GREEN-SYNTHESIS AND CHARACTERIZATION OF IRON NANOPARTICLES BY USING *AZADIRACHTA INDICA* LEAVES EXTRACT

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Abstract
Nano-particles possess unique, electrical, optical as well as biological properties and are thus applied in catalysis, cosmetics, and anti-bacterial activity by using leaves extract of *Azadirachta indica* (neem). Under different experimental conditions green-Iron based nanoparticles (FeNPs) were synthesized. Green-synthesized FeNPs were observed qualitatively by color differentiation pattern (color changed from brown to black) and the formation and stability of the reduced FeNPs in the colloidal solution were monitored by UV-Vis spectrophotometer (range of 200-700 nm). Fourier Transform Infrared (FTIR) spectrum (range of 4,000-400 cm^{-1}) established the presence of phenolic compounds in the leaves extract of *Azadirachta indica*. Therefore, bioactive compounds such as saponins, flavonoids, steroids, and glycosides were detected to be present in the leaves of *Azadirachta indica.* Anti-bacterial activity shown by FeNPs against pathogen such as *E. coli* and *S. aureus*, by agar-disc diffusion method. The zone of inhibition (ZOI) by FeNPs on *S. aureus* was 14.8 mm while on *E. coli* was 9.2 mm. Thus, the synthesized FeNPs proved to have very high anti-bacterial activity on both *S. aureus* and *E. coli*. These results show that the green-synthesis of FeNPs using leaves extract of *Azadirachta indica* successfully proved the anti-bacterial potential and phytochemical properties.

Keywords: *Azadirachta indica*, FeNPs, FTIR, UV-Vis spectrophotometer, Anti-bacterial activity, Phytochemical properties.

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INTRODUCTION

Nanoparticles can be synthesized by physical method which is through evaporation, laser ablation, vaporization. As opposed to the chemical technique, which reduces the metal ions in solution under circumstances that encourage the eventual creation of tiny metal clusters or aggregates. This chemical method is group into classical method, biosynthesis is a form of chemical method that chemicals are not harmful and naturally occurring reducing agent such as plant extract is used[1]. It was also reported that the metal nanoparticles formation is as the result of the antioxidant and reducing property of the phytochemicals[2]. Iron nanoparticles are given less attention due to it extreme reactivity which has rather made it difficult to study. However, iron has very potent magnetic and catalytic property[3].

The plant studied in this work is *Azadirachta indica* (Neem), a member of the mahogany family. Various plant parts can be used in the synthesis of metal nanoparticles that is the leaf, stem, root, flower and seeds. A simple one step method for the synthesis of metal nanoparticles by the reduction of aqueous iron ions using leaf extracts of *Azadirachta indica* at room temperature without using any additive protecting the metal nanoparticles from aggregation[4,5]. In this work the potential of Iron nanoparticles was investigated, and the application of plant-based iron nanoparticles (FeNPs) in the development of antibacterial nanoparticles was carried out by agar disc diffusion method against *Escherichia coli*, and *Staphylococcus aureus*[7,8].

The objective was green-synthesis of FeNPs by using *Azadirachta indica* leaves extract and evaluate their anti-bacterial activity and phytochemical analyses. The production of FeNPs through bio-reduction of iron (Fe) ions by the *Azadirachta indica* leaves extract and their antibacterial and phytochemical analyses may provide valuable technical parameters for industrialization of the green-synthesis technique and further application of the nanoparticles.

MATERIALS AND METHOD

Materials

Fresh and healthy leaves of *Azadirachta indica* were collected from ITM University campus (26°08'17.6"N 78°12'25.5"E) in the month of October, 2022. The precursor i.e. Iron (III) Chloride or Ferric Chloride ($FeCl_3$) was obtained from Sigma Aldrich[5]. Chemical was of analytical grade and used without purification.

Method of Leaves Extract Preparation

Fresh and healthy leaves of plant (*Azadirachta indica*) were thoroughly washed multiple times in running tap water and distilled water. For surface sterilization sodium hypochlorite was used to remove the dirt and dust on the surface of the plant leaves. After that, leaves were plucked from branches breaking from petiole. Approximately 800 gm leaves were dried in sun for 3-4 days till the water in them was fully evaporated and finely grind into powder. 100 gm of this prepared powder was put in a glass bottle and mixed with 100 ml of distilled water and placed in an orbital shaker for next two days. The extract was filtered using Whatman filter paper number one in a conical flask and the leaves were discarded. The obtained extract was refiltered multiple times by Whatman filter paper to discard any residues and a clean extract was obtained in a conical flask. This extract was used to synthesis nanoparticles of iron. The green cleared filtered solution of leaves extract was stored at 4^{o}C for further use[4].

Green-synthesis of Iron-based Nanoparticles (FeNPs)

Before using any glassware, every glassware was sterilized respectively. 40 gm of plant leaves extract was taken in a beaker and 40 gm of 0.001 M Iron (III) Chloride or Ferric Chloride ($FeCl_3$) solution was prepared in another beaker. Both the solutions were mixed maintaining a 1:1 ratio by concentration in the temperature range of 50-60 ^{o}C. The color transformation of leaves extracts after mixing $FeCl_3$ solution from dark green to greenish black indicated the formation of iron-nanoparticles (FeNPs). Then centrifuge the solution at 5000 rpm for 10 minutes. After centrifugation it was observed that the pellet settled down which contained nanoparticles in it. The supernatant was kept in a separate beaker and the pellet is removed in a Petri dish and kept for drying. Repeated the centrifugation process with the supernatant and kept the pellet for drying in a Petri dish which is obtained again. After the pellet that is, iron nanoparticles got dried stored them covered in a Petri dish or Eppendorf tubes for further analyses and characterization[4,5].

Characterization of Iron-based Nanoparticles (FeNPs)

The synthesized FeNPs were subjected to various characterization methods to identify their specific properties. For optical properties, Shimadzu's UV-1800 UV-Vis spectrophotometer was used to record the absorption spectrum to identify the surface plasmon resonance (SPR) band with a resolution of 1 nm between 200-700 nm to confirm

the formation of FeNPs. The functional groups were identified by recording the FTIR spectrum using an Alpha BRUKER Transmission Spectrometer from Pike Technologies. Fourier transform infrared spectroscopy (FTIR, Thermo Fischer Scientific) recorded the absorption spectra in the range of 4,000-400 cm^{-1} to identify the presence of functional groups involved in bio-reduction[5,9].

Phytochemical Analysis of *Azadirachta indica* Leaves Extract

Extract of *Azadirachta indica* leaves were subjected for different phytochemical tests such as saponins, glycosides, flavonoids, steroids, and anthraquinones to identify the presence of these phytochemicals[10,8].

Test for Saponins: A quantity of 1 gm of extract mixed with 5 ml of distilled water in a test tube and filtered. In filtrated solution 3 ml distilled water was further added and shaken it vigorously for 3-5 minutes. The foam obtained on warming indicated the presence of saponins[8].

Test for Glycosides: Benedict's test was applied to detect glycosides in a plant leaves extract. 10 ml plant extract and 10 ml of benedict's reagent was mixed and heated for 2-3 minutes. A brown colored ring formed at the junction indicated the presence of glycosides[11].

Test for Flavonoids: A quantity of 2 ml of 2% sodium hydroxide was added in plant leaves extract, after few minutes the solution turned pale-yellow in colour indicated the presence of flavonoids[8].

Test for Steroids: In a clean test tube 2 ml of chloroform was added with plant leaves extract and allowed the mixture to cool followed by the addition of 2 ml concentrated sulfuric acid carefully in the mixture. The lower chloroform layer became red indicated the presence of steroids[10].

Test for Anthraquinones: Borntrager's test was applied as confirmatory test for anthraquinones presence. 10 ml of benzene was added to 6 gm plant leaves extract, it is soaked. After 10 minutes 10 ml of 10% ammonia solution was added in it and shaken vigorously. The pink/violet formation showed the presence of free anthraquinones[12].

Anti-bacterial Activity Test

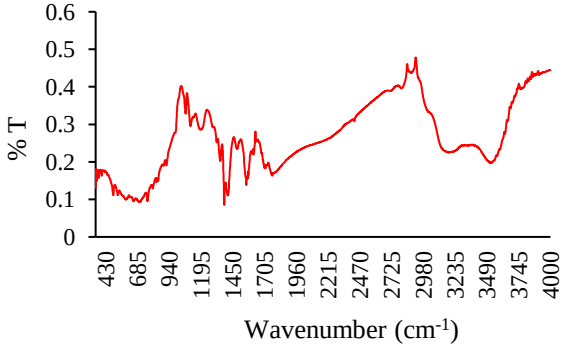
The *Azadirachta indica* leaves extract were tested on the culture strains of *E. coli* and *S. aureus* on agar plates. The sensitivity of the tested pathogenic organisms to plant leaves extract was shown by zones of inhibition after incubation. The zones of inhibition were measured and the Minimum Inhibitory Concentration (MIC) for the extract was determined by the agar-disc diffusion method[7,8].

RESULTS AND DISCUSSION

UV-Vis Spectroscopy

Transformation of the color from pale yellow to black of the solution was due to the presence of FeNPs formed by the reduction of iron salts. It was suggested that compounds like flavanones acts as reducing agent when *Azadirachta indica* leaf extract was used[4]. It was noticed that the instantaneous color change took after about 10 min, thereafter no further color of the reaction mixture changed. This indicated that iron salts present in the reaction mixture had been reduced completely. Following this, the formation of FeNPs was further confirmed by the UV-vis spectral analysis (Figure 1). The formation and stability of FeNPs from their respective salts which gave characteristic peaks at 24 hrs. time interval at different wavelengths using UV-vis spectroscopy. UV-vis spectroscopy analysis was done in the range of 200-700 nm and the maximum absorbance was observed at around 300 nm and 350 nm regions for the formation of FeNPs due to the excitation of surface plasmon vibrations and the absorption peak at 320 nm is 2.3[5]. Thus, the FeNPs can be synthesis within 10 min by this green method using fresh neem leaves make this synthesis method suitable for biomedical applications and biological studies.

Figure-1: UV-vis absorption spectra of iron-nanoparticles (FeNPs).

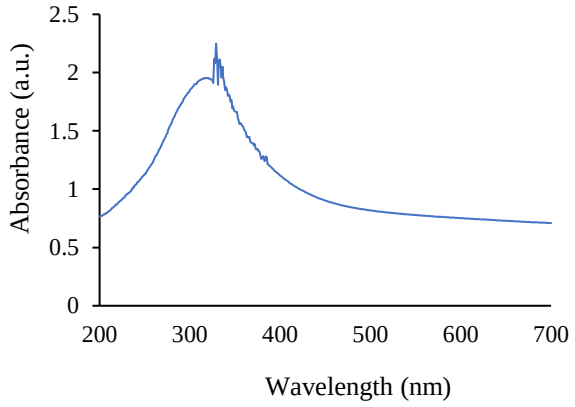


Fourier Transform Infrared (FTIR) Spectroscopy

FTIR analysis was carried out to identify the presence of secondary metabolites in the *Azadirachta indica* leaf extract which are accountable for the stabilization of FeNPs. The representative FTIR spectra of fresh *Azadirachta*

indica leaf extract and the synthesized FeNPs is manifested in figure 2. Results represented the spectra of FeNPs with peaks at 2911.9 cm^{-1} and 1036.3 cm^{-1} strong broad absorption peak at 2911.9 cm^{-1} was assigned to O-H stretching of carboxylic acid and the peak at 2920 cm^{-1} was assigned to C=O stretching in amides arisen due to the carbonyl stretching protein or N-H bend from amides (primary amide). The possible secondary metabolites included saponins and flavanones etc[5].

Figure-2: FTIR spectra of iron-nanoparticles (FeNPs).



Phytochemical Analyses

The results of phytochemical screening of aqueous leaves extract of *Azadirachta indica* confirmed the presence of saponins, glycosides, flavonoids, and steroids (Table 1). These results bear similarities to ones obtained in other studies[13,10], in aqueous *Azadirachta indica* leaves extracts saponins occurred the most, steroids and glycoside are moderate while flavonoids are low and anthraquinones is not present. The medicinal values of the secondary metabolites are due to the presence of chemical substances that produced by the plant extracts against particular organism produce a definite physiological action on the body[11].

Saponins have many health benefits such as the beneficial effects on blood cholesterol levels, cancer, bone health and stimulation of the immune system[11]. Aqueous leaf extract of *Azadirachta indica* showed the presence of saponins which have nematocidal properties. Phytochemicals like saponins are known to induce analgesic mechanisms and minor anti-oxidant activity[14]. Glycosides play numerous important roles in living organisms and are used in medications. Many plants store chemicals in the form of inactive glycosides which can be activated by enzymes breaking off the sugar part and making the glycoside available for use. In higher plants, flavonoids are involved in UV filtration, symbiotic nitrogen fixation and floral pigmentation. They may also act as chemical messengers, physiological regulators and cell cycle inhibitors[10]. Steroid and their metabolites are frequently used as signaling molecules. Steroids along with phospholipids function as components of cell membranes with steroids such as cholesterol decrease membrane fluidity[15].

Table-1: Phytochemical analysis of leaves extract of *Azadirachta indica*.

S. No.	Name of Phytochemicals	Aqueous Extract of *Azadirachta indica*
1	Saponins	+++
2	Glycosides	++
3	Flavonoids	+
4	Steroids	++
5	Anthraquinones	-

+++: Most present; ++: Moderately present; +: Least present; –: Not present.

Anti-bacterial Activity Test

FeNPs were established against both the gram negative and gram-positive pathogenic bacteria such as *S. aureus* (gram positive), *E. coli* (gram negative) using agar-disc diffusion method. However, the zone of inhibition (ZOI) was observed to be more in *S. aureus* when compared to *E. coli*. This is mainly due to the differences in bacterial pathogen's membrane structures. The ZOI by FeNP on the *S. aureus* is 14.8 mm while on *E. coli* is 9.2 mm (Table 2). FeNPs showed significant inhibition of urinary tract infection (UTI) pathogens such as *S. aureus* and *E. coli*[16].

Table-2: Zone of inhibition of FeNPs against gram-positive and gram-negative pathogenic bacterial species.

S. No	Bacterial Species	Zone of Inhibition (ZOI)
1	*E. coli*	9.2 mm
2	*S. aureus*	14.8 mm

CONCLUSION

In this present study, the successful use of *Azadirachta indica* reported as a green method for the synthesis of FeNPs. The color change, observed instantaneously suggested that the formation of black colored solution indicated the formation of FeNPs. FTIR showed that the interactions that existed between *Azadirachta indica* and FeNPs. In addition, the synthesized nanoparticles (FeNPs) were further analyzed using UV-vis spectrophotometer. Therefore, bioactive compounds such as saponins, flavonoids, steroids, and glycosides were detected to be present in the leaves of *Azadirachta indica*. Since this plant had been used in the treatment of different ailment such

as malaria, dysentery, diarrhea, skin burn etc., the medicinal roles of this plant could be related to such identified bioactive compounds. Further antimicrobial tested by the FeNPs, the zone of Inhibition by FeNPs on the *S. aureus* observed maximum when compared with *E. coli*. Overall, the proposed green synthetic method was simple and eco-friendly, because it did not require any extra surfactants or reductants. Achievement of such rapid time scales for synthesis of FeNPs could be a competitive alternative to the conventional chemical protocols and a low-cost candidate as reductant for synthesis of FeNPs, and thus, it has the potential to use in biomedical applications and will play an important role in magnetic targeting drug delivery in near future.

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CONFLICT OF INTEREST
The authors have no conflicts of interests.

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