CFU/mL count, suggesting effectiveness in reducing the bacterial population.The 25 µg/mL Fe_2O_3NP treatment group shows a slight decrease in CFU/mL count, indicating some antimicrobial activity, but not as pronounced as the higher concentrations.The 50 µg/mL Fe_2O_3NP treatment group shows a more noticeable decrease in CFU/mL count compared to the 25 µg/mL group, suggesting increased efficacy with a higher concentration.The 100 µg/mL Fe_2O_3NP treatment group shows the most significant decrease in CFU/mL count, indicating the highest level of antimicrobial activity among the tested nanoparticle concentrations.

Figure 6: Analysis of Time kill curve assay of Fe_2O_3NPs against C. albicans



In figure 6, The 25 µg/mL Fe_2O_3NP concentration had a modest antimicrobial effect, with a slight decrease in CFU/mL count over time.The 50 µg/mL Fe_2O_3NP concentrations demonstrated a more pronounced antimicrobial effect than the 25 µg/mL concentration but was less effective than the 100 µg/mL concentration. The Fe_2O_3 nanoparticles exhibited dose-dependent antifungal activity against C. albicans. Higher concentrations of Fe_2O_3 nanoparticles were more effective in decreasing the CFU/mL count of C. albicans over time. The 100 µg/mL concentration of Fe_2O_3NPs showed the most substantial antimicrobial effect.

Figure 7: Analysis of Time kill curve assay of Fe_2O_3NPs against Lactobacillus sp.



In figure 7, The standard antibacterial treatment group shows a decrease in CFU/mL count, suggesting that the treatment is effective in reducing the Lactobacillus sp. bacterial population.The 25 µg/mL Fe_2O_3NPs treatment group shows a slight decrease in CFU/mL count, indicating some antifungal activity, but not as pronounced as the higher concentrations.The 50 µg/mL Fe_2O_3NP treatment group shows a more noticeable decrease in CFU/mL count compared to the 25 µg/mL group, suggesting increased efficacy with a higher concentration.The 100 µg/mL NP treatment group shows the most significant decrease in CFU/mL count, indicating the highest level of antifungal activity among the tested nanoparticle concentrations.

4. Discussion

In previous studies C.albicans shown resistance against miconazole based on chitosan-coated iron oxide by bacterial cellsbut in this study the synthesis of iron oxide nanoparticles using Hydroctyle umbellata L have shown inhibition of growth of C.albicans and also other oral pathogens (Arias et al., 2020). The synthesised Hydrocotyle umbellata L. mediated iron oxide nanoparticles showed potent antibacterial effects with a maximum inhibition zone of average 9mm at 100 µg/mL concentrations against all the four oral pathogens. In all the other previous study the inhibition zone was more than 9mm at 100 mg/ml and the Fe_2O_3 Nanoparticles have antimicrobial activity against both Gram-positive and Gram-negative bacteria (Masadeh et al., 2015). In the current study iron oxide nanoparticles have done tests against only Gram-positives. In previous studies Fe_2O_3 NPs were synthesised using chemical methods such as sol–gel methodical precipitation method etc. which all possess threats to the environment as well as being expensive (Arias et al., 2018). But as the green synthesis method was used for synthesising iron oxide nanoparticles using the Hydrocotyle umbellata l extract it is eco-friendly and cost effective.

In UV visible spectroscopy analysis the Surface plasmon resonance (SPR) peak with maximum absorbance at 350 nm, provides preliminary confirmation of Fe_2O_3 NPs formation. In a previous study the ideal wavelength of 375 nm was observed in the UV–vis spectroscopy readings for Fe_2O_3NPs synthesised (Shimpi et al., 2018). The green synthesised iron oxide nanoparticles using the Hydrocotyle umbellata l exhibited better antibacterial activity than the standard drugs available. In a previous study, The antibacterial activity of FeNP derived from seedless pods of Acacia nilotica was tested against four species of human pathogenic bacteria: Salmonella, Marsa, Escherichia coli, Staphylococcus aureus, and Candidia. FeNP antimicrobial propensity observed clearly at higher concentration (20, 40, and 60 µg), while at lower concentrations weak activity was detected (Da'na et al., 2018).

5. Conclusion

This study has demonstrated an ecofriendly and cost-effective synthesis of iron oxide nanoparticles using Hydrocotyle umbellata L. In UV visible spectroscopy analysis the Surface plasmon resonance (SPR) peak with maximum absorbance at 350 nm, provides preliminary confirmation of Fe_2O_3 NPs formation. The synthesized Hydrocotyle umbellata L. Mediated iron oxide nanoparticles showed potent antibacterial effects with maximum inhibition zone of

average 9mm at 100 µg/mL of concentration against all the four oral microbes and hence can be used as an antibacterial agent in various pathological diseases at this optimal concentration.

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Disclosure 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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