Investigation of the *in vitro* antibacterial, cytotoxic and *in vivo* analgesic effects of silver nanoparticles coated with *Centella asiatica* plant extract

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ABSTRACT

In recent years, researchers have shown an increased interest in using medicinal plants for the synthesis of silver nanoparticles (AgNPs) having various therapeutic properties. *Centella asiatica* (CA), a medicinal plant, has been used to treat minor burn wounds, psoriasis, and hypertrophic wounds among many other pathological conditions. The current study aimed to synthesize CA coated AgNPs (CA-AgNPs) with appropriate biocompatibility and various therapeutic properties, including antimicrobial and analgesic activities. The synthesized CA-AgNPs were characterized by ultraviolet-visible (UV-Vis) spectroscopy, zeta potential measurements, and fourier transform infrared (FT-IR) spectroscopy. The formation of spherical CA-AgNPs was confirmed by a single surface plasmon resonance (SPR) peak emerging at 420 nm wavelength by UV-Vis. The average hydrodynamic diameter and zeta potential of the particles were found to be 29.5 nm and -24.5 mV, respectively. The FT-IR analyses showed that the AgNPs were coated and stabilized by bioactive compounds from the CA extract. MTT cytotoxicity assay revealed that CA-AgNPs at ≤1 mM concentrations exhibited biocompatibility for L929 fibroblast cells. The antimicrobial activity of CA-AgNPs was confirmed by significant inhibition of *Staphylococcus aureus* and *Escherichia coli*. In addition, the analgesic effect of CA-AgNPs was investigated for the first time in the literature by tail-flick and hot plate methods, and statistically significant results were obtained for both methods. Taken together, these results suggest that CA-AgNPs can be used as an effective antibacterial and analgesic agent in a variety of biomedical applications, including coating wound dressings.

Introduction

Plants have been used in the treatment of wounds, treatment of diseases, and protection against diseases since the earliest periods of human history. The ethnomedicinal use of plants (in the form of teas, syrups, oils, etc.) in the therapy of wounds and diseases is not only inexpensive and reachable, but also supplies a natural resource of medicinal materials. Studies on medicinal plants have approved that herbal drugs display fewer side effects in comparison to chemical (synthetic) matters, and are more cost-effective. While about 3% of the chemicals listed in the Western pharmacopoeia are effective for the treatment of wounds and skin diseases, herbal drugs are considered to be largely beneficial (23). With the development of science and scientific methods, the chemical structure of many bioactive components in the structure of plants has been clarified and many researches (*in vitro* and *in vivo*) have been made on these molecules (25). Many therapeutic effects of plants, such as antimicrobial, antitumor, antiviral, antiinflammatory,

antimalarial are known (45). In addition, the analgesic effects of some plants used in traditional medicine in recent years have been investigated by ethnobotanical studies. The analgesic effect of a plant is characterized by its activity to prevent, reduce, or relieve pain (44). *Centella asiatica* (CA) has critical importance among medicinal plants with therapeutic and analgesic effects.

In Asian countries, CA has been utilized as a traditional herbal medicine for hundreds of years. This medicinal plant has been reported to enhance wound healing and reduce scar formation (11, 28, 38, 51). CA extract shows wound healing, antioxidant, anticonvulsant, anti-inflammatory, antidiabetic, anti-psoriatic, anti-ulcer, sedative, immunostimulant, and cardioprotective properties (35). It has also been reported that CA improves memory and cognitive functions in rats (14, 43). Besides all these features, a methanolic extract of this plant demonstrated *in vitro* antiproliferative property in mouse fibrosarcoma cells, human gastric adenocarcinoma cells, human liver cancer cells, murine melanoma cells, MK-1, B16F10, SVK-14, keratinocytes, and *in vivo* tumor model test systems (3, 4, 6, 54). CA extract contains major triterpenoid components (madecassoside, asiatic acid, asiaticoside, and madecassic acid, etc.), and mixtures of them can stimulate collagen synthesis in *in vitro* human fibroblasts (5). Among the components of CA extract, asiaticoside has strong wound healing properties and reduces wound formation (27, 50). Asiaticoside increases fibroblast proliferation and extracellular matrix synthesis in wound healing (19). Madecassoside's effect on wound healing can include many mechanisms, including antioxidant activity, collagen synthesis, and angiogenesis (27, 50). Asiatic acid has antioxidant, anti-inflammatory, and neuroprotective properties (30, 52).

Nanoparticles research is an area of important scientific attention because of their large surface area to volume ratio and various physicochemical characteristics (33, 34). Many nanoparticles, such as copper, zinc, gold, and silver, have been synthesized by researchers. However, among these, silver nanoparticles (AgNPs) have proven to be highly effective against bacteria, viruses, and other eukaryotic microorganisms (31). AgNPs show the highest antimicrobial activity among all silver forms due to their large surface areas and sizes (42). AgNPs are synthesized by physical, chemical, and biological/green methods (21). The synthesis of AgNPs with plant extract (green synthesis) has attracted great interest in recent years because it is a low-cost and environmentally friendly method (46). The most important advantage of AgNPs synthesized from plant extracts is that they contain therapeutic molecules of plants used in traditional medicine (29).

The analgesic effects of extracts of *Centella asiatica* prepared with various solvents were investigated in the literature, but no such study was found with CA-coated AgNPs (CA-AgNPs). Therefore, the aim of this study is to investigate the analgesic activities of *Centella asiatica* coated AgNPs, which we produced by the green synthesis method, in rats. Additionally, the cytotoxic and antibacterial effects of the synthesized CA-AgNPs were also investigated. We will use the obtained CA-AgNPs as an active agent in wound dressing materials due to their antimicrobial, biocompatibility and analgesic properties.

Materials and Methods

Materials: The dried CA leaves were purchased from a local market (Şifa Market, Bursa, Türkiye). For high-performance liquid chromatography (HPLC) analyses, the analytical standards of asiatic acid, asiaticaside, madecassic acid and madecassoside were purchased from Sigma-Aldrich (Germany). For AgNPs synthesis, $AgNO_3$ was supplied from Sigma-Aldrich (Germany). For antibacterial analyses, the cultures of *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were taken from Kırıkkale University Scientific and Technological Research Laboratories (KÜBTUAM). The solid and liquid broth used in antibacterial tests and bacterial culture were obtained from Sigma-Aldrich (Germany). For cytotoxicity test, cell culture chemicals (DMEM, FBS, Trypsin/EDTA solution, Penicillin/Streptomycin) were obtained from Biochrom (Merck, Germany) and other cell culture materials were taken from Greiner (Austria).

Preparation of CA Plant Extract: Approximately 10 grams of dried CA samples were taken and extracted in a mixture of 100 mL methanol-water mixture (10:90% v/v) with an automatic extraction apparatus for 1 hour. After extraction, the solvent was removed with the aid of an evaporator. The resulting solid plant extract was dissolved in water and the solution was filtered with a 0.45 µm filter. The prepared solution was analyzed by HPLC (41).

Synthesis of CA-AgNPs: For CA-AgNPs synthesis, 1% solution of the plant extract obtained in the above-mentioned method in methanol:water was prepared and the pH of the solution was adjusted to 11 with 5 M NaOH to form negative charge groups (COO^- groups) in the plant extract. 5 mL of this solution was taken and added to the $AgNO_3$ solution (20 mL) which was prepared at different concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 4, 6, 8 and 10 mM). With the addition of plant extract, the color of the $AgNO_3$ solutions immediately turned yellow-brown. The resulting mixtures were stirred at room temperature until this color change was constant (approximately 24 h). The synthesized CA-AgNPs were then purified by passing through 0.45 µL filter and stored at +4 ºC in falcon tubes.

Characterization of CA-AgNPs: The SPR peaks of the synthesized CA-AgNPs were measured by UV-Vis spectrophotometer between 350-600 nm to evaluate the

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production of AgNPs. The size and zeta potential of CA-AgNPs were found by a Zetasizer (Malvern Instruments, Malvern, UK). The chemical structure of the CA-AgNPs was investigated by a FTIR (Vertex 70V, Bruker) with ATR method after the CA-AgNPs powders obtained by drying the samples at room temperature.

Evaluation of Antibacterial Properties of the Synthesized CA-AgNPs: A minimal inhibition concentration (MIC) assay was performed to determine the antimicrobial activity of the synthesized CA-AgNPs. Fresh 24-hour cultures of *E. coli* and *S. aureus* strains were used for the experiment. Bacteria concentration equivalent to 0.5 McFarland turbidity solution (OD 0.08-0.13 at 600 nm) was prepared with sterile phosphate buffer solution from 24-hour fresh cultures and spectrophotometric measurements were made to check the turbidity. The microorganisms whose concentration was adjusted were diluted 1:10 before MIC test. Microdilution method was used in the experiment. For the microdilution method, 100 µL of Mueller Hinton broth and 100 µL of material were added to the 96-well plates to be tested. Then, 5 µL of the prepared microorganism was added to the suspension. For sterility control only wells with medium and antibiotic wells (Gentamicin-64 µg mL^{-1}) were assayed for experimental control. The plate was incubated at 37 °C for 16-20 hours. After incubation, the turbidity was checked with visually. The growth was observed only in wells containing microorganisms, while turbidity was not observed in wells containing antibiotic and materials. Sterility control was considered successful due to the absence of turbidity in the sterility wells. Since CA-AgNPs have a brown color, the turbidity of samples could not be properly checked. To evaluate if bacteria have grown in suspensions containing CA-AgNPs, 10 µL was taken from each well and inoculated on Mueller Hinton agar. The samples were incubated at 37 °C for 18-24 hours. The reproduction was checked after incubation.

Cytotoxicity Tests: The cytotoxicity effects of CA and CA-AgNPs samples were investigated by MTT test. MTT is a very sensitive test to determine cell viability using 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) salt. L929 fibroblast) cells were inoculated into 96-well plates at a concentration of $10x10^3$ per well. Cells were incubated at 37 ºC for 24 hours in a 5% CO_2 conditioned incubator. Then, CA and CA-AgNPs solutions at various concentrations (between 1 mM and 10 mM) were applied to L929 fibroblast cells and incubated for 24 hours. For the control group, just cell medium was used. CA and CA-AgNPs were studied in triplicate. At the end of the 24-hour incubation, the cell medium was discarded from the wells and 50 µl of MTT solution (1 mg/ml) was added each. After 2 hours of incubation, the MTT solution was removed from the wells and 100 µl of isopropanol was added to each well and read at 570 nm wavelength in the ELISA plate reader. Cell viability (%) was obtained by comparing the results from the ELISA reader with the control group (15, 32).

Analgesic Effect: In this study, 14 healthy male Wistar albino rats aged 4-12 weeks were used. The number of animals to be used in the experiments was determined by performing a t-test with the G-power program according to the number of groups, 80% power expectation, and the expected effect width between groups on the basis of previous studies (46, 53). The rats were kept in separate cages at specific ambient conditions (22±3 ºC, 55±2% humidity, 12 hours dark-12 hours light). Rats were fed ad-libitum with commercial feed and water without dietary restrictions. Before the study, all animals were weighed. The animals were divided into two groups, seven in each group. The tail flick method was used in one group and the hot plate method was used in the other group. Equal amounts (1 mL) of saline and 1 mM CA-AgNPs solutions were applied transdermally to both groups. All studies were carried out in Kırıkkale University Hüseyin Aytemiz Experimental Research and Application Laboratory. The study was approved by Kırıkkale University Animal Experiments Local Ethics Committee with the decision dated 26.05.2021, numbered 2021/05, meeting numbered 24.

The tail flick method, which is used to determine the analgesic effect, was first suggested by D'Amour and Smith (9). In this method, the analgesic effect is characterized by the time elapsed after thermal heat is applied to the tail of the animal until it withdraws its tail. In this study, the animals' tail pulling response was monitored by applying heat to approximately 2 cm proximal part of the tail. Then, the time (seconds) was determined when the animal pulled its tail from the point where the radiant heat was applied. Experiment time was limited to a maximum of 15 seconds in order to avoid injury to the animal tail.

The hot plate test was used to figure out analgesic effect by the method explained by Eddy and Leimbach (10). Rats were kept on a hot plate at 53±1 °C. The reactions of animals (such as pulling their hind legs, licking, kicking or jumping) placed on the hot surface were monitored from the first moment. The maximum experimental time was limited to 30 seconds to avoid injury to the animals (39). To measure of animals' response to temperature, basal measurement was made 30 minutes before serum and CA-AgNPs application.

Statistical analysis: The data was first analyzed for the parametric test assumption. The distribution of differences (normality test) between saline and CA-AgNPs application was tested by Shapiro Wilk test in both methods. Levene test was applied to inhibition percentage for homogeneity of variances in both methods. The test

results revealed that the data was normally distributed and variances are homogeneous. The paired t was applied to test the difference between saline and CA-AgNPs application in both methods. Student t test (independent t test) was applied to see if there is a difference between inhibition percentages in both methods. The $P < 0.05$ was accepted as statistical significance level. IBM SPSS Statistics for Windows, Version 25.0 (Armonk, NY: IBM Corp.) was used for analysis. All results were expressed as mean ± SE.

Results

Synthesis and characterization of CA-AgNPs: In this study, biosynthesis of AgNPs was performed by using green synthesis method using CA plant extract. According to HPLC analysis results, it was determined that CA extract contains four bioactive molecules that play a role in wound healing and analgesic effect (Figure 1). When silver ions were added to the plant extract, they were reduced to AgNPs through the functional groups in the structure of the plant. The color change of the solution from yellow to dark brown indicates the formation of AgNPs.

The formation and stability of AgNPs in colloidal solution were analyzed by UV-VIS (Perkin Elmer Lambda 35) spectrometer. It was observed that the solution showed maximum absorbance at 420 nm and the absorbance value increased with increasing $AgNO_3$ concentrations (Figure 2a). The sizes and zeta potentials of the synthesized CA-AgNPs were analyzed with Zeta sizer and the results are shown in Table 1. It was found that all of the synthesized AgNPs had negative zeta potential and these results showed that produced AgNPs were stable. The formation of a single peak in the zeta potential distribution graph (Figure 2b) proves that the solutions remained stable and were not subjected to sedimentation and aggregation. It was observed that the zeta diameters of the synthesized CA-AgNPs were different from each other, but all AgNPs had a hydrodynamic diameter below 100 nm. This is a desired result because in previous studies, it was showed that with the reduction of the diameter of AgNPs, their surface areas increased and thus their antimicrobial activity increased (42).

Table 1. The zeta potential (mV) and zeta diameters (nm) of the synthesized CA-AgNPs.

Concentration (mM)	Zeta Potential (mV)	Zeta Size (nm)
0.1	-16.4	26.64
0.2	-39.7	64.95
0.3	-23.0	40.15
0.4	-21.0	36.17
0.5	-19.7	19.33
1.0	-20.4	25.18
2.0	-26.9	26.82
4.0	-25.0	32.39
6.0	-24.4	26.46
8.0	-28.2	27.58
10.0	-27.2	28.42

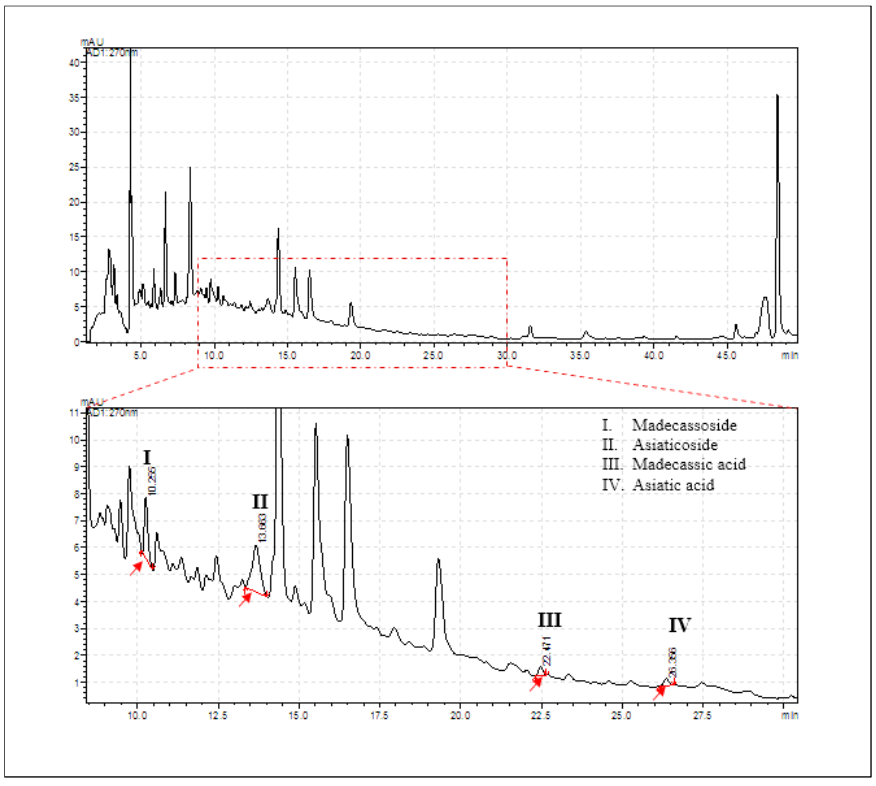

Figure 1. HPLC chromatogram of the CA extract.

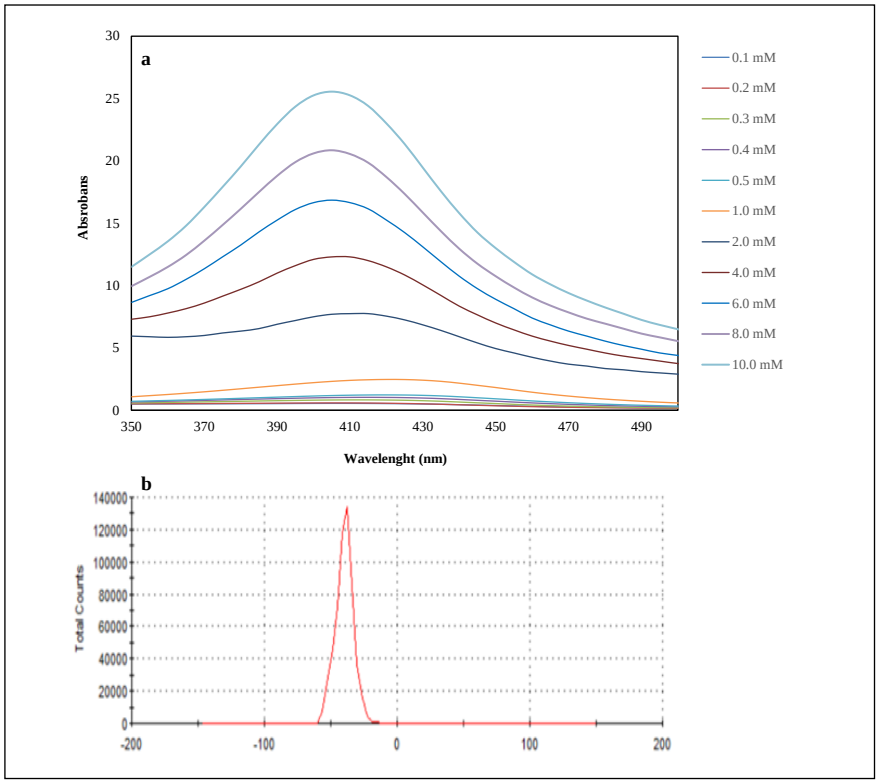

Figure 2. UV-VIS spectrums of CA-coated AgNPs at different Ag^{+} concentration between 350-600 nm wavelength (a) and zeta potential distributions of synthesized 1.0 mM CA-AgNPs group (b).

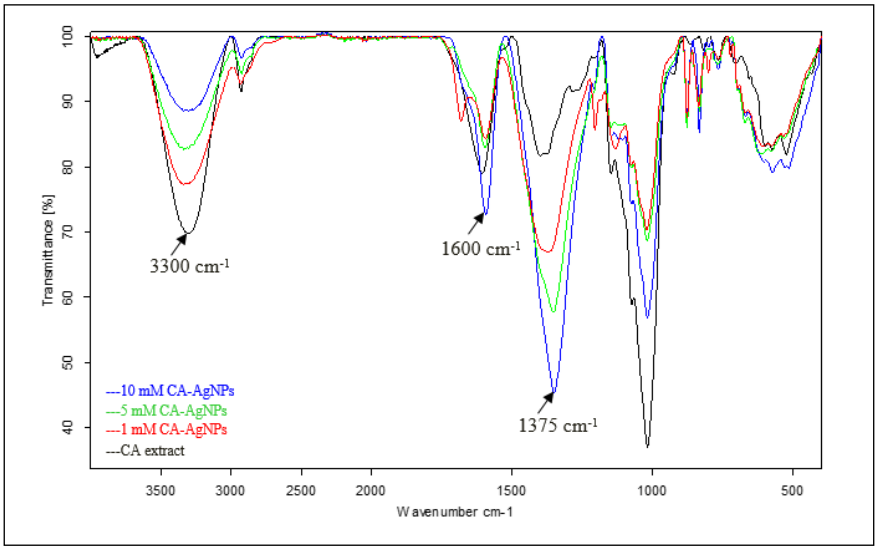

Figure 3. FTIR spectra of the CA extract and CA-AgNPs at different concentration.

Since *Centella asiatica* leaf has a rich content in terms of saponin and phenolic compounds, it contains quite a lot of hydroxyl, carbonyl and carboxyl groups in its structure. Therefore, FTIR analysis was performed to investigate the presence of plant functional groups in both the plant extract and CA-AgNPs synthesized at different $AgNO_3$ concentrations, and the results are shown in Figure 3. In FTIR spectrum of pure plant extract, vibrations were observed at 1375 cm^{-1} and 1600 cm^{-1} due to C=O groups of carboxylic acids and phenols. The stretching at 1375 cm^{-1} was assigned to C-O stretching and O-H deformation, possibly found in the acid groups found in CA leaf extract. Wide stretching at 3300 cm^{-1} was assigned free O-H groups in phenols. When FTIR spectra of the synthesized CA-AgNPs solutions of 1, 5 and 10 mM are examined, the change has emerged in the absorption bands in 1375 cm^{-1} and 3300 cm^{-1} depending on the silver concentration in the absorption bands with the formation of silver nanoparticle. In addition, a change in the frequency of 1600 cm^{-1} carbonyl stretching was observed in CA-AgNPs samples and the increase in the peak intensity of 1375 cm^{-1} may be caused by the occurrence of carbonyl groups during reduction in alkaline medium. The decrease in the intensity of the O-H stretching peaks at 3300 cm^{-1} indicates that OH groups of plant extract were involved in reduction and stabilization of AgNPs (2, 15).

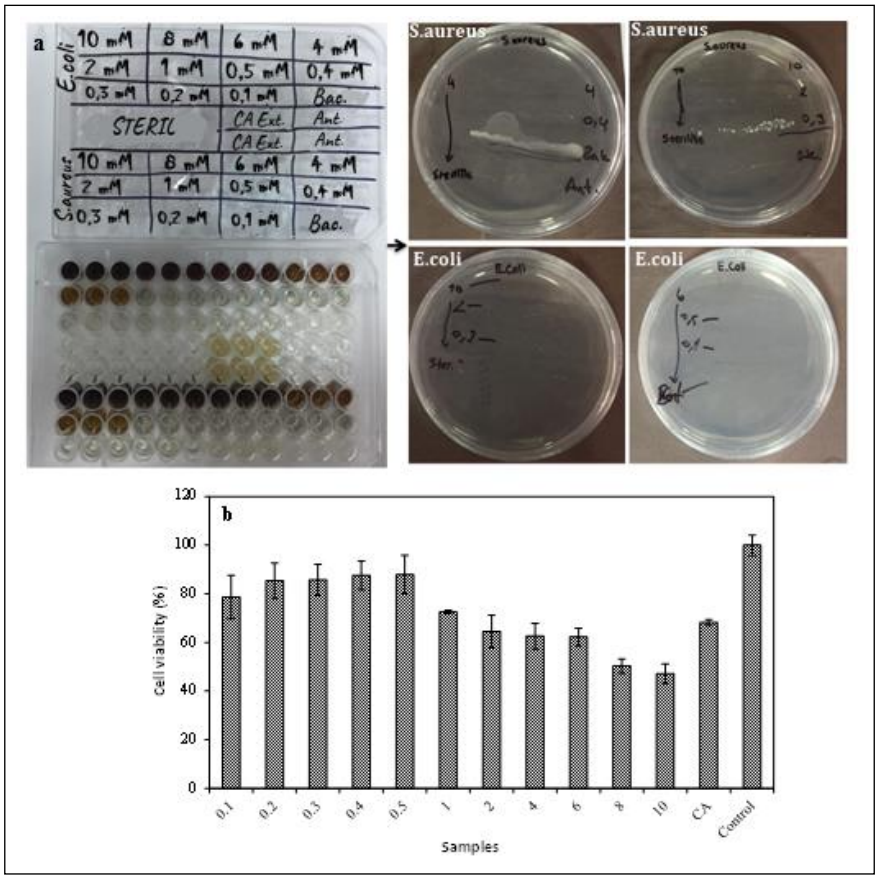

Figure 4. Antibacterial test results; colonies of *S. auresus* and *E. coli* bacteria at different concentrations after incubation with CA-AgNPs (a) and cell viability values of the synthesized CA-AgNPs groups and CA extract determined by MTT test (b).

Antibacterial and cytotoxicity testresults: For antibacterial test, microdilution method was used and the performed test image and results were given in Figure 4a. For *S. aureus* bacteria, 0.4 mM AgNPs was determined as the minimal inhibitory concentration and it was observed that bacteria incubated with concentrations lower than this concentration grew on agar. For *E. coli*, it was observed that bacteria did not grow at all AgNPs concentrations and all AgNPs groups were found to have a 100% lethal effect on this bacterium. In addition, there was no growth in the wells kept sterile and containing antibiotics. It was observed that bacteria that were incubated in sterile medium were grown on agar.

The cytotoxic effect of synthesized CA-AgNPs on L929 fibroblast cells was examined by MTT test. The viability of cells incubated with CA-AgNPs at different concentrations for 24 h was calculated according to the control groups and the results obtained are given in Figure 4b. According to the MTT test results, no significant toxicity was observed in L929 fibroblast cells, where CA-AgNPs having concentration between 0.1 and 1 mM. In L929 fibroblast cells, where CA-AgNPs having concentration between 2 and 10 mM were applied, cell viability fell below 70%. According to the ISO 10993-5 standard evaluation criteria, we can say that the synthesized CA-AgNPs with a concentration higher than 1 mM show cytotoxic properties.

Analgesic effects: In this study, the analgesic effects of CA-AgNPs prepared from the extract of *Centella asiatica* plant on rats were evaluated using tail flick test and hot plate test (Table 2). In these studies, the duration of pain sensation after the administration of CA-AgNPs to experimental animals was examined.

As shown in Table 2, while the average time for animals to feel pain after transdermal saline application in the tail flick test was 4.38±0.34 seconds, this time increased to an average of 6.81±0.45 seconds after the administration of CA-AgNPs. Accordingly, CA-AgNPs inhibited pain by an average of 35.71 ± 2.16%. Statistically, the difference within and between groups was found significant ($t=-11.20$, $df=6$, $P<0.001$).

In the hot plate method, the average time to feel pain after transdermal saline application to the right hind leg of the animals was 16.71±0.58 seconds, while this time increased to an average of 23.33±0.99 seconds after the application of CA-AgNPs. Accordingly, CA-AgNPs inhibited pain by an average of 27.80±3.04%. Statistically, the difference within and between groups was found to be significant ($t=-7.22$, $df=6$, $P<0.001$).

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Table 2. Analgesic effect of *Centella asiatica* coated AgNPs by tail flick and hot plate methods in rats.

	Tail flick				Hot plate			
Animal	**Animal weight (g)**	**After Saline (sec)**	**After CA-AgNPs (sec)**	**Inhibition (%)**	**Animal weight (g)**	**After Saline (sec)**	**After CA-AgNPs (sec)**	**Inhibition (%)**
1	161.9	3.5	5.2	32.7	150.8	15.3	19.1	19.9
2	149.4	3.4	5.7	40.4	156.7	14.6	25.1	41.8
3	158.4	4.7	7.1	33.8	159.1	17.5	21.3	17.8
4	152.5	5.4	8.6	37.2	161.2	18.8	25.1	25.1
5	159.1	4.1	7.3	43.8	154.5	16.7	23.7	29.5
6	153.7	3.9	6.1	36.1	153.7	18.2	26.8	32.1
7	153.1	5.7	7.7	26.0	160.8	15.9	22.2	28.4
Value	155.4±4.4	4.38 ±0.34	6.81±0.45[a]	35.71±2.16	156.7±3.9	16.71±0.58	23.33±0.99[a]	27.80±3.04[b]

Values are mean ± SEM (n=7). [a]P<0.001 represented significant compared to control (saline). [b]P>0.05 represented no significant difference compared to tail flick inhibition percentage (P=0.055).

Tail flick and hot plate methods measure the delay of analgesic response of animals to thermal stimulus. In principle, these two methods are similar to each other. However, while tail flick is a spinal response, the hot plate is predominantly supraspinal (44). In both methods, it was determined that CA-AgNPs prolong the time for animals to feel pain and thus have an analgesic effect. Even though there was no statistical difference between the two methods in terms of inhibition percentages according to P=0.055 value, the difference may be significant level (t=2.12, df=12, P=0.055).

Discussion and Conclusion

The present study clearly shows that CA extract can be utilized favorably to produce AgNPs with an easy green method. Increasing the Ag concentration (between 0.1-10 mM) in the synthesis procedure yielded more AgNPs in an aqueous environment. The obtained CA-AgNPs at different concentration were characterized with many different techniques and it was found that the synthesized CA-AgNPs were stable and in spherical shape, have a SPR peak at around 420 nm. In the literature, AgNPs were reported to be yellow-brown solutions and showed SPR in the range of 420-430 nm (16, 17). This SPR peak observed in the nanoparticle samples we obtained shows that the silver ions were reduced to AgNPs with the used plant extract. Also, negative zeta potentials of AgNPs shows the stabilizer effect of CA plant extract, because, the negatively charged CA-AgNPs repels each other in solutions (13, 48).

The fact that the spectrum of the synthesized CA-AgNPs according to the FTIR results is highly similar to the spectrum of the plant extract shows that the CA-AgNPs are stabilized by phenolics, saponins or carbohydrate-derived biomolecules containing high OH groups in the plant (12).

According to the antibacterial test results, the synthesized CA-AgNPs have been shown to have antibacterial effects on both Gram positive and Gram negative bacteria and certain groups of AgNPs can have potential to be used as antimicrobial agents in many applications. The antibacterial effects of AgNPs have been studied in many previous studies and AgNPs show the high antimicrobial activity compared to other silvers salts due their small size and large surface area (42). In an aqueous environment, AgNPs are oxidized in the presence of oxygen and protons, and released Ag^+ ions show antimicrobial effects (24, 26, 37). The release rate of these ions depends on the some parameters, such as shape, size, concentration and coating agents (26, 42, 47). AgNPs with a particle size of 1-100 nm have been showed to inhibit all bacterial strains at a concentration of 75 µg mL^{-1}. It has also been observed that nanoparticles with a particle size of 1-10 nm have a high affinity to adhere to the surface of the cell membrane compared to larger nanoparticles. Therefore, small sized AgNPs can interact more with the bacterial cell membrane thanks to their large surface area and can cause more damage to the bacteria (42, 47). Ivask et al. (22) investigated the toxic effects of AgNPs with sizes ranging from 10 to 80 nm on bacteria, yeast, algae, crustacean and mammalian cells. At the end of this research, it has been confirmed that small size nanoparticles show highly toxic effects. According to another study that was performed to research the antimicrobial effects of AgNPs at different shape, it was observed that triangular nanoparticles have more

antimicrobial effects compared to spherical and rod-shaped nanoparticles (36).

The cytotoxic effect of all produced CA-AgNPs on L929 fibroblast were investigated with MTT test and it was found that the CA-AgNPs have cytotoxic effect when the concentration of CA-AgNPs was higher than 1 mM. Due to their high antimicrobial properties, AgNPs are frequently used in medicine, medical devices, health products, hygiene products, food and cosmetics industry. However, the strong oxidative activity of silver ions released from AgNPs induces cytotoxicity, genotoxicity, immunological responses and even cell death, causing various adverse effects on biological systems (1, 7, 8). The cytotoxic effects of AgNPs on human cells depend on the concentration, shape, synthesis method and coating agent used (1). Therefore, in our study, we investigated the cytotoxic effects of CA-AgNPs that we synthesized at different Ag concentration.

Centella asiatica extract is a medicinal plant known to have analgesic effects as well as other therapeutic properties (46, 49). Inamdar et al. (20) stated that terpene acids (madecassic acid and asiatic acid), which are mainly responsible for the analgesic and anti-inflammatory activity of *Centella asiatica*, are effective in controlling inflammatory conditions or rheumatism. Somchit et al. (49) reported that CA extracts showed significant analgesic activity in hot plate and acetic acid-induced writhing tests. According to the study, intraperitoneal administration of CA significantly reduced PGE2-induced paw edema. The extract showed significant anti-inflammatory activity even at 2mg/kg compared to the control and the larger dose was found to be more effective than mefenamic acid. Saha et al. (46) reported that both chloroform and methanol extracts of CA showed significant analgesic effects in mice.

In this study, we also proved that CA-AgNPs, which we synthesized for use as a therapeutic and antimicrobial agent in a wound dressing material, have analgesic effects transdermally by thermal (tail flick and hot plate) methods. Pain is one of the symptoms that patients find particularly bothersome during the wound healing process (40). Therefore, the specific concentration of CA-AgNPs we synthesize is a biocompatible agent with properties that will increase the comfort of the patient during the wound healing process. Further studies are also recommended in *in vivo* infected and non-infected wound models.

Acknowledgment

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

OB, HE, ZGG, EA, SE, MY and İV conceived and planned the experiments. OB, HE and EA carried out the experiments. OB, HE and EA planned and carried out the simulations. OB, HE, EA and SE contributed to sample preparation. OB, HE, ZGG, EA, SE, MY and İV contributed to the interpretation of the results. O.B. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

The study was approved by Kırıkkale University Animal Experiments Local Ethics Committee with the decision dated 26.05.2021, numbered 2021/05, meeting numbered 24.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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