

Antibacterial Innovation of Iron Nanoparticles from Pomegranate Peel Extract (*Punica granatum* L.): an In Vitro Study

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Abstract

Iron nanoparticles (Fe-NPs) were synthesized from pomegranate peel extract using ethanol as a solvent. The antibacterial activity of the synthesized Fe-NPs against pathogenic bacteria (*S. aureus* and *E. coli*) was assessed using the well diffusion method. The morphology, structure, size, and atomic distribution on the surface of Fe-NPs were characterized using SEM-EDS, UV-Vis, and FTIR spectroscopy. Different pH conditions (5, 7, and 9) were investigated as experimental factors for Fe-NPs preparation. The results revealed mostly irregular spherical shapes with particle sizes ranging from 1 to 89 nm, with the smallest size observed at pH 9. UV-Vis spectra confirmed the formation of Fe-NPs, appearing in the UV region around 230-295 nm. FTIR spectra indicated the presence of polyphenolic compounds from the pomegranate peel extract, which facilitated Fe-NPs stabilization and acted as bioreductants. Upon reduction of Fe(III) to Fe(0), oxidation of O-H and C-O functional groups occurred. The diameters of inhibition zones against the tested bacteria were 16 mm at pH 5, 13 mm at pH 7, and 15 mm at pH 9, indicating strong antibacterial activity.

Keywords: Antibacterial; In Vitro; Iron nanoparticles; Pomegranate peel extract; *Punica granatum* L.

INTRODUCTION

Iron nanoparticles (FeNPs) are type of nanomaterial widely used in therapeutic and environmental applications (Nury et al., 2023). One of its applications is in the treatment of water contaminated by organic or inorganic pollutants and environmental remediation. Iron nanoparticles are synthesized chemically and physically, for example, using NaBH_4 as a reducing agent. However, this method uses toxic chemicals, requires special equipment, and incurs high costs. Moreover, the use of this method can result in a lack of reactivity and stability of the nanoparticles. Therefore, in recent years, a simple, effective, inexpensive, and environmentally friendly method has emerged as an alternative through the biosynthesis of iron nanoparticles using natural product extracts (A. Safitri et al., 2020).

The biosynthesis of metal nanoparticles is developing rapidly due to the environmentally friendly nature of nanoparticle synthesis. One alternative for nanoparticle synthesis is using plants or plant materials. Biosynthesis of iron nanoparticles (FeNPs) using plant extracts is an alternative because it is environmentally friendly, non-toxic, and fast (Fazlzadeh et al., 2017). To date, many plant extracts, such as tea (oolong tea, green tea, and black tea) (Zhu et al., 2018), *euphorbia cochinchensis*

leaves, strawberry leaf extract, grape leaf extract, pomegranate, oak, and eucalyptus leaf extract have been used to synthesize FeNPs (Ramadhan et al., 2019).

Most research focuses on certain plants and specific plant parts to synthesize nanoparticles. One of these is the pomegranate, which is a dicotyledonous plant from the Punicaceae family and originates from the Middle East. Pomegranate has been known as a natural medicinal plant since 1550 BC. The utilization of pomegranate has focused only on its fruit, with the peel becoming waste material. Based on pharmacological tests, pomegranate peel (*Punica granatum* L.) has been experimentally proven to contain flavonoids, anthocyanidins, gallic acid, phenolic acids, tannins, quercetin, ellagic acid, catechins, and antioxidants from vitamin C. The antioxidant activity of pomegranate peel is due to the pomegranate peel extract containing polyphenol compounds, one of which is punicalagin isolated from pomegranate peel, which has strong antioxidant activity both in vitro and in vivo (Thitipramote et al., 2019).

The synthesis of iron nanoparticles through the bioreduction process of Fe^{3+} using pomegranate peel extract (*Punica granatum* L.) has not been reported before. Seeing the potential of bioactive compounds from

pomegranate peel (*Punica granatum* L.), it is expected that its biological activity as an antibacterial can be determined. Therefore, the researchers intended to conduct research on the in vitro antibacterial innovation of iron nanoparticles from pomegranate peel extract (*Punica granatum* L.).

MATERIALS AND METHODS

Materials

This research was conducted from July to August 2024 at the Integrated Laboratory of the Universitas Islam Malang. The tools used in this research are Thermo Scientific GENESYS Ultraviolet-Visible (UV-Vis) Spectrophotometer, knives, ovens, blenders, 300-400 mesh sieves, autoclave, Laminar Air Flow (LAF), 1000 μ l micropipette, incubator, refrigerator, spatula, scissors, beaker glasses, measuring cylinders, test tubes, test tube racks, erlenmeyer, petri dishes, bunsen burners, matches, hotplate and magnetic stirrer (Thermo Scientific), analytical balance (Ohaus), corkborer, sterile cotton swabs, alcohol sprayer, permanent markers, pipettes, inoculation needles and loop needles. The materials used were pomegranates, nutrient agar (NA), nutrient broth (NB), 90% ethanol, FeCl₃ solid, distilled water, NaOH, large plastic bags, small plastic wrap, aluminum foil, and label paper.

This research consisted of simplicia preparation, simplicia characterization, preparation of pomegranate peel extract, iron nanoparticle preparation, characterization of iron nanoparticle mixture with pomegranate peel extract, and antibacterial testing of pomegranate peel extract (*Punica granatum* L.) and iron nanoparticles.

Procedures

Preparation of Pomegranate (*Punica granatum* L.) Peel Simplicia

The samples used were pomegranate peels obtained from Pasar Induk Among Tani Batu, Malang, East Java. The samples then underwent wet sorting by separating the peels from contaminants. Pomegranate peel samples were dried by aerating and then oven for 2 \times 24 hours at 70°C. Then blended or pounded into powder. The powder is then sieved to get a powder size of 400 mesh (Mphahlele et al., 2016).

Extraction

Sixty gram of simplicia were weighed and then 600 mL of 96% ethanol solvent was added so that the simplicia was completely submerged with a ratio of 1:10 for 3 \times 24 hours in a light-protected state, and stirring was carried out for 1 hour every 24 hours. It was then filtered using cotton to obtain the macerate. The macerate was concentrated with a rotary evaporator at 50°C to obtain a thick ethanol extract of pomegranate peel (Coronado-Reyes et al., 2023).

Yield calculation

The yield was obtained by comparing the weight of pomegranate peel extract and the weight of simplicia before extraction. Then the extract yield was calculated using the following formula (Nofita et al., 2022):

$$\% \text{ rendement} = \frac{\text{extract weight (g)}}{\text{powder weight (g)}} \times 100$$

Preparation of Iron Nanoparticles with Pomegranate Peel Extract

The preparation of iron nanoparticles was carried out by reacting FeCl₃ and Pomegranate Peel Extract with a concentration ratio of 1:15 in 40 mL. Pomegranate Peel Extract was made with a concentration of 1.5% (w/v) (in distilled water) then added to 20 mL of FeCl₃ solution (0.1 M in distilled water) slowly (dropwise). It was then stirred for 60 minutes, and a greenish-black precipitate would form, which was then washed with distilled water. 0.1 M NaOH was added to adjust the pH of the solution to 5, 7, and 9, then stirred for 60 minutes, and a brownish-black precipitate would form. The brownish-black precipitate was then washed with distilled water several times, and the final wash was done using ethanol. After that, the mixture was centrifuged at 10000 rpm for 15 minutes, then dried in an oven at 60°C for 24 hours, and finally characterized using UV-Vis, FTIR, SEM-EDS, and antibacterial testing (Dianti et al., 2019).

Characterization of Iron Nanoparticle Mixture with Pomegranate Peel Extract (*Punica granatum* L.) Ultraviolet-Visible (UV-Vis) Spectrophotometry

The results of Fe nanoparticle synthesis were analyzed using a UV-Vis spectrophotometer branded Thermo Scientific GENESYS. In this analysis, the tool must be baselined first with the solvent to be used, namely ethanol. Measurements were taken at a wavelength of 200-800 nm (Ramadhan et al., 2019). The sample was pipetted as much as 1 ml and diluted with 9 mL of distilled water solvent and then put into a cuvette. Next, the cuvette was inserted into the sample chamber to start the analysis. From this analysis, a spectrum will be obtained showing the absorption peak with absorbance and sample wavelength (Willian, 2022).

Fourier Transformation InfraRed (FTIR)

Fourier Transform InfraRed (FTIR) characterization was performed to identify potential biomolecules in the results of the preparation of iron nanoparticles from pomegranate peel extract. The functional groups of the Fe nanoparticles formed were characterized with an IR spectrophotometer. Fourier transform infrared (FT-IR) spectra were analyzed using a spectrometer in the range 4000-400 cm⁻¹. Measurements were made using a pressed transparent KBr pellet, then the pellet was placed on the IR and the wavelength was determined. The mixture of KBr and the results of the manufacture of Fe

nanoparticles were also made transparent pellets pressed, then measured in an FTIR tool with SHIMADZU 8400S type (Willian, 2022).

Scanning Electron Microscope-X-ray Energy Dispersive Spectroscopy (SEM-EDS)

The nanoparticles to be characterized are glued to a metal cylinder using a double adhesive. Furthermore, the surface of the sample is coated with conductive material (gold metal) under vacuum conditions. The sample is photographed with a certain magnification (5000-200000 kX) so that characterization data showing the surface morphology of the material will be obtained. Quantitative analysis elemental analysis of the sample is determined from the EDS data (Molina et al., 2024).

Antibacterial Activity Test

Autoclave Tools and Materials Sterilization

The glassware used was washed thoroughly. Tools such as tweezers, scalpels, Petri dishes, bottles, and Erlenmeyers were covered with paper. Sterilization of tools was carried out using an autoclave with a pressure of 1 atm at a temperature of 121°C for 15 minutes (Puluh et al., 2019; Mulangri et al., 2020).

Preparation of Nutrient Agar Plate (Solid Media)

A total of 8 g of nutrient agar (NA) powder was dissolved in 400 mL of distilled water and heated to boiling using a water bath (Oklo et al., 2023). The nutrient agar solution was put in the required container (test tube for rejuvenation of bacterial culture). Then the tube was covered with cotton while the Petri dish was wrapped with brown paper. Next, the nutrient agar solution in test tubes and Petri dishes was sterilized in an autoclave at 121°C for 15 minutes with a pressure of 15 Psi. Then the test tubes were placed in an inclined position for 24 hours (Dima et al., 2016).

Preparation of Nutrient Broth (Production Media)

A total of 2 g of nutrient Broth (NB) powder was dissolved in 100 mL of distilled water and heated until dissolved, then the media that was ready was put into a test tube and plugged with cotton. The lid was wrapped in paper and tied with mattress thread. Furthermore, the media was sterilized with an autoclave at 121°C for 15 minutes (Rahma et al., 2023).

Rejuvenation of Pure Culture

Each pure culture of *S.aureus* and *E.coli* was aseptically streaked with an ose needle on a slanted agar solid medium and the agar medium tube was covered with cotton. Furthermore, *S.aureus* and *E.coli* cultures were incubated for 24 hours at 37°C (Oktavia & Pujiyanto, 2018).

Antibacterial Activity Testing Using the Well Diffusion Method

S. aureus and *E. coli* were swabbed onto the solid media to be used with a sterile cotton bud on different plates. Next, a metal borer or corkborer was prepared to make wells on the solidified agar media. The extract and nanoparticles (samples) were inserted into the wells, which were clarified against gram-positive and gram-negative bacteria. Chloramphenicol and ampicillin antibiotics were used as positive controls. They were then incubated for 24 hours at 37°C. Measurement of the inhibition zone was done by measuring the diameter of the clear zone using calipers (Erhonyota et al., 2023).

Data analysis

The research was conducted using a Completely Randomized Design (CRD) and replications were carried out 3 times. UV-Vis spectrophotometry was used to determine the electronic transitions from the results obtained. The appearance of characteristic absorption of several functional groups can be known from the results of the infrared spectrophotometer. The shape and size of the synthesized iron nanoparticles were analyzed using SEM, and EDS analysis was used to determine the distribution of constituent atoms on the surface of the nanoparticles. Meanwhile, the determination of antibacterial activity was analyzed by the well diffusion method by observing the presence or absence of inhibition zones measured using calipers

RESULTS AND DISCUSSION

The plant material used in this study was the peel of *Punica granatum* L., commonly known as pomegranate peel, which belongs to the Punicaceae family. Pomegranate peel is known to contain various bioactive compounds, including polyphenols ((García et al., 2021)), flavonoids, phenolic acids (Kaderides et al., 2020), tannins, anthocyanidins, ellegic acid, quercetin, gallic acid, catechins, and vitamin C (Thitipramote et al., 2019; Muthmainnah, 2019). Besides that, pomegranate peels include many types of mineral elements, including K, P, Na, Ca, Mg, and N, all of which are crucial for preserving the body's regular physiological processes (Singh et al., 2018; El-Hadary & Ramadan, 2019). In addition, plant materials rich in flavonoid compounds are one of the materials that can be used to make nanoparticles (Ramadhan et al., 2019).

Extract Yield

The extract yield value indicates the amount of bioactive compounds contained in the extract. Generally, the greater the yield value, the greater the value of the resulting extract. The requirement for the yield of thick extracts is that the value is not less than 10% (Rosa et al., 2023). The following table shows the results of the

calculation of the percentage yield of pomegranate peel extract.

Table 1. Calculation of the percentage yield of pomegranate peel extract

Sample	Extract result (g)	Yield (%)
Pomegranate Peel	24.36	40.6

Based on Table 1, the results obtained, it is known that the percentage of pomegranate peel extract yield is 40.6%, so there are many bioactive compounds contained in the pomegranate peel extract. This relatively high

yield suggests that pomegranate peel is a rich source of phytochemicals, which can play an important role as reducing and stabilizing agents in the green synthesis of iron nanoparticles.

Characterization of Iron Nanoparticles from Pomegranate Peel Extract (*Punica granatum* L)

Ultraviolet-Visible (UV-Vis) Spectrophotometry
UV-Vis spectrophotometric analysis was carried out in the wavelength range of 200-800 nm according to the following graphic image in Figure 1.

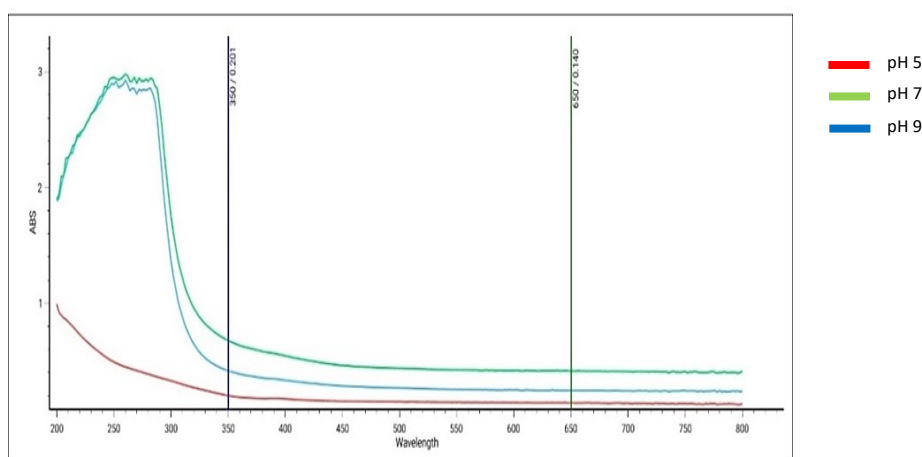


Figure 1. Graph of UV-Vis spectrophotometry test results of iron nanoparticles.

Based on Figure 1, the wavelength of the two peaks at pH 7 and pH 9 shows a range of 230-295 nm which corresponds to the typical flavonoid spectrum. The absorbance peak increases with increasing pH. The increase in absorbance peak can be caused by an increase in iron nanoparticle products formed (Ramadhan et al., 2019).

Fourier Transformation InfraRed (FTIR)

The results of observations using FTIR can be seen in the following figure 2.

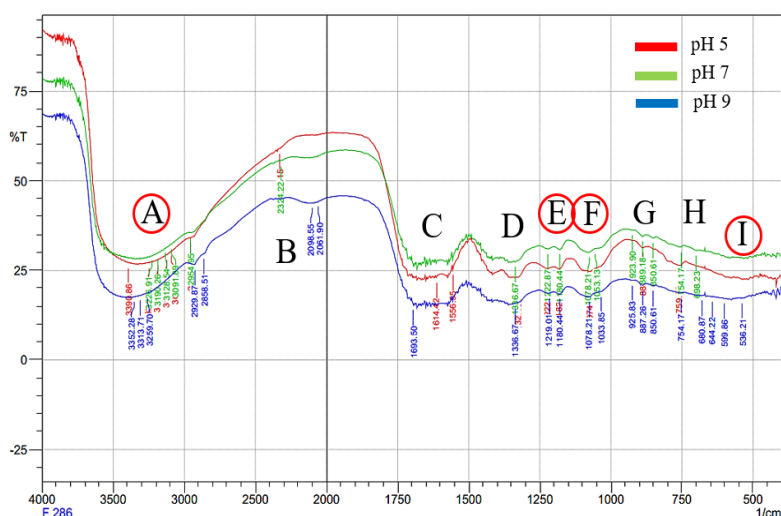


Figure 2. Graph of FTIR test results of iron nanoparticles.

Based on Figure 2, the iron nanoparticles synthesized using pomegranate peel extract exhibited characteristic

absorption bands corresponding Fe-O vibrations in region I, along with several functional groups derived

from phytochemical constituents of the extract, such as hydroxyl (–OH) and carbonyl (C=O) groups. The detailed interpretation of functional groups observed at different pH conditions is summarized in Table 2.

Table 2. Results of the interpretation of FTIR spectra of Fe nanoparticles at various pH levels.

Area	Wave number (cm-1)			Functional group
	pH 5	pH 7	pH 9	
A	3390cm-1	3226cm-1	3352cm-1	OH
E	1182cm-1	1180cm-1	1180cm-1	CO
F	1074cm-1	1078cm-1	1078cm-1	CO
I	536cm-1	536cm-1	536cm-1	Fe-O

The broad peaks observed in the region around 3226–3390 cm^{-1} correspond to O–H stretching vibrations, indicating the presence of hydroxyl groups, which are typically associated with polyphenolic compounds such as flavonoids and tannins in pomegranate peel extract. The peaks in the range of 1074–1182 cm^{-1} correspond to C–O stretching, further confirming the involvement of alcohols, phenols, and flavonoid derivatives in the nanoparticle synthesis and stabilization process. Meanwhile, the absorption band at $\sim 536 \text{ cm}^{-1}$ is characteristic of Fe–O vibrations, confirming the successful formation of iron nanoparticles. These results suggest that phytochemicals present in the pomegranate

peel extract, particularly flavonoids and polyphenols, act as reducing and capping agents in the green synthesis of iron nanoparticles, thereby stabilizing the particles and preventing aggregation.

These findings are consistent with previous reports of green-synthesized nanoparticles using plant extracts, where hydroxyl and carbonyl groups of polyphenols were identified as the main functional groups responsible for reducing Fe^{3+} ions into Fe nanoparticles and acting as capping agents to enhance stability (Dheyab et al., 2025). As previously reported that flavonoids and tannins in plant extracts provide both electron-donating functional groups and steric stabilization, preventing nanoparticle aggregation. The similarity of functional groups observed in this study with those in earlier works confirms that pomegranate peel extract acts not only as a reducing agent but also as a stabilizer during nanoparticle synthesis (Ahmed et al., 2016).

Scanning Electron Microscope-X-ray Energy Dispersive Spectroscopy (SEM-EDS)

The morphology of the synthesized iron nanoparticles was observed using SEM at a magnification of 100,000 \times , as presented in Figure 3. The SEM images revealed that the nanoparticles exhibited irregular shapes.

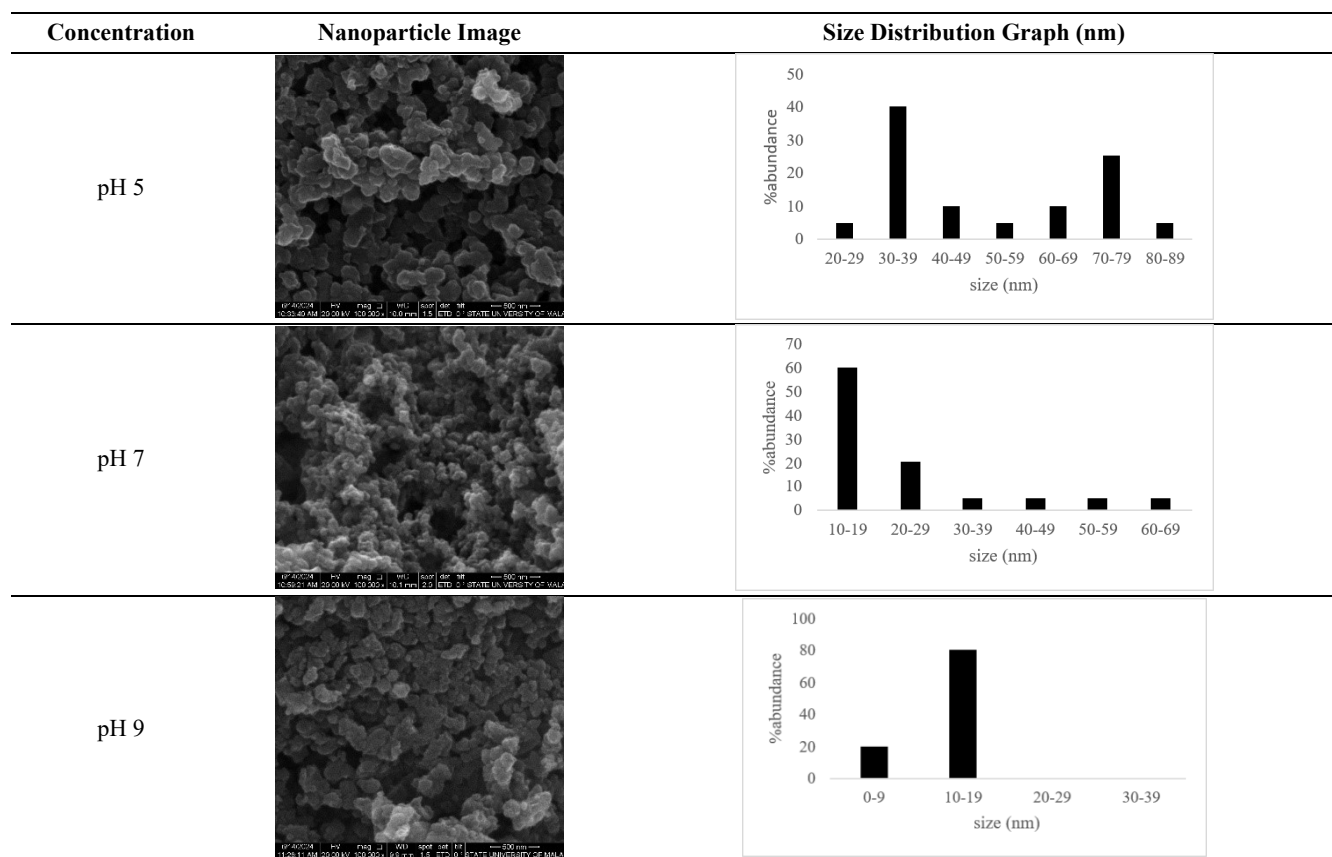


Figure 3. SEM results with a magnification of 100,000 \times (left), size distribution graph of Fe nanoparticles at various pH (right).

Based on Figure 3, the resulting measurement results show that the majority of particle sizes are below 100 nm. This shows that the analysis results are nanoparticles, because nanoparticles are particles that are between 1-100 nm in size (Abd Karim et al., 2021; Safitri et al., 2021). Based on the table above, it can be seen that the more alkaline the pH concentration of nanoparticles, the smaller the size of nanoparticles with a high abundance. This finding supports the hypothesis that alkaline conditions enhance the nucleation process during nanoparticle formation, thereby producing smaller and more uniformly distributed particles. The elemental composition of the nanoparticles was further analyzed using EDS, and the results are shown in Table 3.

Table 3. EDS results of iron nanoparticles from pomegranate peel extract

Variation	Element	% Weight	% Atomic
pH 5	OK	34.26	60.1
	FeK	41.75	20.9
	ClK	23.99	19
	Matrix	Correction	ZAF
pH 7	OK	30.16	54.6
	NaK	2.35	3
	FeK	42.56	22
	ClK	24.93	20.4
pH 9	OK	26.85	51.6
	NaK	0.15	0.2
	FeK	47.73	26.3
	ClK	25.27	21.9
	Matrix	Correction	ZAF

Based on Table 3, the results of the EDS analysis showed that there were Cl and Fe signals originating from the FeCl₃ precursor used in the synthesis of iron nanoparticles. The Na signal was attributed to NaOH, which was used to adjust the pH during the nanoparticle synthesis process. The O signal was associated with polyphenol groups and other compounds containing O-molecules from pomegranate peel extract. In addition, the O signal can also indicate the formation of several iron oxides that were formed (Ramadhan et al., 2019). This is in accordance with the FTIR results, where the iron nanoparticles produced were not only Fe with zero charge, but also produced iron oxide.

Antibacterial Activity of Iron Nanoparticles of Pomegranate Peel Extract

Observations of antibacterial activity can be seen in Table 4.

Table 4. Antibacterial activity of the extract and iron nanoparticles of pomegranate peel extract.

Concentration of iron nanoparticles from pomegranate peel extract	Inhibition zone (mm)	
	<i>E. coli</i>	<i>S. aureus</i>
pH 5	-	16mm
pH 7	-	13mm
pH 9	-	15mm

Based on table 4, the antibacterial activity of iron nanoparticles synthesized from pomegranate peel extract showed inhibition zone of 16 mm at pH 5, 13 mm at pH 7, and 15 mm at pH 9 against *S. aureus*. This is because the size of the nanoparticles is smaller so it can more easily lyse bacteria. Iron nanoparticles of pomegranate peel extract have no inhibition against *E. coli* bacteria. The antibacterial activity of iron nanoparticles is known to be influenced by several factors, including nanoparticle concentration, shape, size, type of bacteria strain, number of bacterial colonies and contact time between iron nanoparticles and bacteria (Prasetiowati et al., 2018). In this study, the inhibition zones observed against *S. aureus* can be categorized as strong according to the classification standard (Rahayuningsih et al., 2023). This suggests that pomegranate peel extract-mediated iron nanoparticles have potential as antibacterial agents, particularly against Gram-positive bacteria.

CONCLUSIONS

The extract yield shows the amount of bioactive compounds present in the extract. In UV-vis analysis, iron nanoparticles show results in accordance with the typical flavonoid spectrum. FTIR analysis confirmed the presence of functional groups, including alcohol and flavonoids. An increase in pH toward alkaline conditions resulted in a decrease in the size of the iron nanoparticles. Furthermore, the antibacterial activity of iron nanoparticles exhibited a larger inhibition zone compared to that of the extract.

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Authors' Contributions: The first author prepared the manuscript, designed the research, and analyzed the data together with the second and third authors, while the fourth and last authors conducted the experiments and the supervising lecturer provided guidance on the research and experimental design.

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