PREPARATION OF ANTIBACTERIAL IRON-BASED NANOPARTICLES USING *Ruellia tuberosa* L. ROOT EXTRACTS AS BIOREDUCTOR

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**ABSTRACT**

In the current work, iron nanoparticles (Fe-NPs) were prepared from extracts of *Ruellia tuberosa* L. extracted with ethanol and water, and their antibacterial activities toward pathogenic bacteria (*S. aureus* and *E. coli*) were investigated. The morphologies, structures, sizes and distribution of atom on the surface of Fe-NPs were checked by SEM-EDS, UV-Vis and FTIR spectroscopy. The pH conditions (3, 5, 7, and 9) were chosen as experimental factors for the preparation of Fe-NPs. Formation of results in mostly spherical shapes with particle size found within the range 15-50 nm, and the smallest particle size was obtained from pH 9. UV-Vis spectra confirmed that the formation of Fe-NPs emerged in the UV region around 275 nm. FTIR spectra showed the presence of polyphenolic compounds from extracts of *R. tuberosa* L, that decreasing aggregations of Fe-NPs and act as bioreductors. As Fe(II) reduced to Fe(0), oxidation of $\text{O}^-$, $\text{OH}^-$, and $\text{CHO}$, $\text{COOH}$ occurred. The optimal condition for antibacterial activities was at pH 3, resulted in the 16.70 and 13.40 mm of inhibition zones, for *S. aureus* and *E. coli*, respectively.

**Keywords**: Iron Nanoparticles, Antibacterial Agent, *Ruellia tuberosa* L., SEM-EDS

**INTRODUCTION**

Iron nanoparticles (Fe-NPs) can be easily prepared by different techniques, both chemically and physically. For instance, salts of Fe(II) or Fe(III) reacted with NaBH\(_4\) as reducing agents, vacuum sputtering, and the use of organic solvents as iron precursors.\(^1,2\) Chemicals and physical methods have been applied to produce nanoparticles with certain sizes and morphology. However, these techniques involve toxic, corrosive, and flammable compounds, *i.e.* NaBH\(_4\) or organic solvents.\(^2\) The reactivity and stability of nanoparticles using these methods are usually reduced due to agglomeration of the product.\(^3\)

Therefore, biosynthesis of iron nanoparticles using natural product extracts has appeared as an unpretentious, inexpensive, and eco-friendly approach these days.\(^4,5\) Biological synthesis of nanoparticles can be carried out with green reagents, microorganisms, and plant biomaterials. Plants can reduce metal ions on their several parts such as leaves, roots, and tissues.\(^6,4\) Several secondary metabolites are playing a role in nanoparticle formation such as flavonoids, terpenoids, and polyphenols.\(^5,8\) They are accountable for the size and morphology of the nanoparticles formed.\(^5,8\)

Using plant extracts making nanoparticles is a much simpler, scalable and less expensive method compared to other biological methods. They are responsible for both reducing and stabilizing activities. Several published studies have shown that extracts of the plants can be used to replace chemicals used and physical approaches for the preparation of iron nanoparticles.\(^5,9\) Organic functional groups such as amines, carboxylic acids and phenols can reduce Fe(II) or Fe(III) in aqueous solution. Recently, plant extracts, such as tea (oolong, green, and black tea),\(^5,7\) extracts of strawberry, grape, and *Euphorbia cochinichensis* leaves, pomegranates, oaks,\(^7\) and eucalyptus leaf extracts\(^8\) have been effectively used to synthesize iron nanoparticles.

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Most studies focus on the whole or parts of plants for the preparation of metal nanoparticles and their applications for numerous purposes.\textsuperscript{9-11} Ruellia tuberosa Linn., a flowering plant species in the Acanthaceae family, locally known as pletekan, can be used in iron nanoparticle synthesis. This plant is widely grown in Indonesia, and also in other South East Asia countries.\textsuperscript{12} R. tuberosa L. has been used as an anti-inflammatory,\textsuperscript{13} anti-oxidants,\textsuperscript{14} antibacterial,\textsuperscript{15} and anticancer.\textsuperscript{16} The phytochemical and LC-MS studies revealed that hydroethanolic extracts from \textit{R. tuberosa} L. mostly contained flavonoids and phytosterols.\textsuperscript{14,15}

The synthesis of Fe-NPs through the Fe(II/III) bioreduction process using \textit{R. tuberosa} L. root extracts has not been previously reported. This is for the first time that \textit{R. tuberosa} L. extracts are used for the synthesis of nanoparticles. Therefore, it is important to synthesize Fe-NPs using root extracts of \textit{R. tuberosa} L. in a simple step, with the use of non-toxic chemicals and environmentally friendly. Furthermore, in vitro biological activities of the resulted Fe-NPs are investigated through the determination of their biological activity against \textit{S. aureus} and \textit{E. coli} by the good diffusion method. For the positive control, the antibacterial activity of commercially available antibiotics, chloramphenicol and ampicillin are also tested.

**EXPERIMENTAL**

**Materials**
The main material used in this research was \textit{R. tuberosa} L. roots powder that acquired from UPT Materia Medica (Batu, East Java). The species determination, \textit{Ruellia tuberosa} Linn, has been conducted by a botanist from the Materia Medica. All additional chemicals were ethyl alcohol (pure, \( d = 0.789 \text{ g/}\text{mL} \)), \( \text{FeCl}_2\cdot4\text{H}_2\text{O} \geq 99.0\% \), analytical grade), ammonium buffer solution (pH 10-11, \( d = 0.978 \text{ g/cm}^3 \)), \( \text{NaOH} \geq 97.0\% \), pellets, analytical grade), and HCl (37\%, analytical grade), purchased from Sigma-Aldrich, demineralized water (Hydrobatt), nutrient broth (NB) and nutrient agar (NA) were purchased from Merck. Cultures of \textit{Staphylococcus aureus} (Gram-positive) and \textit{Escherichia coli} (Gram-negative) were acquired from the Department of Microbiology, State University of Malang. Chloramphenicol and ampicillin (meets USP testing specifications) as a positive control for antimicrobial assay were obtained from Merck.

**Instrumentation**
The synthesized Fe-NPs were characterized by UV-Vis (Shimadzu UV-1601 UV-VIS spectrometer), FTIR spectrophotometer (sample was analyzed using a KBr plate, Shimadzu FTIR 8400S) in the wavenumbers of 4000-400 cm\(^{-1}\) and Scanning Electron Microscopy (SEM) (FEI, Type: Inspect-S50).

**Preparation of \textit{R. tuberosa} L. Root Extracts**
Distilled water and ethyl alcohol in 1:1 were used to extract 1 kg of \textit{R. tuberosa} L. root powder (size of approximately 90 mesh) in the room temperature for 72 h. The extracts were separated through filtration, a rotary evaporator vacuum was used to further concentrated extracts at a low speed at 120 rpm, with a temperature of 50 \(^\circ\)C. The concentrated extracts were kept at 4 \(^\circ\)C for subsequent use.

**Preparation of Fe-NPs**
To prepare Fe-NPs, the concentration ratio applied for 0.1 M \textit{FeCl}_2 aqueous solution and \textit{R. tuberosa} L. root extracts were 1:15. Moreover, the synthesis of Fe-NPs also was carried out in the different pH values at 3, 5, 7 and 9, by addition of 0.1 M HCl or 0.1 M NaOH. The obtained Fe-NPs were separated by centrifugation for 10 min, at a speed of 10,000 rpm, and the resulting precipitates were washed with demineralized water and ethanol several times. Lastly, Fe-NPs were desiccated for 24 h, in the temperature of 80 \(^\circ\)C, using an oven, and stored at desiccator for further analysis.

**Characterization of Fe-NPs**
The optical properties of Fe-NPs were analyzed by using a UV-Visible spectrophotometer. The surface morphology of the synthesized Fe-NPs was characterized by performing scanning electron microscopy, and energy-dispersive X-ray spectroscopy (EDS) was applied to confirm the purity of the sample. To identify functional groups in the Fe-NPs, a typical Fourier transform infrared spectroscopy was used.
Antibacterial Assay
The Fe-NPs antibacterial activity was analyzed using a good diffusion technique using a previously published method. The antimicrobial assessment of Fe-NPs was conducted by using *S. aureus* (Gram-positive) and *E.* (Gram-negative) test pathogens. Cultures of bacterial were kept in nutrient broth. A sterile cotton bud was used to spread bacterial culture on nutrient agar plates. The wells of 7 mm diameter dimensions were formed into the agar medium plates with aid of sterile gel puncture, and the samples were added into the well. The positive controls were chloramphenicol and ampicillin, commercially available antibiotics. The incubation of cultures of the bacteria was conducted overnight at 37 °C, in an upright position. The Fe-NPs were used in the different concentrations: 1%; 1.5%; 5%; 10%; 20%; 50%; and 100% (v/v). The detail procedures for the determination of antibacterial activity were described in our preceding paper. After the incubation period, the diameter of zone inhibitions was quantified with a meter ruler (Vernier calliper) around each disk, and the inhibition average value was determined and stated in millimeter.

RESULTS AND DISCUSSION
Characterization of Fe-NPs
After addition of root extracts to 0.1 M aqueous FeCl\(_2\) solution, the nanoparticles synthesis reaction started, resulted in the color change from yellow solution (Fig.-1b) to blackish solution (Fig.-1c), indicating the formation of Fe-NPs. A previous study has been reported visual color changes in the metal complexes solutions due to the synthesis of metal nanoparticles. Characterization of metallic nanoparticles due to such surface plasmon resonance (SPR) phenomenon can be analyzed using a UV-Vis spectroscopy technique. The conduction of electrons interaction on the surfaces of metal nanoparticles with incident photons may lead to a resonance effect in SPR. The interaction depends on the composition and nature of the dispersion medium, and also the shape and size of the metal nanoparticles. Accordingly, a characteristic optical absorption spectrum of metallic nanoparticles is shown in the UV–vis region.

![Fig.-1: The Solutions of (a) Hydroethanolic *R. tuberosa* L. Root Extracts, (b) FeCl\(_2\), and (c) Fe-NPs.](image)

![Fig.-2: Results of Fe-NPs From UV-Vis Spectroscopy prepared from *R. tuberosa* L. Root Extracts at Different pH Conditions.](image)

The absorption peaks were observed around 275 nm owing to the surface plasmon vibrations excitation in Fe-NPs (Fig.-2). An increase in the sharpness of the absorption peak observed with an increase in the pH of roots extract. The absorption peaks were larger and also shifted to some extent toward the long-
wavelength region. An earlier study showed similar results that the synthesized iron nanoparticles using tea extract showed an absorption peak around 275 nm, showing the formation of iron nanoparticles. The maximum absorption peak in this study was found at the highest pH, pH 9. This is consistent with research conducted by Saif et. al., that the optimum condition of formation of Fe-NPs was achieved at alkaline pH.

Samples of the Fe-NPs were examined by SEM coupled with EDS, to confirm the results of the UV-vis spectral results. The SEM image of Fe-NPs synthesized using root extract of R. tuberosa L. is displayed in Fig.-3, together with particle size distribution. As shown in Fig.-3a, the morphology of Fe-NPs formed in pH 3 was rough and uneven, with some irregular and large spherical shapes. These mean that the aggregation of Fe-NPs had occurred and this pH condition was not optimal for the formation of Fe-NPs. As pH increases, Fe-NPs show more uniform shapes, and there are decreases in particle sizes of the synthesized metal nanoparticles (Fig.-3b to 3d). At pH 3, Fe-NPs have average particle sizes of 10 nm, and size distribution between 5 and 29 nm. In the higher pH values, pH 5 to pH 9, Fe-NPs have smaller average particle sizes of 5 to 12 nm, and lower size distribution of 5-23 nm.

Many studies of the metal nanoparticles biosynthesis with plants extract have been reported. The effect of the pH in the formation of Fe-NPs has been associated with the phytochemical contents within R. tuberosa L. root extracts. Our previous study indicated that hydroethanolic roots extract from R. tuberosa L. positive contained phenolic compounds, importantly flavonoids and phytosterols. The compounds in the plant extracts contributed to the reduction of positively charged metal ions to their ground state. This is achieved by donating electrons to the ions with the bio-reductors; as in the case of the Fe(II) to Fe(0) atoms, and small Fe(0) particles at the beginning of the reaction. This is followed by these Fe(0) atom growing into structures of spherical-like, and these Fe-NPs subsequently merging into a stable structure, with the assistance of bio-capping agents from the plant extracts, as illustrated in Fig.-4.

Reduced metals usually undergo nucleation; this process fast occurs at high pH since the –OH ions are abundant, accordingly, the formation of small size particles is more likely. Moreover, the aggregation can be prevented because the same phytochemicals responsible for starting the formation of the Fe-NPs coated the nanoparticles, supplying electron-rich hydroxyl capping agents onto the particles. Additionally, decreasing in particle sizes and constriction of particle sizes distribution can also be related to the nature of phytochemical compounds (e.g. flavonoid compounds) that deprotonated in alkaline conditions. Flavonoid compounds are more reactive to interact with Fe-NPs and covering more surfaces of Fe-NPs. Subsequently, the formation of aggregation or large clusters on the surface of Fe-NPs is prevented. On the other hand, in acidic pH conditions, flavonoid compounds may undergo hydrolysis, hence, becoming less reactive and, as a result, is not optimal in preventing aggregation or cluster formation in Fe-NPs. As a result, in this current study, the finest formation of Fe-NPs resulted in pH 9.

The synthesis of Fe-NPs prepared from R. tuberosa L. root extract was subsequently characterized by EDS method, which provides the supplementary indication for the reduction of Fe(II) to ground state Fe(0). The spectrum displays intense iron signal alongside with weak signals that can be risen from X-ray emission from organic molecules like phytosterols or flavonoids bound to the NPs or in the neighborhood of the particles (Fig.-5). In general, EDS signal shows the presence of Fe, Na, O and Cl signals. The percentage of Fe atoms is getting higher along with increasing pH value (Table 1). The Na and Cl signals may be originated from NaOH and FeCl₂ precursors used in the preparation of Fe-NPs. The oxygen signals are mostly associated with the polyphenol groups and or/ oxygen-based groups, such as –COO and –OH in the roots extract of R. tuberosa L. Nonetheless, the formation of some iron oxide nanoparticles may also be indicated in the O signal.

| No | pH | %Fe | %Cl | %Na | %O  |
|----|----|-----|-----|-----|-----|
| 1  | 3  | 64.30| 6.01| 1.42| 19.65|
| 2  | 5  | 71.73| 1.79| 1.36| 33.91|
| 3  | 7  | 74.34| 1.18|-    | 25.67|
| 4  | 9  | 74.41| 0.59|-    | 23.64|

Table-1: The Elemental Distribution of the Fe-NPs at Different pH Conditions
Iron-based nanoparticles using *Ruellia tuberosa* L. root.

**Fig. 3:** SEM Images of the Fe-NPs at Various pH Conditions, together with Particle Size Distribution: (a) pH 3; (b) pH 5; (c) pH 7; and (d) pH 9. Magnification was 100,000×.

**Fig. 4:** Proposed Mechanism of Fe-NPs Formation with the Aid of Roots Extract of *R. tuberosa* L as Capping Agents.
The characteristics of Fe-NPs from FTIR spectra (wavenumbers 400-4000 cm$^{-1}$) are provided in Fig.-6. The possible functional group responsible for the interaction between Fe-NPs and capping agent were assessed by conducting an FTIR analysis. The most distinct peaks shown for the Fe-NPs are labeled differently (A to G). The functional group corresponding to each label is listed in Table-2.

All synthesized nanoparticles at different pH have relatively similar peaks. The intense and broad absorption peak emerged at 3500-3400 cm$^{-1}$, in region A, relates to the O-H stretching vibrations of phenolic compounds and or/ carboxylic acid$^{25}$, indicating that O-H functional groups involved in the preparation of Fe-NPs. The phenolic compounds can act as a reductant for Fe$^{2+}$ ions to Fe$^{0}$, in addition, to be the capping agents on Fe-NPs surfaces. Peaks in region D (1440-1420 cm$^{-1}$) relate to aromatic compounds, organics; and those compounds derivatives, such as alcohol, polyphenol, or organic acids, terpenoids, and proteins contained in the extracts.$^{25,26}$ These suggest an interaction between hydroxyl groups or oxygen-containing groups from the $R$ tuberosa L. roots extract with the surface of Fe-NPs.
second intense absorption peak in region C, found in 1650 -1640 cm\(^{-1}\), corresponds to C=C in alkene groups from non-saturated hydrocarbon compounds. Consequently, the FTIR spectra of Fe-NPs showed another prominent peak in region E (1050-1030 cm\(^{-1}\)). This peak may link to heterocyclic compounds from plant extract, importantly from the carbonyl group. Another peak was detected in F area, in the range of 680–660 cm\(^{-1}\), by C-H stretching from aromatic compounds, and or/ alkenes. Finally, absorption bands at region G, 590-570 cm\(^{-1}\) belonged to Fe-O stretches, confirming the formation of Fe-NPs. This data was in agreement with results from a prior study\(^\text{23}\), that confirms the successful use of plant extracts to synthesize Fe-NPs, using FTIR analysis.

| Table-2: The FTIR Absorption Peaks Assignment from Fig.-5 |
|-----------------|-----------------|-----------------|
| Label | Wavenumber (cm\(^{-1}\)) | Assignment\(^\text{24-28}\) | Compound indicated |
| A | 3500-3400 | –OH stretching | Polyphenols |
| B | 1900-2000 | C–H stretching | Alkanes |
| C | 1650-1640 | C=C stretching | Alkenes |
| D | 1440-1420 | CH\(_3\) bending | Alkenes |
| | | C–C stretching | in aromatic ring compound |
| E | 1050-1030 | C–O–C stretching | Carbonyl compounds |
| F | 680-660 | C–H bending | Alkenes, Aromatics |
| G | 590-570 | Fe–O stretching | Fe(0) |

The antibacterial activity of Fe-NPs prepared from R. tuberosa L root extracts was examined against pathogenic bacteria, S. aureus and E. coli using well diffusion method. The zone of inhibition formation at different pathogenic bacteria at different pH values is shown in Fig.-7, and the zone of inhibition from positive controls (ampicillin and chloramphenicol) is displayed in Fig.-8. The Fe-NPs used were in different concentrations, 1.5 to 100% (v/v), however, the 100% concentration resulted in the highest inhibition against both bacteria. Therefore, the zones of inhibition in mm diameter around each well with Fe-NPs solution listed in Table-3 are at 100% concentration. The synthesized Fe-NPs have shown prominent anti-bacterial activities against S. aureus and E. coli. Nonetheless, anti-bacterial studies elucidate that S. aureus, gram-positive bacteria, is more sensitive to Fe-NPs, in comparison with E. coli, gram-negative bacteria. Similar trends were observed in the anti-microbial capacity of ampicillin and chloramphenicol, which resulted in the higher numbers in inhibition zones against S. aureus, rather than E. coli (Fig.-9).

It is known that gram-negative bacteria are less sensitive to the action of an anti-microbial agent, compared to gram-positive bacteria. Because there is a monolayer cell wall in gram-positive bacteria, that has lower lipid content, allowing any compounds from outside their cell wall entering the cell. On the other hand, there is a three-layer cell wall structure in gram-negative bacteria, comprising of outer, lipopolysaccharide, and peptidoglycan layers with higher lipid content, and, hence, it is quite tough to intercept their cell walls.\(^\text{27,28}\)

| Table-3: Zone of Inhibition of Fe-NPs prepared From R. tuberosa L. Root Extract at Different Ph |
|-----------------|-----------------|-----------------|
| pH | Zone of Inhibition against |
| | S. aureus (mm) | E. coli (mm) |
| 3 | 16.70 | 13.40 |
| 5 | 15.20 | 11.85 |
| 7 | 10.80 | 9.20 |
| 9 | 7.20 | 7.40 |
| Ampicillin | 25.70 | 25.40 |
| Chloramphenicol | 30.20 | 29.85 |
It is interesting that in the antibacterial test, the lowest pH used (pH 3) for the preparation of Fe-NPs, resulted in the maximum inhibition zones for both bacteria, *S. aureus* and *E. coli*. This suggests that the antibacterial activity of metal nanoparticles is influenced by pH conditions. The toxicity of nanoparticles depends on a combination of several factors such as temperature, precursor concentration, type of bacteria used for testing, and pH conditions. In this study, results from SEM images indicated that agglomeration and bigger size particles on Fe-NPs occurred to the lowest pH (pH 3), and, thus, resulted in the highest toxicity to the bacteria. The Fe-NPs showed efficient antibacterial property at pH 3, compared to other pH conditions attributable to their large surface area, which offers better interaction with bacteria.
Another possible explanation is that the compounds acting as a coating molecule or capping agent also influenced the antimicrobial activity of the resulted Fe-NPs. The LC-MS results from the extracts used showed that the extracts contained mostly flavonoid compounds, including sorbifolin, cirsimaritin, cirsimarin, and cirsiliol 4'-glucoside. Based on the pKa values of each compound, there are 3 compounds (sorbifolin, cirsimaritin, and cirsiliol 4'-glucoside) with a pKa value greater than acidic pH conditions, therefore, the ionization of the three compounds is favorable, allowing those compounds to interact easily with the membrane on cell wall bacteria.

However, in alkaline conditions, there is only one compound (cirsimarin) having pKa value almost the same with the pH, thus, reducing its ionization capacity, and as a result, does not interact strongly with the bacteria. These may cause antimicrobial activity is more significant in acidic conditions than in alkaline conditions.

Overall, the proposed mechanism of action of Fe-NPs as an antibacterial agent is the nanoparticles attached to the membrane or cell wall, then also infiltrated inside the bacteria. The bacterial membrane comprises of sulfur-containing proteins; and the iron nanoparticles react with these proteins in the cell, as well as with the DNA. Once Fe-NPs enter the bacterial cell, they block cellular channels and as a result causing membrane damages, or inhibit the intake of the nutrients and cell mobility. It is, consequently, resulted in cell lysis (Fig.-9).

**CONCLUSION**

The Fe-NPs have been successfully prepared from roots extract of *R. tuberosa* L., using an inexpensive, effective, and environmentally friendly technique. The UV–vis spectrophotometry, SEM-EDS, and FTIR
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spectroscopy analyses suggest the reduction of Fe(II) to Fe(0). In this investigation, *R. tuberosa* L. roots extract was determined to act as a reducing agent and to form capping layers around the nanoparticles. The particle sizes of Fe-NPs were dependent on pH condition, pH 9 contributed to the most homogenous surface and the smallest size. FTIR spectra result demonstrated that carboxylate and hydroxyl groups of the extracts interacted with the iron nanoparticles and lead surface stabilization. The inhibition zones shown in the antibacterial screening test indicating that the Fe-NPs prepared in the current work has the effective antibacterial activity against pathogenic *S. aureus* and *E. coli*. In contrast, pH 3 resulted in the highest inhibition zones of Fe-NPs. The Fe-NPs that prepared biologically could be of convenient use in they for their proficient antibacterial activities.

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