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Synthesis and Characterization of Silver Nanoparticles from *Ashyranthus aspera* Extract for Antimicrobial Activity Studies

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ABSTRACT: Development of biologically inspired experimental processes for the synthesis of nanoparticles is evolving into an important branch of nanotechnology. Plant-mediated synthesis of nanomaterials has been increasingly gaining popularity due to its eco-friendly nature and cost-effectiveness. In the present study, we were synthesized silver (Ag) nanoparticles using aqueous extracts of fresh leaves of *Ashyranthus aspera* medicinal plants as bio-reducing agents. UV-Vis spectrometer used to monitor the reduction of Ag ions and the formation of AgNPs in the medium. UV-Vis spectra and visual observation showed that the color of the fresh leaf extracts of *Ashyranthus aspera* turned into grayish-brown respectively, after treatment with Ag precursors. XRD and SEM have been used to investigate the morphology of prepared AgNPs. The peaks in the XRD pattern are associated with that of the Face-Centered-Cubic (FCC) form of metallic silver. TGA/DTA results associated with weight loss and exothermic reaction due to the desorption of chemisorbed water. FTIR was performed to identify the functional groups which form a layer covering AgNPs and stabilize the AgNPs in the medium. Moreover, silver nanoparticles using aqueous leaf extracts of *Ashyranthus aspera* were separately tested for their antibacterial activity against Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Enterobacter*). The results showed that the bacterial growth was inhibited by the extracts containing AgNPs Nanoparticles. The biosynthesized nanoparticle was prepared from *Ashyranthus aspera* leaf extracts exhibits potential applications as broad-spectrum antimicrobial agents.

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Nanoparticles represent a particle with a nanometer size of 1–100 nm. The nanoscale material has new, unique, and superior physical and chemical properties compared to its bulk structure, due to an increase in the ratio of the surface area per volume of the material/particle (Cushing, *et al.,* 2004). The most widely studied nanoparticle materials are metal nanoparticles especially copper and silver because they are easier to synthesize and have diverse applications (Sasidharan *et al.,* 2020). Moreover, these materials have a wide range of applications: detectors, catalysts, surface coating agents, and antibacterial/antimicrobials, among many others. Despite the existence of numerous metals in nature, only a few of them such as gold, silver, palladium, and platinum are synthesized extensively in the nanostructured form (Yang *et al.,* 2017; Sathishkumar *et al.,* 2009; Gan *et al.,* 2018). Among the above-mentioned metals, silver nanoparticles have attracted much attention due to their unique characteristics for utilizing in the various application (Arvizo *et al.,* 2012; Bhattacharya *et al.,* 2008; Awwad, *et al.,* 2013). Conventional physical and chemical methods presently have limited use in preparing metal nanoparticles due to toxic chemicals (Singhal *et al.,* 2013). Moreover, these methods are associated with high-energy input and costly downstream processing (Anupam Roy *et al.,* 2019). Green synthesis of nanoparticles makes use of environmentally friendly, non-toxic, and safe reagents. Nanoparticles synthesized using biological techniques or green technology have diverse natures, with greater stability and appropriate dimensions since they are synthesized using a one-step procedure. Plants provide a better platform for nanoparticles synthesis as they are free from toxic chemicals as well as contain natural capping agents (Henry *et al.,* 2018). The synthesis of metal nanoparticles elicited high interest among the researchers and nanotechnologists. Efforts are made to prepare green synthesized nanoscale objects with different metals like copper, zinc, titanium, magnesium, gold, and silver. Other green synthesized metals like gold with their low-dimensional structure (Solomon *et al.,* 2007). Ag nanoparticle is an

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interesting metal to be studied, especially in the field of health and medicine. Ag is a strong antibacterial and also toxic to cells. Ag can damage bacterial cell walls, inhibits bacterial cell growth, and disrupts cell metabolism because of the interaction between Ag ions with macromolecules in cells, such as proteins and deoxyribonucleic acid (DNA). The ion Ag that interacts with the cell prevents protein synthesis, further decreases the membrane permeability, and eventually leads to cell death. The Ag nanoparticles are chemically more reactive than Ag in their bulk. Therefore, Ag nanoparticles are indicated to have stronger antibacterial capabilities (Jirovetz, *et al.,* 2003). Green synthesis methods for synthesizing nanoparticles using natural products can be used to address the problem by utilizing plants or microorganisms (Chandran *et al.,* 2006). The utilization of plants in the biosynthesis of nanoparticles involves the content of secondary metabolites as reducing agents (Ashok *et al.,* 2018). Allegedly, biological agents act as reducers, stabilizers, or both in the process of forming nanoparticles (Das *et al.,* 2013). In this context, plant-mediated NPs synthesis seems to be cost-effective as well as eco-friendly method. Moreover, NP synthesis from plants with medicinal properties proves to be beneficial in treating various ailments in a better and easy way. On such a plant is Achyranthes aspers, which is distributed as a weed throughout India, tropical Asia, and other parts of the world. Ayurvedic, Yunani practitioners, and Kabirajes use different parts of this plant to treat leprosy, asthma, fistula, piles, arthritis, wound, insect and snake bite, renal and cardiac dropsy, kidney stone, diabetes, dermatological disorders, gynecological disorders, gonorrhea, malaria, pneumonia, fever, cough, pyorrhea, dysentery, rabies, hysteria, toothache, etc. The plant is a popular folk remedy in traditional system of medicine throughout the tropical Asian and African countries. The plant is reported to be used as an antimicrobial, larvicidal, antifertility, immunostimulant, hypoglycemic, hypolipidemic, anti-inflammatory, antioxidant, diuretic, cardiac stimulant, antihypertensive, antianasacra, analgesic, antipyretic, antinoiceptive, prothyrodic, antispasmodic and hepatoprotective (Qais *et al.,* 2018). The present study synthesized Ag nanoparticles using aqueous extracts of fresh leaves of *Ashyranthus aspera* and then evaluated its antibacterial activity, particularly against the growth of Gram-positive bacteria (*Staphylococcus aureus)* and Gram-negative bacteria (*Enterobacter)*.

MATERIALS AND METHODS

Chemicals and Plant Material Collection: All the reagents purchased were of analytical grade and used without any further purification. Silver nitrate ($AgNO_3$) was purchased from Sigma-Aldrich with ≥ 99.5% purity. Fresh leaves of *Ashyranthus aspera* was collected from the local area land, North Sulawesi, Indonesia. Distilled water was used for preparing aqueous solutions all over the experiments.

Preparation of Leaf Extract: Aqueous leave extracts were prepared by the following procedure: fresh leaves of *Ashyranthus aspera* were collected and washed with tap water at first, and then the surface was washed under running water with distilled water until no impurities remained. Then, the fresh leaves were dried in room temperature in open air, shadow five days. Then 50 g of coarse powder was crushed dried plant material by using grinder and 10g was weighed and put into a beaker with 100 ml of distilled water. The mixture was heated for 20 minutes at 60°C while stirring occasionally and then allowed to cool at room temperature (Marslin *et al.,* 2018). The mixture was filtered using the Whatman no:1 filter paper. The extract was stored in the refrigerator for further use to synthesize Ag nanoparticles from the $AgNO_3$ precursor solution.

Synthesis of Ag Nanoparticles: $AgNO_3$ powder was dissolved in distilled water to prepare a 10mM $AgNO_3$ stock solution from which a series of 1 mM, 2 mM, 3 mM, 4 mM, and 5mM $AgNO_3$ solutions were prepared. The $AgNO_3$ solutions were mixed with the aqueous extract of *Ashyranthus aspera* fresh leaves at a ratio of 1:1 (v/v) to a volume of 50mL in a flask. The flask was wrapped with an aluminum foil and was then heated in a water bath at 60°C for 5 hours. After different time intervals, the color change of reaction mixture is observed from transparent yellow to dark brown indicates that the formation of AgNPs and then finally silver nanoparticles were prepared. Further analyzed by using UV - Vis spectrophotometer. Furthermore, the mixture was stored in the refrigerator for the antibacterial activity test.

Antibacterial activity of biosynthesized Ag NPs: The antibacterial activity of the biosynthesized silver nanoparticles against Gram-positive and negative bacteria species was done by agar well diffusion method. Experimented bacteria were Gram-positive bacteria (*Staphylococcus aureus,)* and Gram-negative bacteria (*Enterobacter)*. Preliminary antibacterial activity of silver nanoparticles was evaluated using the agar well diffusion assay as described earlier (Li *et al., 2*004). The bacterial test organisms were grown in the nutrient broth overnight to attain the colony-forming unit (CFU) of ~10^6 per·ml. One hundred microlitres of each bacteria culture were spread on the Muller Hinten agar plates. Agar wells (8mm) were punched

with the help of sterilized micropipette tips and loaded with AgNPs. The plates were then incubated for 24h at 37°C, and diameters of zone of inhibition were recorded in millimeter (mm).

RESULT AND DISCUSSION

UV-Visible Analysis of AgNP Synthesis: The aqueous extract of fresh leaves of *Ashyranthus aspera* change their colors when warmed. The *Ashyranthus aspera* extract changes color from yellowish-brown to brownish yellow, as shown in Fig 1. This warm extract solution changed color again after adding the $AgNO_3$ solution. Color changes are possible because some of the Ag ions begin to be reduced due to the effects of heat and produce the Ag^+ complex.

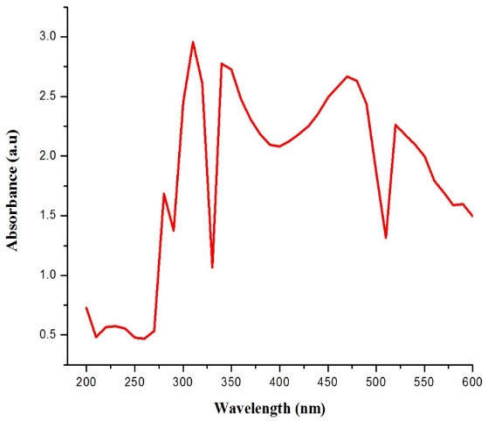

Fig 1: XRD spectrum of silver nanoparticles

This complex was responsible for changing color from brownish-yellow to grayish brown (*Ashyranthus aspera)*. This color change indicates the formation of Ag nanoparticles (Balaji *et al.,* 2009). The brownish color appears due to the coherent oscillation of conduction band electrons at the nanoparticle's surface, resulting in surface plasmon resonance (SPR) (Mandal *et al.,* 2005). UV-visible spectra of the silver nanoparticle's colloidal solution synthesized using *Ashyranthus aspera* as a function of time caused by the reduction of $AgNO_3$ are shown in Fig 1. There was no remarkable absorption peak just after the addition of the extract to silver nitrate solution, while a peak at 300–450nm started to emerge as the color of the solution changed. The SPR band at 450nm indicates the synthesis of silver nanoparticles by the *Ashyranthus aspera* extract that saturated after 4 hours.

Fourier transform infrared spectroscopy (FTIR): Fig 2 shows the sharp FT-IR spectrum of synthesized AgNPs located at about 3784.64cm^{-1}, 3094.34cm^{-1}, 1591.65cm^{-1}, 1401.33cm^{-1}, 1000cm^{-1},703.09cm^{-1} and 3200-3000cm^{-1}. In the IR spectra there is no strong absorption around 3400cm^{-1} to 1700cm-1 to show the absence of hydroxyl group. A band at 3784.64cm^{-1} may indicate N-H stretching (no band) may be 3 amines. 3094.34cm^{-1}→ C-H stretching (aromatic) 1591.65cm^{-1}→ a medium peak indicating C=H stretching. 1401.33cm^{-1} → C-H deformation (in methylene group).

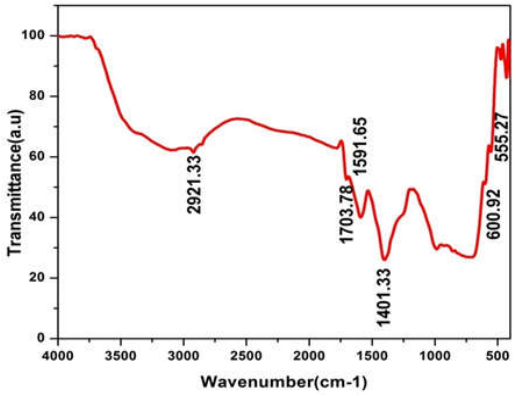

Fig 2: FTIR spectrum of silver nanoparticles

The region below 1000cm^{-1} indicates the C-O deformation and then 703.09cm^{-1} → CH_2 (rocking) 3200-3000cm^{-1}→ the absorption due to C-H stretching and AR-H stretching occurs in their region. The sharp bands of weal to medium intensities are observed. IR spectroscopic study confirmed that hydroxyl group and amines have the stronger ability to bind metal, could form a layer covering the metal nanoparticles (i.e., capping of silver nanoparticles) to prevent agglomeration and thereby stabilize the medium (Shanmuganathan *et al.,* 2018; Oves *et al.,* 2018). These results suggest that the biological molecules perform dual functions of the formation and stabilization of silver nanoparticles in the aqueous medium.

X-ray diffraction pattern (XRD): The XRD pattern of synthesized AgNPs using *Ashyranthus aspera* leaf extract was shown in Fig 3. The XRD was done to determine the crystalline nature of AgNPs and the resulted peaks were found at (2θ) 38.12, 44.30, 64.44, 64.63, 77.40 representing (111), (200), (220) and (311) face-centered cubic structure of silver which were compared with the standard powder diffraction card of Joint Committee on Powder Diffraction Standards (JCPDS), silver file No. 04–0783. Other peaks at 2θ values in Ag NPs pattern can be ascribed to the residues of the organic content of the plant extract. These peaks reveal the crystallization of some plant metabolite moieties on the surface of the Ag NPs, which is in agreement (Welch and Compton, 2006). This is a piece of acceptable evidence to confirm the involvement of the plant extract compositions in the AgNP formation. This result is following XRD analysis.

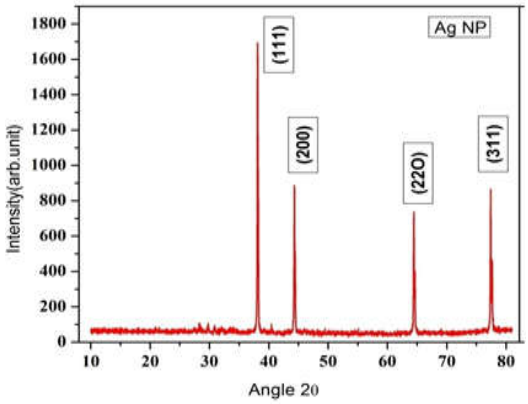

Fig 3: XRD spectrum of silver nanoparticles

Cyclic Voltammetry: Among different silver, the electrochemical properties of biosynthesized silver nanostructures were examined using cyclic voltammetry (CV). Nanoparticles were characterized by cyclic voltammetry (CV) by direct detection of nanoparticles. Moreover, the silver nanoparticle was analyzed by CV to observe the possible presence of electroactive compounds that could interfere with the analysis of nanoparticles.

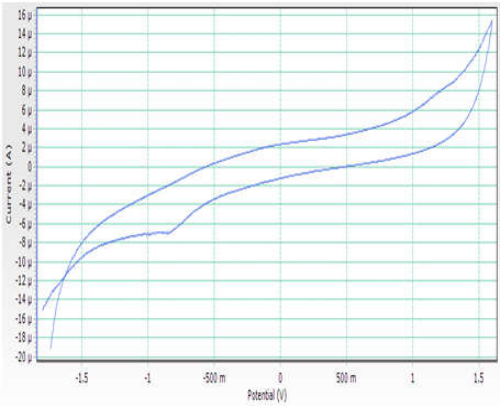

Fig 4: Cyclic Voltammetry of silver nanoparticles

Table 1: Peak Analysis

X1:	-1.029 V
Y1:	-3.473 µA
X2:	-510.359 mV
Y2:	-3.473 µA
Peak I:	-3.689 µA
Peak E:	-979.313 mV
Area:	40.409 µC
Range:	-1 V to -510.3 mV

Results showed that the leaf extract mediated synthesis AgNPs exhibit similar redox properties as solution-synthesized silver nanoparticles by modifying the Brust meth in the literature (Giovanni and Pumera, 2002; Hoda Erjaee and Saeed 2017). Fig .4 shows the *Ashyranthus aspera* extract mediated synthesis AgNPs showed the characteristic CV profile of traditional AgNPs. There is an apparent feature of electrochemical dissolution (stripping) of silver nanostructures on the electrode surface, at 200 mV, and reduction of the generated silver ions in the oxidative part of the cycle from the solution at +100 mV. As it can be seen from the voltammograms, the behavior of *Ashyranthus aspera* extract mediated synthesis nanoparticles significantly differs, depending on the concentration of the nanoparticles, with a broadening of the peak at high concentrations, indicating a probable aggregation established that the electrochemical behavior of various silver nanoparticles significantly differs, depending on the size of the nanoparticles. *Ashyranthus aspera* extract mediated synthesis silver nanoparticles showed a voltammetric profile, for potential peak values and the shape of both peaks, similar to the traditional AgNPs with size higher than 40 nm as shown in Table 1. It can be seen that the oxidation processes of an electrode start at a much higher potential and does not influence silver detection. The presented results demonstrate the applicability of an electrode as a sensor for the detection of equivalence points in the redox reaction between silver (Milardovic *et al.,* 2018).

Particle Size Analyzer: To analyze the particle size distribution in different trials Particle size analyzer was used (Microtrac, USA). The particle size of the synthesized AgNps was analyzed using a dynamic light scattering (DLS) particle size analyzer. The DLS particle size analysis was carried out using a standard analysis time, and the size of AgNps was found to vary between 2.8 to 92.1nm as shown in Table 2. The size, topography, and shape of AgNps were measured using a DLS particle size analyzer. (Kiruba *et al.,* 2012). Particle size measurement was done for all trials to choose the primary optimum size of nanoparticles. Based on the results, the mean size of AgNPs at optimum condition was recorded 43.1 nm and the range of nanoparticles was from 2.8 to 92.1 nm as shown in Fig 5. As expected, the Particle size analyzer measured size is slightly larger than the XRD size. As it has been mentioned previously, Particle sizes were similar to XRD measurements, while the particle sizes were significantly larger than X-ray Diffraction. The differences possibly reflect the fact that XRD only measures a number based size distribution of the physical size and does not include any capping agent, while Particle size analyzer measures the hydrodynamic diameter, which is the diameter of the particle, plus ions or molecules that are attached to the surface and moves with the AgNPs in solution. These

ions or other associated molecules make the particle appear larger to the instrument in comparison to XRD. Hence, the hydrodynamic diameter is always greater than the size estimated by XRD. Nevertheless, many studies proposed the importance of hydrodynamic diameter for understanding and optimizing the size of nanoparticles and their performance in biological assays (Anandalakshmi *et al.,* 2006).

Fig 5: Particle size analysis of silver nanoparticles

Table 2: a) Distribution Results

Peak	Diameter (nm)	Std. Dev
1	2.8	0.9
2	92.1	75.7
3	0	0
4	0	0
5	0	0
Average	85.1	76.5
Residual	3.244e-003	(O.K)

b) Cumulants Results

Diameter (d)	43.1 (nm)
Polydispersity Index (P.I.)	0.430
Diffusion Const. (D)	1.141e-007 (cm²/sec)

Fluorescence Spectroscopy: The FL of the synthesized bio-inspired AgNPs by *Ashyranthus aspera* leaf extract is also studied via fluorescence emission spectroscopy. Fluorescence (FL) spectrum is one of the methods to estimate the optical property of silver nanoparticles as photonic materials. The colloidal silver nanoparticles are dispersed in water and the FL emission spectra are recorded for the excitation wavelength at 647 nm.

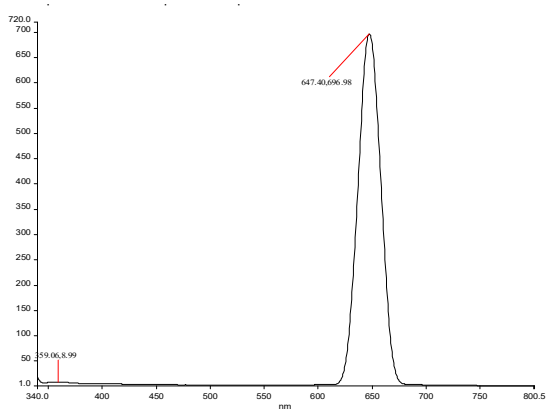

Fig 6: Fluorescence spectroscopy of silver nanoparticles

A broad emission is obtained at 698 nm. The intensity of the fluorescence emission peak is gradually increased up to 359 nm, after which it is slowly decreased up to 700nm. The present peak is redshifted (Ratan, *et al.,* 2004). Linoleic acid during the formation of silver nanoparticles further enhances the intensity of emission. Fluorescence spectra for silver nanoparticles are shown in Fig 6. These spectra infer the possibility of the silver nanoparticle to be used as a 'nanolaser' with optical pumping (Skandalis *et al.,* 2017).

Antibacterial activity: Table 2 shows that the antibacterial activity against *Staphylococcus aureus, Enterobacter* was increased. Fig 7. shown in *Staphylococcus aureus* was indicated by an increase in the inhibit concentration of silver nanoparticle using 25µl, 50 µl, 75 µl, 100 µl and then the zone diameter from this concentration 11mm, 11.2 mm, 11.4 mm,11.8 mm with the increasing Ag concentration in *Ashyranthus aspera* extract as shown in Table 3.

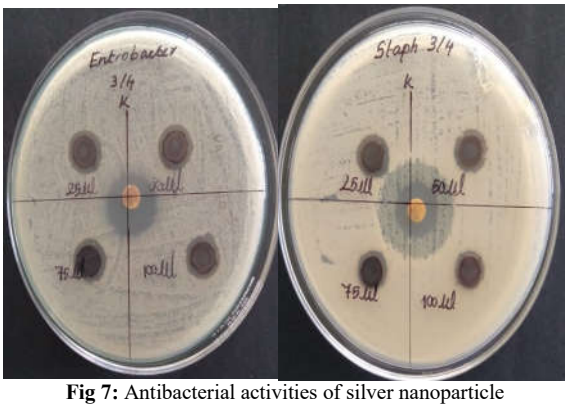

Fig 7: Antibacterial activities of silver nanoparticle

However, the opposite result was shown by *Enterobacter*, which was indicated by an increase in the inhibit concentration of silver nanoparticle using 25µl, 50 µl, 75 µl, 100 µl and then the zone diameter from this concentration 11mm, 12 mm, 12 mm,13 mm with the increasing Ag concentration in *Ashyranthus aspera* extract. Ciprofloxacin positive control showed similar inhibition zone diameter, which averages above 16 mm, for the two bacteria with the increasing Ag concentration in leaves extracts of the plants. This information was supported by data that the average size of Ag nanoparticles synthesized using *Ashyranthus aspera* extract was relatively smaller than that using the extract of *Ashyranthus aspera.* The results of this study were also supported by previous studies that the small size of Ag nanoparticles makes these particles easier to penetrate the outer wall of bacteria, enter the body, destroy the respiratory chain, and thus inhibit cell respiration, causing bacterial death (Franci and Falanga Galdiero, 2015). Regarding the inhibition zone, the antibacterial activity of Ag nanoparticles synthesized in this study was categorized into strong inhibitory activity (inhibition zone of 10–19 mm) according to Davis and Stout (Skandalis *et al.,* 2017).

Table 3: Antibacterial activity of silver nanoparticles

Bacteria	25ul	50ul	75ul	100ul	Control
Staphylococcus	11	11.2	11.6	11.8	15
Enterobacter	11	12	12	13	17

Conclusion: AgNPs began to form within 10min and higher formation yield at 70 min after the addition of leaf extract to silver nitrate as shown by the UV-vis spectrum at 450 nm. It was found that the formation of AgNPs was increased with time. The XRD peaks ascribed with the FCC structure of silver. The FT-IR spectrum ascribed the biological molecules which perform dual functions of formation and stabilization of silver nanoparticles in the aqueous medium. The biosynthesized AgNPs were found to have a pronounced antibacterial activity against *Staphylococcus aureus, Enterobacter*.

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