Drug Discovery

Green Synthesis of Propolis-Ag Nanoparticles Using the Propolis Extract and Their Antimicrobial Activity

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ABSTRACT

Silver nanoparticles are expected to play an important role in solving the problem of multi-drug resistant bacteria emergence in the healthcare sector. Silver nanoparticles are synthesized in various ways, but the preparation of silver nanoparticles using biomass has attracted the attention of many researchers. The preparation of nanoparticles by biological method has many advantages such as low cost, simple fabrication process and environmental safeness. Propolis has been widely used as a folk medicine in the world since ancient times because of their high antimicrobial, antiviral, antioxidant and anti-inflammatory activities. Especially, the bio-synthesized metallic nanoparticles using the Propolis extract could be used to prevent and cure many bacterial and viral diseases due to synergistic effect of antimicrobial and antiviral activity of the propolis extract and the metallic nanoparticles. In this paper, we synthesized Ag nanoparticles by bio-reduction method using Propolis extract. The Surface Plasmon Resonance property of manufactured Ag nanoparticles was 422 nm, the average particle size was 10nm and the zeta potential value was -36.3mV. We also demonstrated that Propolis-Ag nanoparticles had a strong antimicrobial activity against *Escherichia Coli O111*. These results could provide the possibility of propolis-silver nanoparticles as antimicrobial agents.

Keywords: Propolis-extract, Propolis-Ag nanoparticles dispersions, Surface Plasmon Resonance (SPR), Zeta Potential, Antimicrobial Activity

1. INTRODUCTION

Nowadays, several infectious diseases are seriously threatening human health all over the world. Some examples are dysentery, cholera, HIV, monkey smallpox, and COVID-19. In addition, new epidemics have been breaking out continuously (Ephraim, 2022).

Although human beings have tried to develop effective medicine to prevent and cure such diseases, we are still facing several challenges. Firstly, it is important to find out the novel and effective materials against antimicrobial-resistant strains due to the emergency of antibiotic resistance strain (Julia et al, 2017). Secondly, many RNA viruses are very easily variative, which means that the vaccine inoculations might be failed (Yalcin & Yalcin, 2021).

DISCOVERY SCIENTIFIC SOCIETY

Recently, many scientists are interesting about nanomaterials due to their antimicrobial, antiviral, anti-inflammatory and antioxidant activities (Julia et al,2017). The bacteria cannot gain the resistance against metallic nanoparticles such as Ag and Cu nanoparticles. Nanoparticles (NPs) are known to possess superior benefits such as augmented effectiveness against drug-resistant viruses, lower cost, and availability for surface modification, and improved efficacy at low concentrations (Ventola, 2015). Ag nanoparticles are one of the most studied antimicrobial materials (Bart et al., 2010; Bomko et al., 2018; Farimehr et al., 2022). Due to their high antimicrobial activity, Ag nanoparticles also exhibit the inhibitory effects against fungi and viruses (Balagna et al., 2020; Seyyed et al., 2020). The antimicrobial activity of the Ag nanoparticles is much stronger than other nanomaterials such as CuO NPs and ZnO NPs. (Padryk et al., 2021).

Propolis has several pharmacological properties such as antimicrobial, antiviral, antioxidant, and anti-inflammatory activities (Sedef et al., 2020). If the metallic nanoparticles are synthesized using biological and pharmacological properties of the propolis, their antimicrobial and antiviral activities will be much stronger.

Therefore, a large number of studies on biosynthesis of nanoparticles using the propolis have been published. Samir et al. synthesized Ag nanoparticles using silver nitrate and Egyptian Propolis extract, and evaluated their antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* (Samir et al., 2021). And also, Gayathri et al. prepared the propolis-Ag nanoparticles, and studied their antimicrobial activity against pathogenic bacteria like *Staphylococcus aureus, E. coli* and *Aspergillus flavus* obtained from clinical samples (Gayathri et al., 2022). Faizah et al. synthesized gold nanoparticles using the propolis extract, and the biosynthesized gold nanoparticles exhibited a strong inhibitory effect on *Staphylococcus epidermidis*, *Candida albicans, Klebsiella pneumoniae* and *Listeria monocytogenes.* (Faizah et al., 2022). And also, another study showed that biosynthesized selenium nanoparticles using the propolis ethanolic extract were used for human healthcare (Ndwandwe et al., 2020).

In this paper, we have synthesized the propolis-Ag nanoparticles dispersions using the propolis extract as a reductant and dispersant. In addition, we have characterized some properties and evaluated their antimicrobial activities against *E. coli*.

2. MATERIALS AND METHODS

2.1. Materials

Propolis samples were collected during summer 2022 from Rangrim County in Jagang Province (41°00′ N/127°15′ E), DPR Korea. The samples were kept in sterile dark glass containers until further use. The microbial culture media and silver acetate ($CH_3COOAg{\cdot}3H_2O$, 99.9% trace metals basis) were purchased from Sigma-Aldrich Chemicals. The ethanol and ultra water (UW) were all of analytical grade.

2.2. Preparation of the Propolis extract

Propolis was extracted in ethanol (70%, w/v) using an ultrasound bath for 2h. The propolis ethanolic extract was filtered through Whatman filter paper No.1 and stored below 4℃ till further experiments (Ghramh et al., 2019; Anita et al., 2023).

2.3. Synthesis of the Propolis-Ag nanoparticles dispersions

Propolis-Ag nanoparticles dispersions were synthesized by bio-reduction of silver acetate using the propolis ethanol extract as a reducing and stabilizing agent. The reaction was carried out by adding 0.1 mL propolis ethanol extract immediately after adding 0.4 mL of 0.01 M silver acetate solution to a flask containing 20 mL of ultrawater under continuing stirring for 5 min. When the color of the solution changed to red brown indicating the formation of silver nanoparticles, the reaction was considered to be fully developed and the light absorption property of the reaction solution were characterized by UV-Vis spectrophotometer. (Ghramh et al., 2019).

2.4. UV-Vis spectroscopy

The Surface Plasmon Resonance property of the propolis-Ag nanoparticle solution was characterized using UV-Vis spectroscopy (ScanDrop250, Analytik jena, Germany) at wavelength 200–800 ㎚ (Koperuncholan, 2015; Ghramh et al., 2019; Corciova et al., 2019).

2.5. Optimization of the biosynthesis of the Propolis-Ag nanoparticles

We have used several experimental parameters such as the propolis extract type, extract volume, pH and reaction time to optimize the synthesis of Ag NPs. In this experiment, three kinds of the propolis extract that were prepared at different ultrasonic exposure times (1, 2 and 3h) were used. The propolis extract volume was varied at different ranges (0.05, 0.1, 0.15 and 0.2 mL) and the reaction pH was maintained at 6.0, 7.0, 8.0 and 9.0, respectively by using K_2CO_3. The reaction time was monitored at different times (2, 5, 10 and 30 minutes).

2.6. Zeta potential analysis

Malvern Zetasizer Nano ZS 300 HAS (Malvern Instruments, Malvern, UK) was applied to assess the stability and size of synthesized the propolis-Ag nanoparticles at 25 °C and at 12° angle based on photon correlation spectroscopy. Analysis time was 60s and the average zeta potential was determined. The zeta potential of the nanoparticles solution was determined without dilution (Koperuncholan, 2015; Terica et al., 2021).

2.7. Antimicrobial activity

The Standard agar well-diffusion method is used to test the antimicrobial activity of biosynthesized AgNPs against *Escherichia Coli O111,* which was kindly identified and provided by the Korean Center of Culture Collection, Pyongyang. The strains were grown overnight and diluted in Mueller-Hinton broth (MHB) to a cell density of 10^5 Colony Forming Unit (CFU)/mL. 100 μl of the resulting mycelium was plated on Mueller-Hinton agar (MHA) plates and evaporated under sterile conditions. Wells of 3mm diameter were punctured in MHA plates using sterile cork borer and each well was loaded with different solution (propolis-Ag nanoparticles dispersions, propolis extract, Silver acetate and Levofloxacin). The plates were then incubated at 37ºC for 24 hours, and the antimicrobial activity was calculated by measuring the inhibition zone (Corciova et al.,2019).

3. RESULTS AND DISCUSSION

3.1. Optimization of the biosynthesis of the Propolis-Ag nanoparticles

UV-Vis spectroscopy is very important for examining the size and shape of nanoparticles in aqueous suspensions (Kumar et al., 2017). To optimize the synthesis conditions of the Ag NPs dispersions, the synthesis reaction results were evaluated by setting the kinds of the propolis extract, the amount of essential oil, the pH of the reaction solution and the reaction time as the main reaction parameters, and measuring the absorption maximum of the reaction solution and absorbance by UV spectrophotometer.

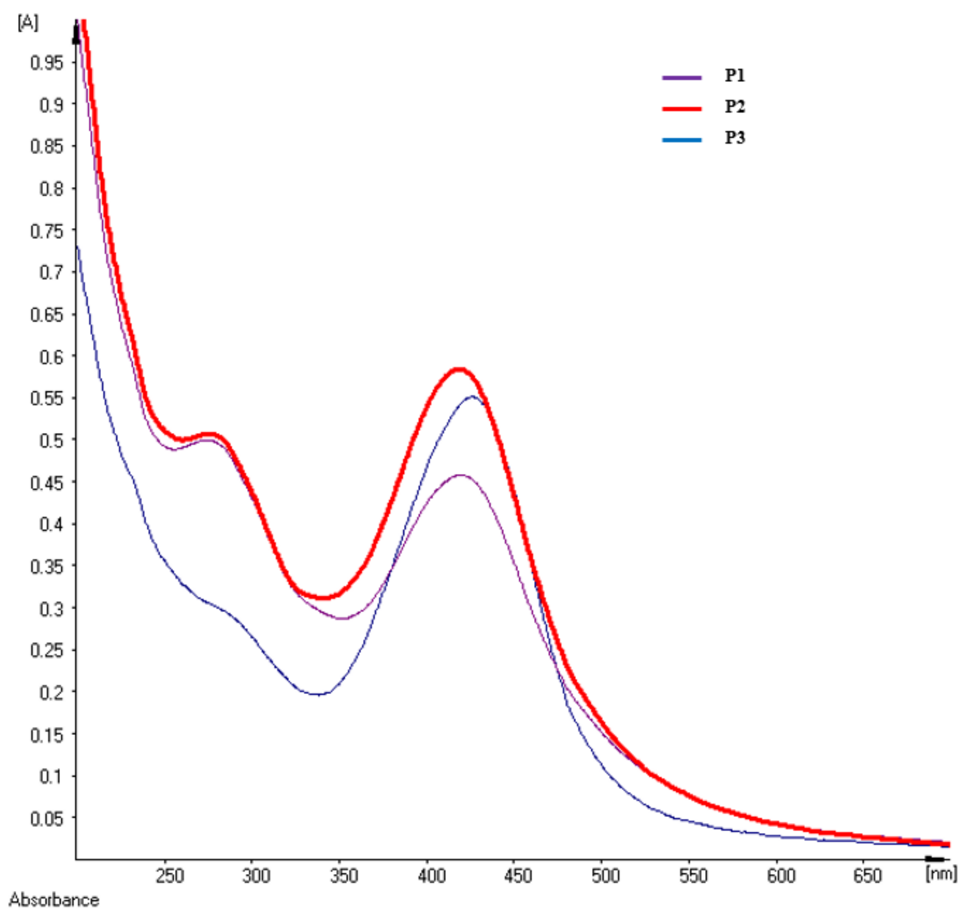

Figure 1. UV-Vis absorption spectra of the biosynthesized propolis-Ag nanoparticles dispersions using different types of the propolis extract; (P1-1h exposure, P2-2h exposure, P3-3h exposure)

3.1.1. Effect of the Propolis extract type

The three kinds of the propolis-Ag nanoparticles dispersions were synthesized using the various the propolis extracts with varying ultrasonic exposure times.

Figure 1 illustrates the UV-Vis spectra of these samples. There was no clear difference in wavelength using the three different the propolis extracts. The maximum absorbances of the SPR peaks were found almost at the same wavelength (between 421 and 425 nm).

The maximum peak intensity was obtained in case of P2, that its ultrasonic exposure time was 2 h. But in case of P1 and P3 that their ultrasonic exposure times were 1 and 3 h, the heights of absorption peaks are much lower. This result showed that various biomolecules could not be extracted adequately, or released molecules might be destroyed by excessive ultrasonic waves.

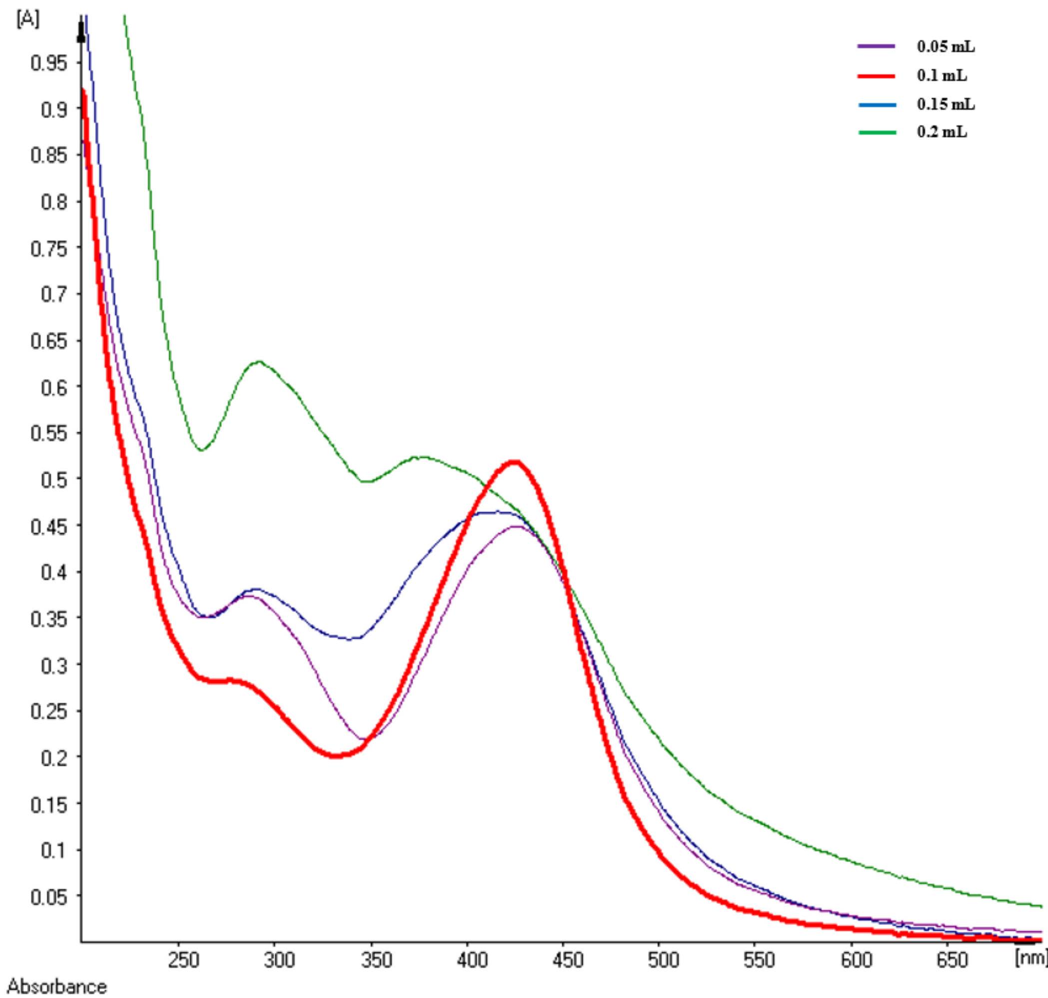

Figure 2. UV-Vis absorption spectra of the biosynthesized the propolis-Ag nanoparticles dispersions at different volumes of the propolis extract

3.1.2. Effect of the propolis extract amounts

Extract concentration affects the size, shape and color of synthesized Ag NPs as reported in earlier papers for different nanoparticles (Kamrun et al., 2020).

Different amounts of the propolis extract were added and four samples were prepared separately. Figure 2 shows the UV-Vis spectra of samples with varying extract amounts (0.05, 0.1, 0.15 and 0.2 mL). The UV-Vis spectra of prepared the propolis-Ag nanoparticles showed that 0.1mL of Propolis extract provides maximum yield with the uniform distribution of spherical shaped Ag NPs.

However, the other amounts possess an obscure peak, and the heights of absorption peaks are lower. When the extract amount is 0.05mL, the nucleation process for the formation of Ag NPs cannot be fairly progressed or may be slow. And then, in case of 0.2 mL of extracts, the aggregation of nanoparticles may be enhanced by excessive extracts, and also the larger NPs may be formed by the secondary reduction of silver ions on the surface of the nanoparticles (Sunita & Rajkuberan, 2020).

3.1.3. Effect of pH

Furthermore, the Ag NPs were synthesized at varying pH ranges (6.0, 7.0, 8.0 and 9.0). The results are showed in Figure 3. Solution pH affects the shape and size of the nanoparticles, and also has the ability to change their capping as well as stabilizing abilities (Kumar et al.,2017).

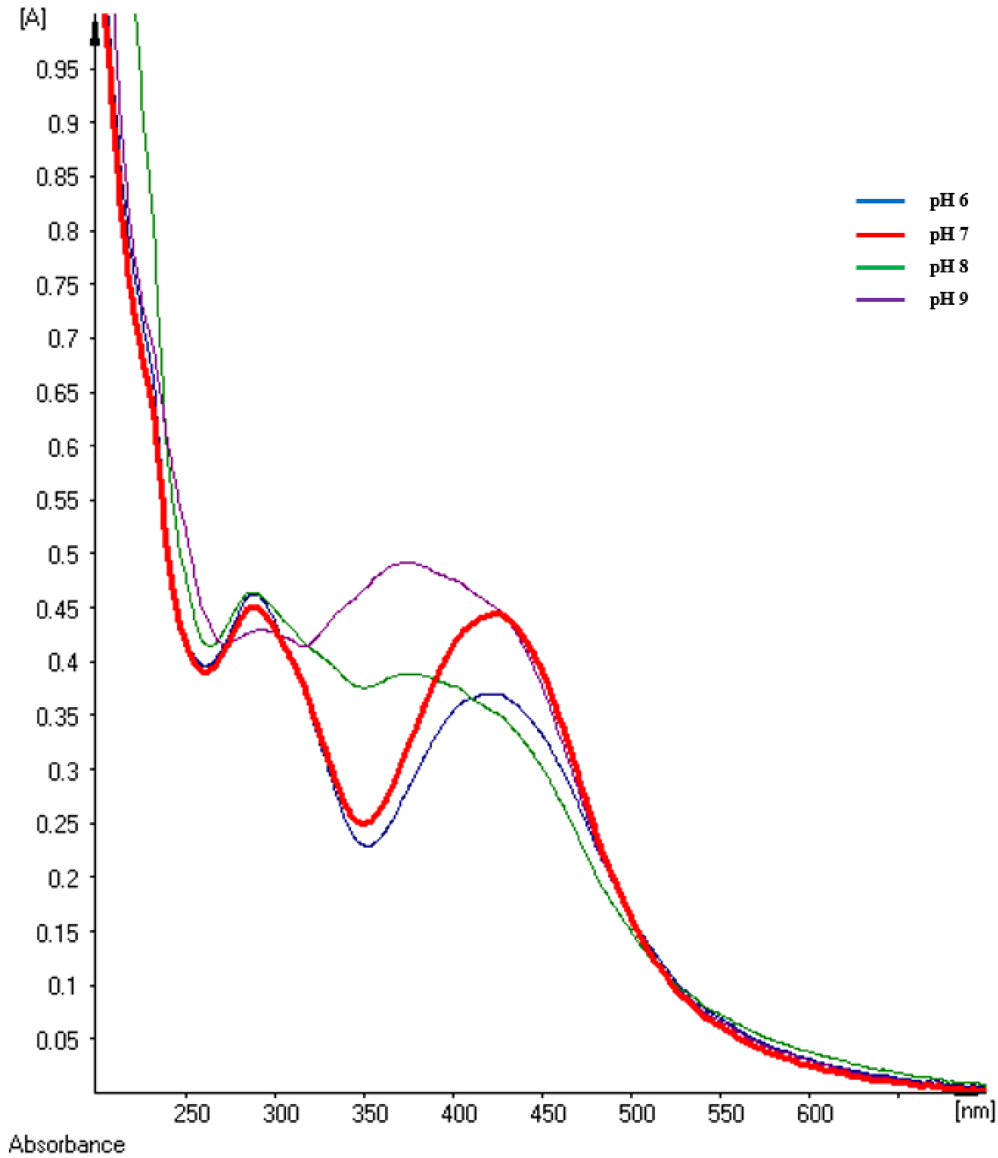

Figure 3. UV-Vis absorption spectra of the biosynthesized the propolis-Ag nanoparticles dispersions at different pH levels

The UV-Vis spectra provide evidence for the enhanced formation of Ag NPs at pH 7.0, whereas the formation of nanoparticles is suppressed at acidic pH(pH=6) and basic pH (pH=8, 9). By increasing the pH from acidic pH to neutral pH, the height of the absorption peak increases. And also, by increasing the pH from neutral pH to basic pH, the absorption intensity decreases.

This result showed that the bio-reduction process of Ag NPs was completed at neutral pH. And then it suggested that basic pH (8.0, 9.0) leads to the inactivation of biomolecules, which help in the reduction and stabilization of NPs. The height of the peak was much lower at pH 6.0, as the nucleation process for the formation of Ag NPs at acidic pH was slow (Chetan et al., 2022).

3.1.4. Effect of reaction time

In Figure 4, results are presented of UV-Vis spectroscopy of biosynthesized Propolis-Ag nanoparticles dispersions at different reaction times. By varying the reaction time (2-30 min), it was found that maximum reduction of silver ions and higher potential of Propolis extract biomolecules was observed at 5 min of reaction time. When the reaction time was 10 or 30min, the heights of SPR peaks were much lower. It may be because the reaction isn't adequately progressed or the biosynthesized Ag NPs are much larger by the secondary reduction on the surface of the formed NPs (Sunita & Rajkuberan, 2020).

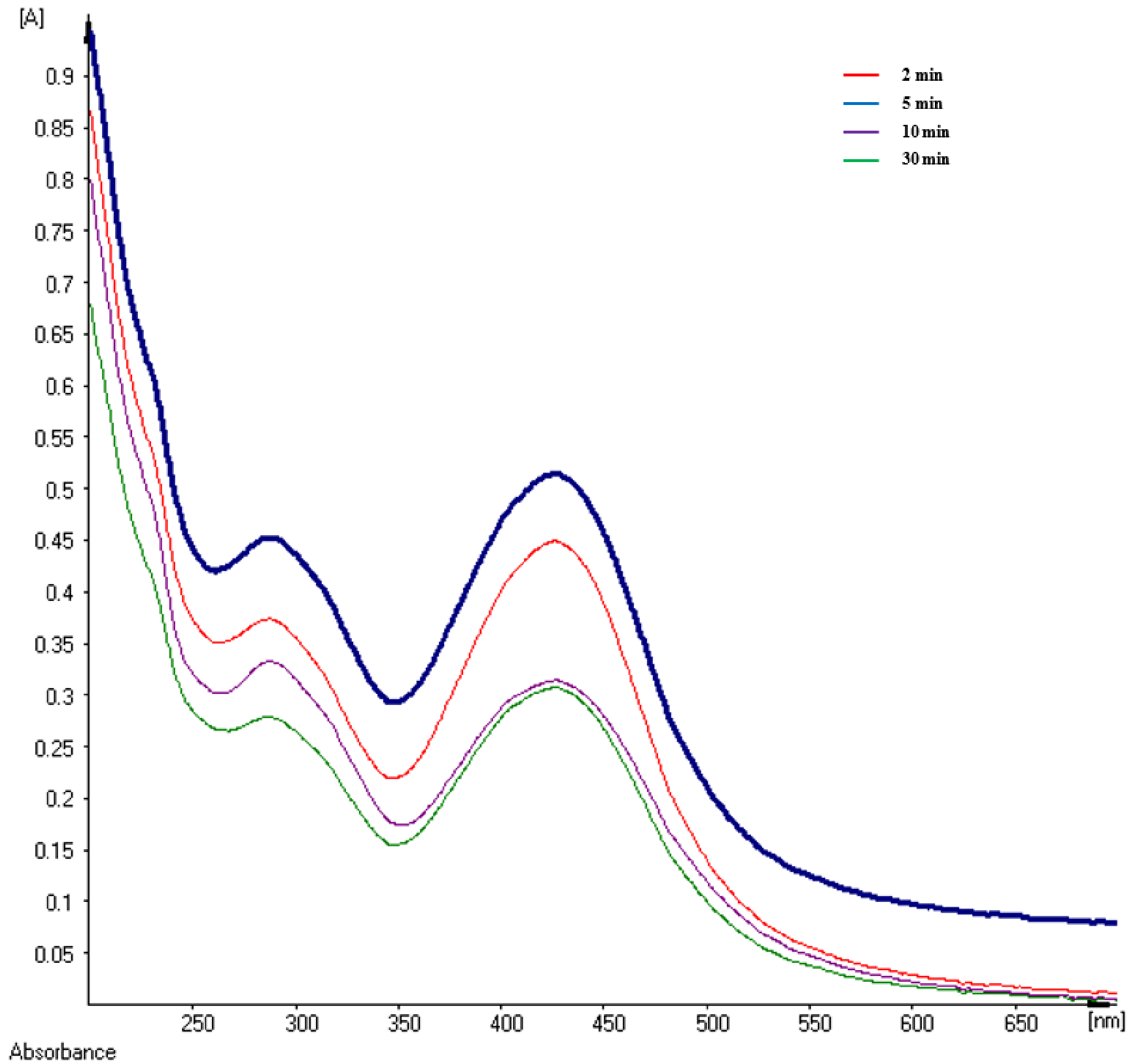

Figure 4. UV-Vis absorption spectra of the biosynthesized Propolis-Ag nanoparticles dispersions at different durations

3.2. Characterization of the Propolis-Ag nanoparticles dispersions

3.2.1. Surface Plasmon Resonance property

Figure 5 shows the UV-Vis spectra of Propolis-Ag nanoparticles biosynthesized at rational conditions. The synthesized nanoparticles exhibited the characteristic surface plasmon resonance (SPR) band at 422 nm, expected for Ag NPs, therefore it would be known that Ag nanoparticles were correctly formed by effects of reductant and dispersant of the Propolis extract. UV-Vis spectra in low wavelength region exhibited an absorption band at around 300 nm and it was attributed to biomolecules of Propolis extract.

3.2.2 Analysis of the particle sizes and zeta potential

In the present study, we have analysed the Propolis-Ag nanoparticle dispersions using Zeta potential analyzer (Malvern Zetasizer Nano ZS 300 HAS) to evaluate the particle size and the stability (Figure 6-7).

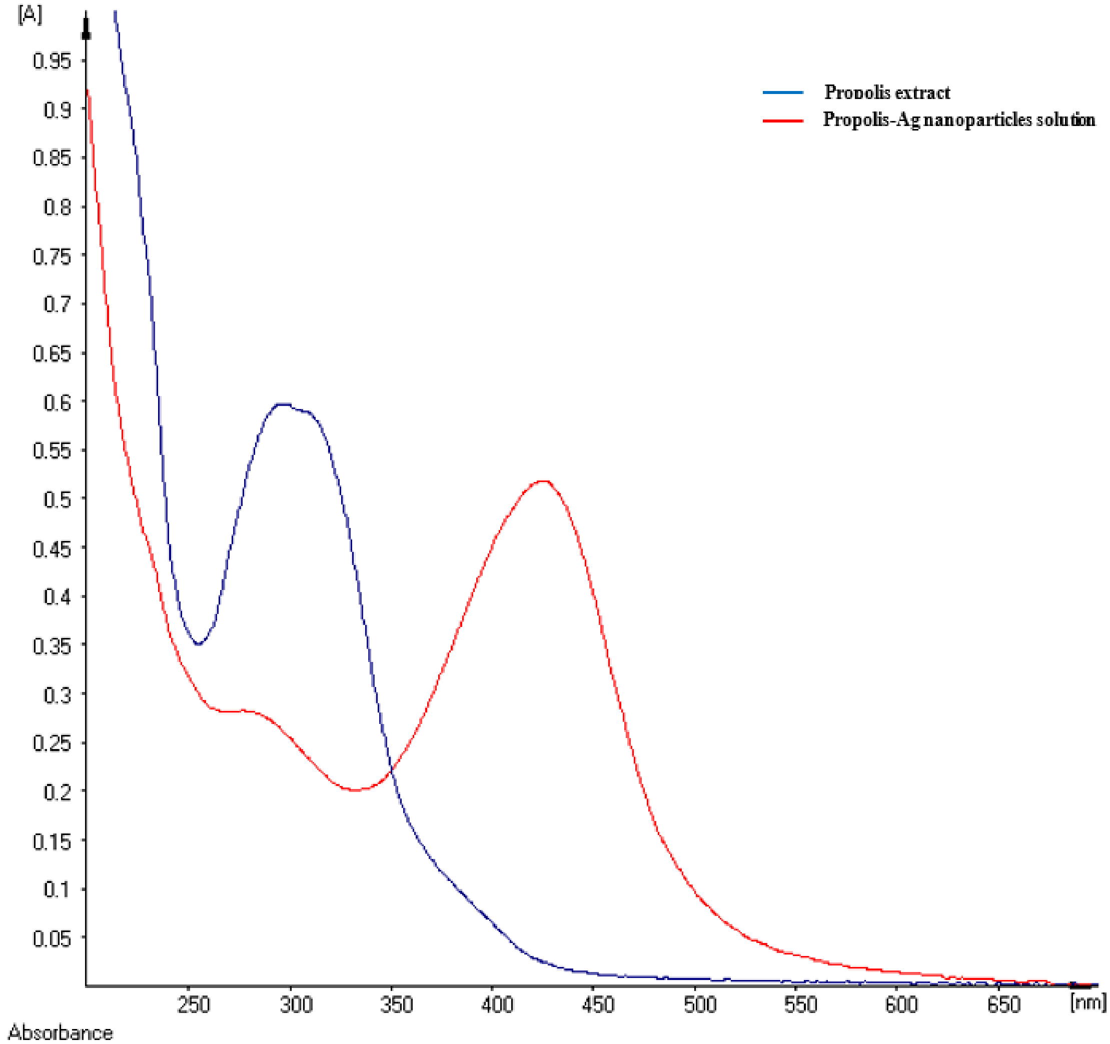

Figure 5. UV-Vis absorption spectra of the Propolis extract and the Propolis-Ag nanoparticles dispersions

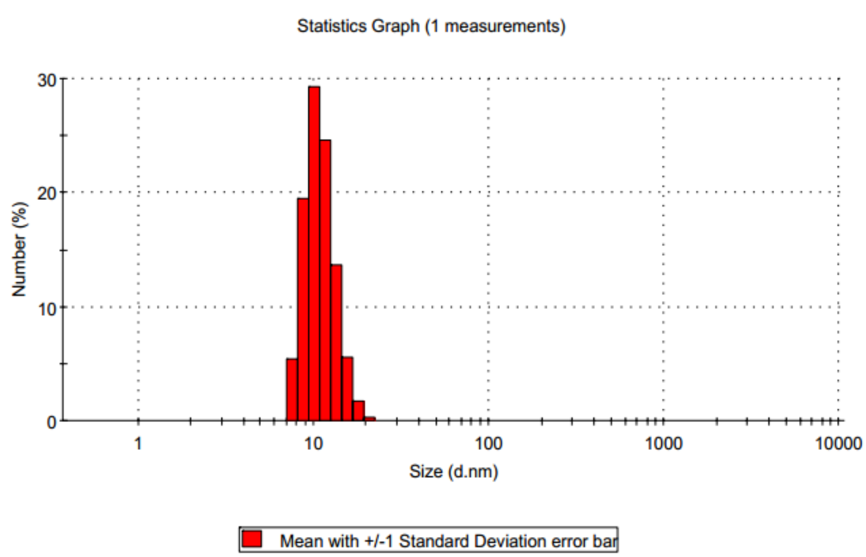

Figure 6. Distribution of sizes of Ag nanoparticles in the Propolis-Ag nanoparticles dispersions

Ag nanoparticles were in the range of 7.5~15.7nm as shown in Figure 6. The zeta potential value of Ag nanoparticle in Propolis-Ag nanoparticles dispersions was -36.3mV which suggested that the Ag NPs have very strong stability at room temperature (Figure 7).

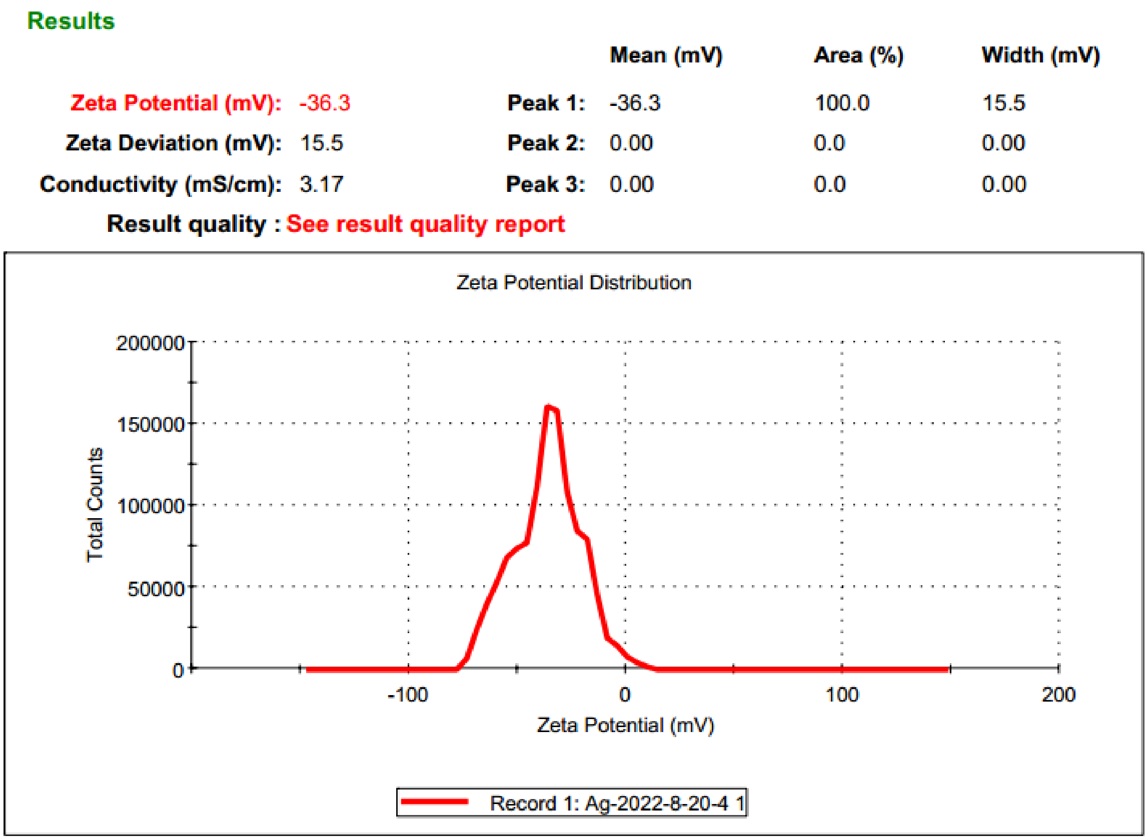

Figure 7. Zeta potential of Ag nanoparticles in Propolis-Ag nanoparticles dispersions

3.3. Antimicrobial activity of Propolis-Ag nanoparticles dispersions

The biosynthesized Ag NPs exhibited a strong inhibitory effect against the pathogens *Staphylococcus aureus* (Gram-positive), *Escherichia coli*, and *Pseudomonas aeruginosa* (Gram-negative) (Kumar et al.,2017).

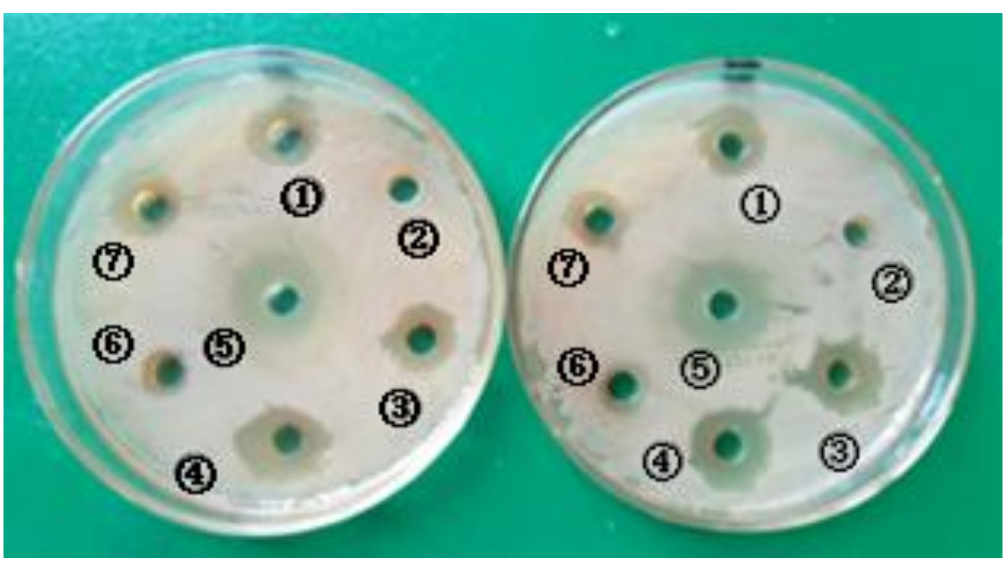

Figure 8. Antimicrobial activity of Propolis extract (1,7), Silver acetate (2), Propolis-Ag nanoparticles (3,4), Levofloxacin (100μg/ml, 5), Diluted propolis Ag NPs dispersions (1/10, 6 of the stock solution) against *E. coli* at 37 ℃ for 24 h.

Table 1. Antimicrobial activity of Propolis-Ag nanoparticles against *E. coli.*

	Inhibition zone ($\overline{X}$ ±SE, mm)
Group 1	8.9±0.13
Group 2	-
Group 3,4	13.6±0.25
Group 5	14.1±0.12
Group	7.2±0.16

Note. Group 1 and 7- Propolis extract, Group 2- Silver acetate, Group 3 and 4- Propolis-Ag nanoparticles, Group 5- Levofloxacin (100μg/ml, 5), Group 6- Diluted propolis Ag NPs dispersions (1/10, 6 of the stock solution)

Antimicrobial activities of Ag NPs are influenced by the size and shape of the particles. The smaller particles have a stronger inhibitory effect against microbes (Yaqoob et al., 2020). Group 3,4 with Propolis-Ag nanoparticles dispersions showed a significantly high inhibitory zone against *E. coli* compared to Group 1 (Propolis extract) and Group 2 (Silver acetate) in the inhibitory zone after 24h incubation (Figure 8, Table 1). The inhibition was reduced in case of the Group 6 with a concentration of propolis Ag NPs dispersions of 1/10 of the original solution. Also, the size of the inhibition zone of Propolis-Ag nanoparticles against *E. coli* was similar to Group 5 with Levofloxacin. Therefore, we can know that the antimicrobial activity of the propolis-Ag nanoparticles dispersions is more powerful.

Like the previously conducted studies, our results have confirmed the synergistic effect of Ag NPs and Propolis extract against microbes (Sougata et al., 2012; Gayathri et al., 2022). And it also has confirmed that the Propolis-Ag nanoparticles might be used in development of the novel antimicrobial and antiviral medicines.

4. CONCLUSION

The synthesis of Ag NPs using propolis, known as a drug, as a reducing, and dispersing stabilizer, has important implications for the preparation of eco-friendly nanomaterials. In this paper, propolis Ag NPs dispersions were synthesized using propolis extract and several properties were characterized. Propolis extract obtained from 2 h of ultrasound treatment during propolis alcohol extraction was found to be the most suitable for Ag NPs preparation, and the amount of propolis extract suitable for the preparation process, pH of the reaction solution, and reaction time were determined. Furthermore, the SPR characteristics, particle size, and Zeta potential of propolis Ag NPs dispersions were measured to suggest that the silver nanoparticle dispersions with particle size 7.5-15.7 nm are very stable. The antibacterial activity of propolis silver nanoparticle dispersions green synthesized with propolis alcohol extract was analyzed to find that the antibacterial activity against *Escherichia Coli* O_{111} was high compared to that of antibiotics. This result would be of help in development of the novel antimicrobial and antiviral medicines for anti-epidemic work.

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Author Contributions:

Song-Sik Kim: study design. Song-Jin Pak: Conceptualization and performing experiment, Chol-Hong, Chol-Jin Hwang and Kwang-Il To: performing experiment and data collection. Su-Chol Rim, Jong-Chol Son and Il-Song Pang: data analysis, draw-ing figure, writing and reviewing.

Ethical Approval

Not applicable. This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent

Not applicable.

Conflicts of interests

The authors declare that they have no conflicts of interest, competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Data and materials availability

Data that support the findings of this study are embedded within the manuscript.

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