Antimicrobial activity of biologically synthesized silver and zinc nanoparticles using *Allcemilla vulgaris* (Layd's mantle) leaf extract

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Abstract

In this study, the extract of *Allcemilla vulgaris* ~~(Layd's mantle)~~ *was used in the* production of silver and zinc nanoparticle without use of any chemical agent was investigated. Biologically synthesized nanoparticles were characterized by Scanning Electron Microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), UV-Vis spectroscopy, X-ray diffraction (XRD) and Zeta potential analysis. The silver (AgNPs) and zinc nanoparticles (ZnNPs) showed strong antibacterial activity against both tested *Escherichia coli* O157:H7 (Gram negative) and *Staphylococcus aureus* (Gram positive) bacteria. The antibacterial activity of bio-synthesized nanoparticles against two pathogens at ~~was assessed by~~ minimal inhibitory concentration (MIC) assays values of 0.06 ug/ml and 16.2 ug/ml for *E.coli* O157:H7 AgNPs and *Staphylococcus aureus* against ZnNPs were at 4.25 ug/ml and 6.25 ug/ml for, respectively. This study concludes that the bio- synthesized AgNPs and ZnNPs may be used as an effective antimicrobial activity, so it can be projected as future generation antimicrobial agents and designing newer drugs.

Comment [WU1]: Added

Comment [WU2]: powdered extract

Comment [WU3]: Delete since author name has been suggested to be included in the research title

Comment [WU4]: Synthesis would be preferred word to production

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Comment [WU7]: And then evaluated for antibacterial potential using micro dilution broth method.

Comment [WU8]: Delete

Comment [WU9]: The whole of this sentence must be rewritten to carry any meaning. It is so vague as of now!

Comment [WU10]: This must be supported with scientific statistical data which you did not provide for reference!

Key words: Biological synthesis, silver, zinc, nanoparticle, *S.aureus*, antimicrobial activity

1. Introduction

Nanomaterials have got a special place and various applications in technology [1]. The metallic nanoparticles that have large surface area so; they can be used as antibacterial agent [2]. Nanoparticles can be synthesized by different physical, chemical and biological methods [3]. The risk of toxic compounds used chemical and physical methods limits the biomedical applications. So, the using of biological materials i.e. plants, plant components, bacteria for synthesis are safe and eco-friendly [4]. Plant based synthesis has some advantages such as it is faster and stable. It is possible to get different sizes and shapes of NPs comparable to microorganisms. Many investigations showed silver and zinc nanoparticles are exhibit antibacterial, antioxidant and photocatalytic properties [5, 6]. Many studies showed that silver and zinc nanoparticles are a superior product from the field of nanotechnology because of their exclusive properties such as the most important antibacterial, anti-viral, antifungal and anti-inflammatory activities and good stability [7, 8]. For these properties, silver and zinc nanoparticles have been used most widely in the different industries ie. health, food packaging, textile and other applications.

Alchemilla vulgaris ~~(Lady's mantle)~~ is a perennial plant belonging to the Rosaceae family, which comprise of more than 300 species and common in Africa, Asia, Europe, and the Americas. Lady's mantle benefits include treating muscle spasms, swelling and inflammation, digestive problems, water retention, mild diarrhea, and diabetes. This study, easy and biological synthesis of silver and zinc nanoparticles using *A.vulgaris* plant powders considered to be an environmentally friendly procedure was investigated. ~~For this purpose, *A.vulgaris* plant powders were used for the bio-synthesis of silver and zinc nanoparticles~~. Additionally, the evaluation their antibacterial activity against *E.coli* and *S.aureus* which are known as human pathogenic bacteria was investigated.

Comment [WU11]: What about the other bacterium species? You would rather use antibacterial activity to cater for both species

Comment [WU12]: This should antibacterial to be more specific. The plant species must be included here as well

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Comment [WU14]: Replace this with 'possessing'

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Comment [WU17]: Replace with 'associated with'

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Comment [WU20]: Rather 'other studies'

Comment [WU21]: Compared to what?

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Comment [WU23]: What is this? You would rather use the plant species itself not the author

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2. Materials and methods

2.1. Bio-synthesis of silver and zinc nanoparticles

The methodological protocol by [9] was adopted with minor modifications. Briefly fresh *A.vulgaris* plant (10 g) of dried plant was added into 100 ml boiling deionized water for 30 min. The aqueous extract was left to cool at room temperature, and then filtered using Whatman No. 1 filter paper to a final volume of 80 mL. The filtrate was stored at 4 ^{0}C until further use in the green synthesis of AgNPs and ZnNPs. Ten (10) mL of *A.vulgaris* aqueous extract was taken from the stock solution and 3 mM of $AgNO_3$ (90 mL) was dissolved in the *A.vulgaris* extract solution using magnetic stirrer to a final volume of 100 mL. The solution was then heated at 60–80 ^{0}C. The color change of mixture indicated the complete nanoparticle synthesis and this was confirmed with an absorbance peak using a UV–Vis spectroscopy. Another 10 mL of *A.vulgaris* aqueous extract was taken from the stock solution and 2 g of zinc nitrate hexahydrate crystal were added. The solution was mixed with a magnetic stirrer and then heated at 60–80 ^{0}C to a final volume of 100 mL. The color change of mixture indicated the complete nanoparticle synthesis which was validated by absorbance measurement using a UV–Vis spectroscopy.

Comment [WU27]: In the previous sentence you talked about fresh plant and here you are saying dried plant, which one is which?

Comment [WU28]: From which to which?

Comment [WU29]: From what to what

2.2. Characterization of bio-synthesized silver and zinc nanoparticles

Characterization of AgNPs and ZnNPs was done using a dynamic light scattering (DLS), UV-Vis spectrometry, scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FT-IR). Crystalline metallic silver and zinc were examined by X-ray diffractometer. A few drops of the concentrated AgNPs and ZnNPs solution were deposited on a carbon tape covered stub, and left overnight for drying. Then, the stub was coated with gold using a sputter coater to produce clear images from the SEM (ZEISS EVO LS10). The AgNPs and ZnNPs were well dispersed in water and the solution was used to determine the absorbance of the AgNPs and ZnNPs. The well dispersed AgNPs and ZnNPs solution was

also used for measuring the effective diameter of the AgNPs and ZnNPs with a DLS. The formation of the AgNPs and ZnNPs and existence of the plant extract on the surface of the AgNPs and ZnNPs were both proven by FT-IR. For FT-IR analysis, the AgNPs and ZnNPs solution was centrifuged at 10.000 rpm for 15 min and the precipitated AgNPs and ZnNPs were allowed to dry at 60 ^{0}C.

Comment [WU30]: Incomplete procedure for FT-IR analysis methodology

2.3. Culture and growth conditions of microorganisms

The methodological procedure by [10] was adopted. In this study, microorganisms (*E.coli* O157:H7 NCTC 12900, *S.aureus* ATCC 29213) were obtained from the culture collections of Department of Food Hygiene and Technologies, Faculty of Veterinary, Erciyes University, Kayseri, Turkey. Microorganisms were plated on blood agar (Oxoid, CM0271) and incubated at 37 ^{0}C for 18-24 h. After incubation, 2–3 colonies of each organism taken from blood agar were inoculated to 5 ml Mueller Hinton broth (Oxoid, CM0405) and incubated overnight at 37 ^{0}C. The suspension was later adjusted to 0.5 McFarland turbidity (approximately 10^{8} cfu ml^{-1} for bacteria).

Comment [WU31]: What was the use of this adjustment?

2.4. Determination of Antibacterial activity with Micro dilution Broth Method

The method by [10] was followed with minor modifications. Briefly biologically synthesized silver and zinc nanoparticles were tested against pathogenic bacterial strains such as Gram (-) *E.coli* O157:H7 (NCTC 12900) and Gram (+) *S.aureus* (ATCC 29213). The antibacterial activity of these microorganisms were studied on the well broth micro dilution method using 96-well plates. The silver and zinc nanoparticles were diluted in 6 serial two-fold in Muller-Hinton broth (Oxoid, CM0405) in a 96-well microtiter plates. 100 µL of freshly grown bacteria were then standardized until a bacterial number of 1×10^{8} cfu ml^{-1} in Muller-Hinton broth was reached and added to each well. The micro dilution test comprised positive control without extract and negative control lacking microorganisms under the same conditions. Plates

Comment [WU32]: Delete this. It was mentioned earlier in the text

Comment [WU33]: Not clear, rewrite to carry meaning. How can you study on a well broth micro dilution method

Comment [WU34]: Did you mean; were made in two fold concentrations with 6 serial dilution? Please rewrite the phrase

Comment [WU35]: This sentence those not make sense to me. Reconstruct it to enable the reader follow the procedure well. We always use the word 'comprise of'. Not only comprise!

were incubated aerobically at 37 ^{0}C for 24 h. To determine antimicrobial activity and MICs, 10 µL broth was spot inoculated onto Mueller-Hinton agar (Oxoid CM0337) and incubated at 37 ^{0}C for 24 hours. Then the inhibition of bacterial growth was recorded and interpreted as the MIC according to [10,11]. The antibacterial activities of the NPs were compared with commonly used antibiotics including ciprofloxacin and vancomycin as positive control. Tests were performed in duplicate.

Comment [WU36]: Antibacterial

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3.Results and Discussion

3.1.Characterization of AgNPs and ZnNPs suspensions

~~AgNPs and ZnNPs synthesis was found to be successful;~~ Considering the primary indication color change indication (yellow color of extract to dark grey after mixing with $AgNO_3$ solution), nano partices formed. Figure 1 A and B show that the size of the AgNPs is around 35 nm and the size of the ZnNPs is around 40 nm. Small aggregations were observed in the SEM image. The small aggregations of the AgNPs may increase the dynamic size. The ZnNPs show two absorbance peaks at 283 nm and 323 nm, which correspond to the presence of the plant extract on the ZnNPs surfaces and ZnNPs, respectively. The dynamic size of the ZnNPs was measured to be around 200 nm.

Comment [WU39]: Explain the events after this and include the chemical validation with UV-VIS spectroscopy here and SEM. You need to expound on this section to carry more scientific information. This will convince readers about the success of your research even without you mentioning it as you did before

Figure 1 A and B near to here

Nano-particulate silver showed a well-defined absorption peak in visible region at 253 and 355 nm (Fig. 2A). The interaction of AgNPs with extract of *A.vulgaris* validated the reduction of Ag^{+} ions to Ag^{0} by the reactive groups that may get in turn oxidized to other species. Similarly, nano-zinc showed a well absorption peak in visible region at 253 and 351 nm (Fig. 2B). The UV–Vis spectroscopy is generally used in different studies to examine the size and shape

Comment [WU40]: Which extract? Powered or aqueous?

of nanoparticles in aqueous suspension. Sastry et al., (1998) stated that the optical absorption spectrum of metal nanoparticles is dominated by surface plasmon resonance (SPR) [12].

Figure 2A and B near to here

The formation of the AgNPs and ZnNPs and the presence of the plant extract as capping agents on the AgNPs and ZnNPs surfaces were evaluated by FT-IR (Perkin Elmer Spectrum 400, Fig.3 A,B and C). Figure A shows organic content of *A.vulgaris* plant extract. According to results, the O-H of the alcohol ring stretching was clearly observed at ~ 3254 cm^{-1} and the stretching band at ~1311 cm^{-1} was related to the alkyl halide group and C–F. Additionally, the alkylene (-C≡C-) and alkene (C-H) stretching merged peaks appeared at ~2101 cm^{-1} and ~1629 cm^{-1}, respectively. The alkane and alkene C-H stretching merged peaks appeared at ~2917 cm-1 and ~1497 cm^{-1}, respectively. The N-H of the amide ring stretching was clearly observed at ~1606 cm^{-1}, and the stretching band at ~1321 cm^{-1} was related to the alkyl halide group and C–F. The bending vibration at ~833 cm-1 was assigned to the C–H of the carboxylic group. The N-H of the amide ring stretching was clearly observed at ~ 3208 cm^{-1} (Figure 3C) and The O-H of the alcohol ring stretching was clearly observed at ~ 3378 cm^{-1}(Figure 3B). The functional groups from plant tissues can interact with different kind of metal salts and this process determine to nanoparticles formation [13]. In this study, FT-IR results recognized the possible biomolecules are responsible for the reduction and stabilization of AgNPs and ZnNPs synthesized.

Figure 3 A, B and C near to here

X-ray Diffraction technique (BRUKER AXS D8) was used for characterization of the structures of AgNPs and ZnNPs. The AgNPs and ZnNPs precipitate was dried at 80 ^{0}C before XRD analysis to remove all the moisture on AgNPs and ZnNPs surface. The dry AgNPs and

Comment [WU41]: In the text you are using the word figure not fig. Please reconcile this and be consistent

Comment [WU42]: do

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Comment [WU44]: which biomolecules according to you. Secondly how long can it take for your nanoparticles to be stable. Did you do stability studies? How would you convince readers that this would be a better antibacterial agent when you do not mention of the stability of this nanoparticles

Comment [WU45]: And other techniques were used to do what. It does not carry much sense when you write this

ZnNPs were scanned under the range of scattering angle (20) between 10˚ to 90˚. The XRD pattern of AgNPs and ZnNPs shows that the crystallinity of AgNPs and ZnNPs is quite low and most peaks are attributed to amorphous structure of AgNPs and ZnNPs (Fig.4 A and B). The prominent diffraction peaks appeared on 15.82˚, 16.56˚ and 16.74˚ which refers to amorphous structure of AgNPs and ZnNPs. However, some weak diffraction peaks at 16.2˚ and 35.4˚ present t0 (110) and (002) lattice planes of AgNPs and ZnNPs.

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Figure 4 A and B near to here

Zeta potential was taken as the mean value of different measurements. Zeta potential on the surface of AgNPs and ZnNPs was found to be −22.5 mV and – 22 mV, respectively. thereby this can be anticipated that AgNPs and ZnNPs showed good stability in water due to the electrostatic repulsive forces (Fig.5 A and B). This stability and zeta potential were clues for an electrostatic mechanism due to adsorption of seconder metabolites. The obtained zeta potential value for the bio-syntesized AgNPs and ZnNPs prove that they are good stable. These results are agreed with obtained by Kitler et al., (2010) [14].

Comment [WU47]: Rewrite this sentence to carry scientific meaning!

Comment [WU48]: This grammar is far away to acceptance

Comment [WU49]: Which results? With this disjointed information? Mention the results you are comparing with this article.

Figure 5 A and B near to here

3.2 Antimicrobial Assay of AgNPs and ZnNPs

The silver and zinc nanoparticles demonstrated strong antibacterial activity (p=0.05) against both tested bacterial strains in the MIC assay (Table 1). However, AgNPs broadly presented a faintly higher efficacy than ZnNPs. In detail, the MIC values of ZnNPs and AgNPs detected as values of 4.25 ug/ml and 6.25 ug/ml against *S.aureus*, respectively. Also, 0.06 ug and 16.2 ug concentrations per ml were sufficient for killing of *E.coli* O157:H7 with AgNPs and ZnNPs, respectively. Figure 6 and 7 shows the effect of AgNP and ZnNPs on the growth inhibition of *S.aureus* and *E.coli*.

Comment [WU50]: Which statistical tool was this?

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Table near to here

Figure 6 A and B near to here

Similar to the present study, Bhumi and Savithramma (2014) reported that the ZnO-NPs had antimicrobial effect on *E. coli* and *S.aureus* [15]. Navale et al. (2015) detected that the MIC value of ZnO-NPs against *S.aureus* was 40 ug/ml [16]. According to study conducted by Emami-Karvani et al (2011), ZnO nanoparticles have antibacterial effect at the 10 ug/ml concentrations against *E.coli* [17]. Similar observations reported by Mostafa et al. (2015) suggested that the MIC of AgNPs against *S.aureus* and E.coli were 2.5 and 2 ug/ml, respectively [18]. However, Fayaz et al. (2010) showed that AgNPs were effective to Gram negative bacteria at 30–35 ug/ml versus were effective against Gram positive bacteria at 65–80 ug/ml concentrations [19]. Shameli et al.(2012) recorded that AgNPs were effective against *S.aureus* and *Salmonella typhi* at 20 ul of AgNPs [20].

Figure 7 A and B near to here

Different investigations showed that the sizes of zinc nanoparticles are important for antibacterial affectivity [21]. Results from this study indicated that the biological synthesized ZnNPs have improved antimicrobial activity. According to literature, ZnONPs show antibacterial effects on both Gram-positive and Gram-negative bacteria [22]. The efficient antibacterial activity of this nanoparticle is related to nanoparticle's surface area [23,24]. Yamamoto et al. (2001) stated that the antibacterial activity of ZnO increased with reducing particle size [25]. Additionally, different authors indicated that the antibacterial activity increase as the concentration of ZnO NP increased [26]. In another study, Agnihotri et al., 2014 was reported that the MIC of AgNPs at 30 ug/ml for strains of *E.coli* [27]. This value is approximately higher than the MIC determined in our study (0.06 ug/ml) .

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Comment [WU59]: Which sizes?

Comment [WU60]: Mention the MIC values to enable the understanding by the reader

Comment [WU61]: When you include only one reference here? You need more references to support this

Comment [WU62]: Can you suggest the reasons for this disparity?

According to Reddy et al., 2014, the MIC of ZnONPs was found in 40 ug/ml for *Klebsiella pneumonia* [28]. This value is also higher than the MIC determined in our study. Therefore, it can be emphasized here that our green-synthesized nanoparticles displayed better antimicrobial effect than observed in other studies. However, Zarei et al., 2014 reported that the MIC values of AgNPs changed 3.12–6.25 µg/ml ranges for *L.monocytogenes*, *E.coli* O157:H7 and *S.typhimurium* [29]. These results agree with our results. So, AgNPs and ZnNPs may be used as a substitute to commercial antibiotics. Our results are supported by the observations obtained by different author's [30, 31]. According to studies, the differences in bacteria's cell membrane structure can cause the different nanoparticle toxicity. Different studies showed that after contact with the bacterial membrane, nanoparticles generates high rate of reactive oxygen species. So, the death of bacteria results from chemical interactions between hydrogen peroxide and membrane proteins [32].

4. Conclusions

Biological synthesis using by plant extract promises eco-friendly approach for AgNPs and ZnNPs synthesis have wide applications in various domains of science and thereby life. In this study, AgNPs and ZnNPs synthesized by using *A.vulgaris* extract were tested for their antibacterial. AgNPs and ZnNPs formation was justified by simple visual detection of color change in solution and wavelength vs. absorbance spectrum generated in visible region; capping of certain compounds with functional groups on AgNPs and ZnNPs surface were determined by FTIR spectrum; morphology were studied by advance techniques like XRD and SEM. The bio-synthesized AgNPs and ZnNPs have showed good antibacterial activity against pathogenic bacteria if compared to the antimicrobics currently marketed. Thus, we concluded that the present green synthesis route may be considered further to produce antimicrobial agent useful in a wide array of biomedical and pharmaceutical applications.

Comment [WU63]: Which one?

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Comment [WU65]: This conclusion is not supported by enough information. You need to give enough comparision with including earlier reports as supported by the previous reviewers

Comment [WU66]: Not formal

Comment [WU67]: To be specific 'antibacterial'

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Comment [WU69]: Different referencing style of journals

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