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Antibacterial activity on methicillin-resistant *Staphylococcus aureus* (MRSA) and antioxidant properties of silver nanoparticles synthesized by *Hunteria umbellata* (K. Schum.) seeds

Fidelis Ifeakachukwu Okolafor[1]✉, Martyna Chinedu Uba[1], Onoche Vera Okolafor[2] and Salem Kivos Adebiyi[3]

[1]Department of Science Laboratory Technology, Faculty of Life Sciences, University of Benin, Benin City 3002, Nigeria; [2]Edo State College of Agriculture and Natural Resources, Iguoriaki, Edo State Nigeria; [3]University of Central Lancashire, UK
✉Corresponding author, E-mail: fidelis.okolafor@uniben.edu

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Abstract. Silver nanoparticles (AgNPs) have gained significant attention over the years due to their unique physicochemical properties and diverse applications in various areas including medicine, electronics, and so on. *Hunteria umbellata* (*HU*), is a glabrous tree native to West Africa that belongs to the Apocynaceae family with various medicinal properties. The medicinal applications; anti-microbial and anti-oxidant properties of synthesized and characterized silver nanoparticles by using *HU* seeds, were studied. Aqueous and ethanol extracts of *HU* were obtained for the synthesis process. UV-Vis spectra analysis was used to reveal the distinct absorption patterns for AgNPs synthesized by ethanol and aqueous extracts. FTIR spectra exhibited characteristic transmittance peaks, indicating the presence of functional groups such as hydroxyl groups. XRD analysis confirmed the crystalline nature of AgNPs with identifiable peaks at specific planes while SEM/EDX was used to provide insights into the size distribution and morphology of AgNPs, reinforcing the data from other characterization methods. Medicinal properties were assessed through antibacterial assays using Methicillin-Resistant *Staphylococcus aureus* (MRSA) and DPPH radical scavenging activity, showcasing the potential

biomedical applications of AgNPs-*HU* complex. Comparative studies with standard compounds like ascorbic acid validated their efficacy as antioxidants. The findings of this study suggest promising antibacterial and antioxidant properties of AgNPs-*HU* complex synthesized by *HU* seeds extracts and also contribute to the understanding of nanomedicine and underscore the potential of green synthesis for biomedical applications.

Keywords: *characterization, Hunteria umbellata,* plant extracts, silver nanoparticles, synthesis.

Introduction

Over the years, from the 1960s up until today Methicillin-resistant *staphylococcu aureus* (MRSA) has continued to be disseminated globally from human to human among healthcare workers, communities, and individuals. The burden of MRSA dissemination can be attributed to geographical variation with differences that may occur from local infection practices, management of the disease, awareness of the infection, and circulating clones. The epidemiology of MRSA has seen its ravaging effect in some parts of Europe where <5% of *S. aureus* isolates from invasive cases were reported to be MRSA (Köck *et al*., 2010; Tacconelli *et al*., 2018); report in America stated that 50% of all skin and tissue infections are clinical isolates of MRSA (Styers, 2006). MRSA is predominantly endemic in Asia with over 50% of *S. aureus* traced to bloodstream infections (Mendes *et al*., 2011; Chen & Huang, 2014). Nigeria and South Africa are the leading countries with the highest MRSA figures in Africa with over <25% (Falagas *et al*., 2013) prevalence rate, owing to the incidence of HIV infection, lack of infection control practices, and availability/use of antibiotics. In Nigeria for example, MRSA is predominantly identified at the hospital laboratory by chance or cases of patients repeated treatment with antibiotics with little or no effect. Over the years, clinicians have repeatedly used combination therapy of vancomycin with β-Lactams as an alternative treatment regime for MRSA (Bal *et al*., 2017; Purrello *et al*., 2016), however, hospital cases have shown that these combination therapies may no longer be effect again to combat MRSA today because of the clinical manifestation, virulence factors and *S. aureus* new clones (staphylococcal cassette chromosome mec- SCCmec), hence the need to search for alternative treatment regimen to combat MRSA. Andrade *et al*. (2023) suggested the use of targeted Nanoparticles to fight MRSA, however, he concluded that these nanoparticles could be used via antibiotic-independent

activity and/or serve as drug delivery systems (DDSs). We believe that sylva nanoparticles (AgNPs) may be explored in the nanofabrication of antibacterial substances that can be used to combat MRSA. The use of green synthesis particularly plant-based synthesis was preferred for this study because of safety consigns and wide acceptability. *Hunteria umbellata* (*HU*) on the other hand has shown relatively reducing potential and medicinal applications which necessitated its use for this study.

A variety of techniques are combined under the umbrella of nanotechnology to work at the nanoscale to design, create, characterize, and apply materials, systems, devices, and structures (Hornyak et al., 2018). This scale usually applies to sizes less than 100 nanometers. AgNPs have garnered attention across various sectors like medicine, food, and healthcare due to their exceptional physical and chemical attributes, such as high electrical conductivity, and biological, thermal, and chemical properties (Galatage *et al.*, 2021). These distinctive traits make AgNPs suitable for applications such as antimicrobial agents, biosensor materials, and cosmetic products (Bamal *et al.*, 2021). In recent times, researchers have focused on AgNPs due to their effectiveness against a wide range of microorganisms and the emergence of drug-resistant strains against conventional antibiotics (Prasher *et al.*, 2018; Mateo & Jiménez, 2022; More *et al.*, 2023;). The synthesis of AgNPs falls into three main categories; physical methods, chemical methods, and biological methods. Physical methods involve techniques like evaporation-condensation using a tube furnace under atmospheric pressure (Bouafia *et al.*, 2021; Nguyen *et al.*, 2023). In the process of preparing AgNPs, chemical methods use organic solvents or water, whereas biological methods also referred to as "green synthesis" rely on non-toxic substances to function as reducing agents and solvents. (Kanwar *et al.*, 2021; Patel *et al.*, 2023).

Characterization is essential after AgNPs are synthesized because their physicochemical characteristics have a big impact on how they behave biologically. A comprehensive characterization of prepared nanoparticles is necessary for their efficient use in nanomedicine, healthcare, or other applications related to human welfare. Various analytical techniques such as ultraviolet spectroscopy (UV-vis spectroscopy), X-ray diffractometry (XRD), Fourier Transform Infrared Spectroscopy (FTIR), transmission electron microscopy (TEM), scanning electron microscope (SEM), Dynamic Light Scattering (DLS) analysis, energy-dispersive X-ray spectroscopy (EDS), among others, are employed to evaluate synthesized nanomaterial (Catalano *et al.*, 2021; Selvan *et al.*, 2021; Ullah *et al.*, 2024).

H. umbellata (HU), a glabrous tree native to West Africa, is characterized by broad leaves, creamy to pale yellow flowers, and yellow smooth fruits, commonly known as Erinor abeere (Yoruba) Osu (Edo), Nkpokiri (Igbo), this plant

belongs to the Apocynaceae family (Okolafor & Ekhaıse, 2021). Historically, *HU* has been used in traditional medicine to treat various ailments such as malaria, diarrhea, diabetes mellitus, gastric ulcers, and skin conditions. Recent studies have highlighted its antimicrobial (Aderele *et al.*, 2020), anti-inflammatory (Ahajumobi & Anderson, 2022), anti-diabetic (Aderele *et al.*, 2020; Okolafor & Ekhaıse, 2021) and antioxidant properties (Edosuyi *et al.*, 2018; Oboh *et al.*, 2018; Ogunlana *et al.*, 2021), particularly in extracts from its leaves, bark, fruit, pulp and seeds. A report on the synthesis of AgNPs by *HU* seeds and the application of the synthesized NPs as antibacterial and antioxidant properties is lacking in the literature which necessitated this study. This study focuses on the use of aqueous and ethanol seed extracts of *HU* as a biological precursor for the synthesis of AgNPs, and its antibacterial effect on Methicillin-Resistant *Staphylococcus aureus* (MRSA) and antioxidant properties.

Materials and methods

Sample collection and preparation

Dried seeds of *HU* were purchased at the Uwa Market, Benin City, Nigeria, in January 2024. The epicarps of the seeds were removed and air-dried to obtain constant weight. The dried seeds of *HU* were pounded using a mortar and pestle, it was then ground using a manual grinder. Subsequently, the seeds were further pulverized into a powdered form using a electronic blender (Model; KCB239K), and the pulverized seeds were stored in an airtight container.

Extraction of plant materials

Ethanolic extraction. Pulverized dried *HU* seeds (200g) were introduced into a thimble of soxhlet extractors (Model; KEX 250 (F) B00232348) and ethanol was added to a round bottom flask which was placed on the heating mantle of the soxhlet extractor, with a small amount of ethanol poured into the soxhlet extractor column to wet the thimble. The extraction process was monitored for 48 hours until completion. After the extraction, the extracted solvent was concentrated using a rotary evaporator (Model; DUAB RE100-Pro). The extracts obtained from the previous soxhlet extraction phase were transferred into round bottom flasks. Glycerol was applied to the tip of the round flat bottom flask for ease of removal. The rotary evaporator settings were adjusted to 86°C and 276 rpm, and the concentration of samples were monitored for 14 hours. The resultant crude extracts were then stored in cream jars and subjected to freeze-drying to -55°C (Model; BK-FD189). The recovered ethanol was carefully preserved in separate Winchester bottles for future use.

Aqueous extraction. Pulverized *HU* seeds (100g); were measured into a beaker and 800ml distilled water was poured and stirred using a glass stirring rod. The mixture was stirred and heated on a hot plate (Model; Heat-stir-SD162) continuously at 70°C for 3 hrs and was allowed to cool. After cooling, the mixture underwent filtration using a Teflon cloth to remove solid residues. The filtrate was then transferred into a separating funnel setup. The supernatant was subjected to further drying on a hot plate until it attained a paste-like consistency, which was then transferred to cream jars and subjected to freeze-drying for preservation (Okolafor & Ekhaıse, 2021).

Synthesis of Ag nanoparticles

Silver nitrate ($AgNO_3$) solution (1 mM) was prepared by dissolving 0.01697g silver nitrate powder in 1000 ml distilled water. One gramme (1g) of aqueous and methanol extracts of *HU* seed was carefully weighed and dissolved in 5 milliliters of Tris-HCl buffer (Tris hydroxymethyl aminomethane) and the pH was adjusted to 7.0. The prepared $AgNO_3$ solution was mixed with the dissolved *HU* extracts (1:1), and vortexed using a vortex mixer (PV-1 Grant-bio). The mixture was allowed to incubate at room temperature for 24 to 48 hrs. After the synthesis, the mixture was centrifuged using a high-speed centrifuge at 15000 rpm for 10 minutes. The supernatant cells were harvested and used for further characterization and applications.

Preparation of samples for characterization

The supernatant cells harvested from the synthesis were repeatedly washed with Tris-HCl buffer (pH 7.0) and distilled water. The purified harvest cells were smeared on a microscopic slide by emulsification using sterile deionized water. The smear was allowed to air dry on the slide and heat was fixed by passing through flame repeatedly. The heat-fixed slide was used for FTIR, XRD, and SEM characterization.

Characterization of $AgNO_3$ nanoparticles

One milliliter aliquot of the synthesis was collected daily for the UV-Vis characterization, while Trs-HCl (Ph 7.0) buffer was used as the blank. UV-Vis spectroscopy (UV-6300PC double beam spectrometer) studies were performed for analysis of reflectance or absorbance of the sample at different wavelengths (250 to 850 nm). Fourier transform infrared spectroscopy (FTIR Thermo scientific Nicolet iS5) studies were used for the analysis of chemical bonding and functional groups. X-ray diffraction (XRD, Shimadzu XDS 2400H diffractometer with Cu anode control, 40 KV, 30 M.A, optics: Automatic divergence slit), in Bragg-

Brentano configuration, using Cu-Kα radiation (1.54Å) was used for the identification of its crystalline structure and morphology. The XRD pattern was recorded in the 2θ range between 0° to 60° at a scan speed of 0.017°/14s. The particles were coated in gold and were then analyzed using scanning electron microscopy (Hitachi SU 3500 scanning microscope, Tokyo, Japan).

Antimicrobial properties

The antimicrobial properties of AgNPs-*HU* complex synthesized using aqueous and methanol extracts of *HU* were tested against clinical strains of Methicillin-Resistant *Staphylococcus aureus* (MRSA) obtained from the University of Benin Teaching Hospital (UBTH). The antimicrobial properties of the AgNPs were tested using a microplate broth dilution method described by López-Malo *et al.* (2020). The MRSA was standardized to 0.5 McFarland and amoxicillin tablet was used as the control drug. Different concentrations (78.44, 156.9, 313.8, and 627.5, mg/L) of the synthesized AgNPs were introduced into a peptone broth containing standardized MRSA incubated in a microplate at 37^0C under shaking condition for 24 hr. The bacteria inhibition by AgNPs was calculated using the following formula:

$$\%\, Bacteria\ inhibition = \frac{Absorbance\ of\ control\ drug - Absorbance\ of\ sample\ (AgNPs)}{Absorbance\ of\ control\ drug} \times \frac{100}{1} \quad (1)$$

To determine the half-maximal effective concentration (EC_{50}) of AgNPs synthesized by aqueous and methanol extracts of *HU* as an antibacterial agent, a non linear curve fitting was computed on Origin Pro 9.0 using the following derivative formula:

$$y = A1 + \frac{A2 - A1}{1 + 10^{(Logx0 - x)P}} \quad (2)$$

Where the X values are supposed to be the logarithm of the dose and LOGx0 is the center of the curve, that is, the concentration for the half response. So we can compute the EC_{50} by:

$$EC_{50} = 10^{Logx0} \quad (3)$$

Antioxidant properties

The antioxidant properties of AgNP were determined by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging method (Sharma & Kumar, 2011). DPPH power (0.01 g) was dissolved in 50 ml methanol to give 100 µg/ml stock concentration. From the stock concentration, 80, 60, 40, and 20 µg/ml concentrations were

diluted while methanol served as the blank. The prepared 1g AgNPs were mixed with the varied concentration of DPPH preparation and allowed to incubate for 15 minutes. Ascorbic acid was used as the control antioxidant. The antioxidant properties of AgNPs were determined using the formula:

$$\%\ inhibition = \frac{Absorbance\ of\ control - Absorbance\ of\ test\ sample}{Absorbance\ of\ control} \times \frac{100}{1} \quad (4)$$

To determine the half-maximal effective concentration (EC_{50}) of AgNPs synthesized by aqueous and methanol extracts of *HU* as an antioxidant, a non-linear curve fitting was also computed on Origin Pro 9.0 using the derivative formula in equations 2 and 3 above.

Results

UV-Vis Spectroscopy

UV-Vis spectroscopy is one non-invasive and fast real-time method available to monitor and obtain a reliable measurement of nanoscale-related properties. The UV-VIS spectroscopy results (Figure 1) of AgNPs revealed high peaks at 450 and 500 nm at 72/24 hrs for ethanol and aqueous extracts of *HU* respectively.

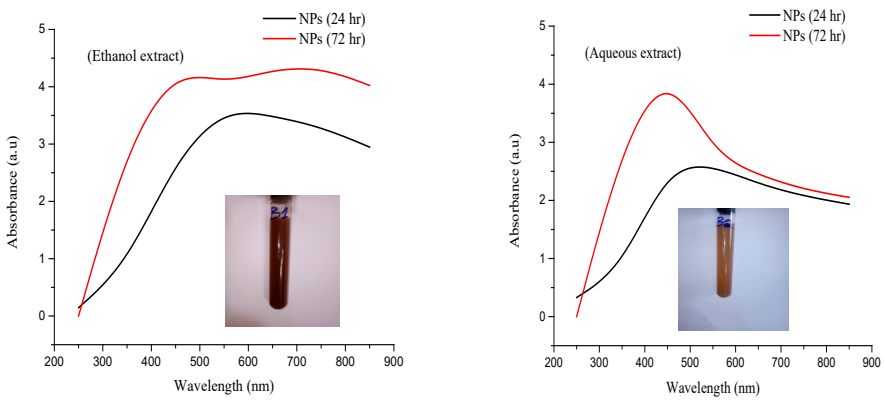

Figure 1. UV-VIS spectra of AgNPs synthesized by ethanol and aqueous extracts of *HU*.

Fourier transform infrared spectroscopy (FTIR)

The FTIR analysis provided insights into the types of molecules and the functional groups present on the surface of AgNPs synthesized. Figure 2 shows variations in light transmission at different wavelengths, indicating the involvement of various molecules in the synthesis process. High % transmission at specific wavelengths suggests the presence of molecules that might contribute to the stability and potential medicinal activity of the AgNPs synthesized by *HU*.

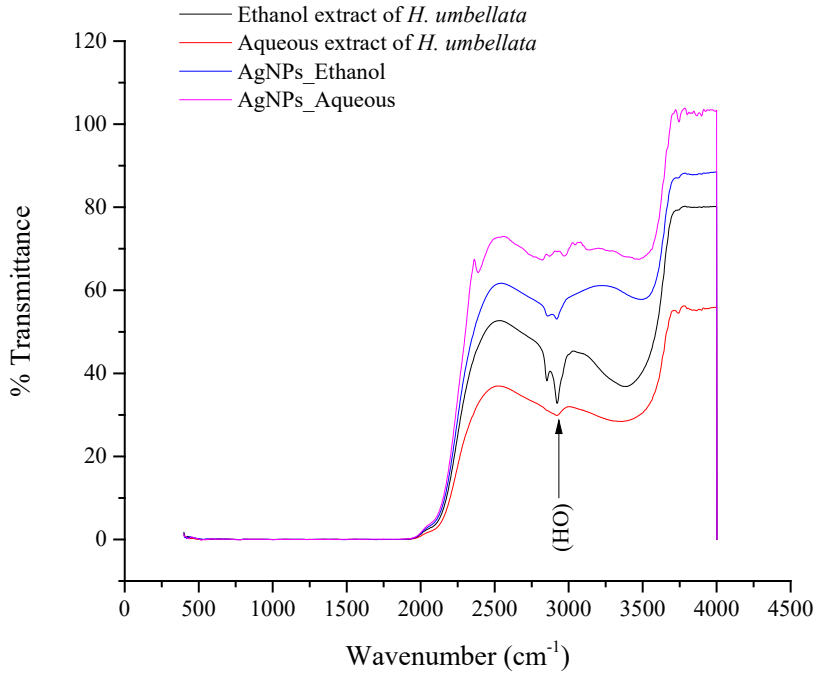

Figure 2. FTIR spectra of AgNPs synthesized by ethanol and aqueous extracts of *HU*.

X-ray diffraction spectroscopy

The X-ray diffraction (XRD) analysis has shorter electromagnetic wavelength radiation that can be used for the determination of the degree of crystallinity, and deviation from a compound of interest. Figure 3 shows the crystallinity of AgNPs by aqueous and ethanol extracts of *HU* which was confirmed by comparing with the standard Joint Committee on Powder Diffraction Standards

(JCPDS) card number 00-004-0783. The peaks at 2θ with the corresponding Miller indices (hkl) in parenthesis for AgNPs by ethanol and aqueous extracts of *HU* were 24.12 (111), 26.1 (200), 32.5 (220), 31.4 (311), 32.4 (222), 45.0 (400) and 32.3 (111), 35.0 (220), 45.0 (222), 51.4 (400) respectively.

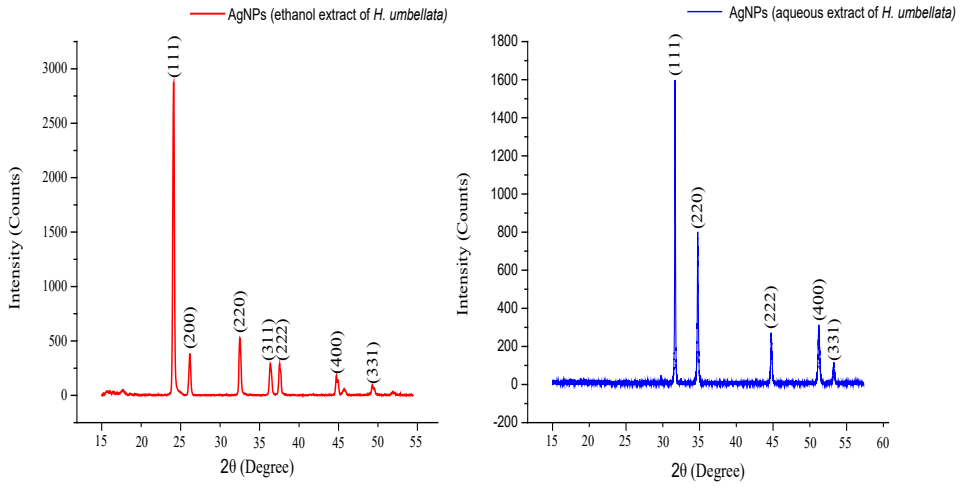

Figure 3. XRD characterization of AgNPs synthesized by ethanol and aqueous extracts of *HU*

Scanning Electron microscopy (SEM)

Scanning electron microscopy (SEM) analysis revealed information about the size distribution and shapes of the AgNPs. Figures 4 & 5 illustrate the surface features of nanoparticles synthesized by ethanol and aqueous extracts of *HU*, complementing the data obtained from other characterization techniques. The EDX characterization confirmed the crystalline and elemental composition of the AgNPs, however, other elements such as O, Na, Al, P, Ca, Cl, Cu, and Fe were captured in trace amounts after the synthesis, whereas Ag was the most prominent element.

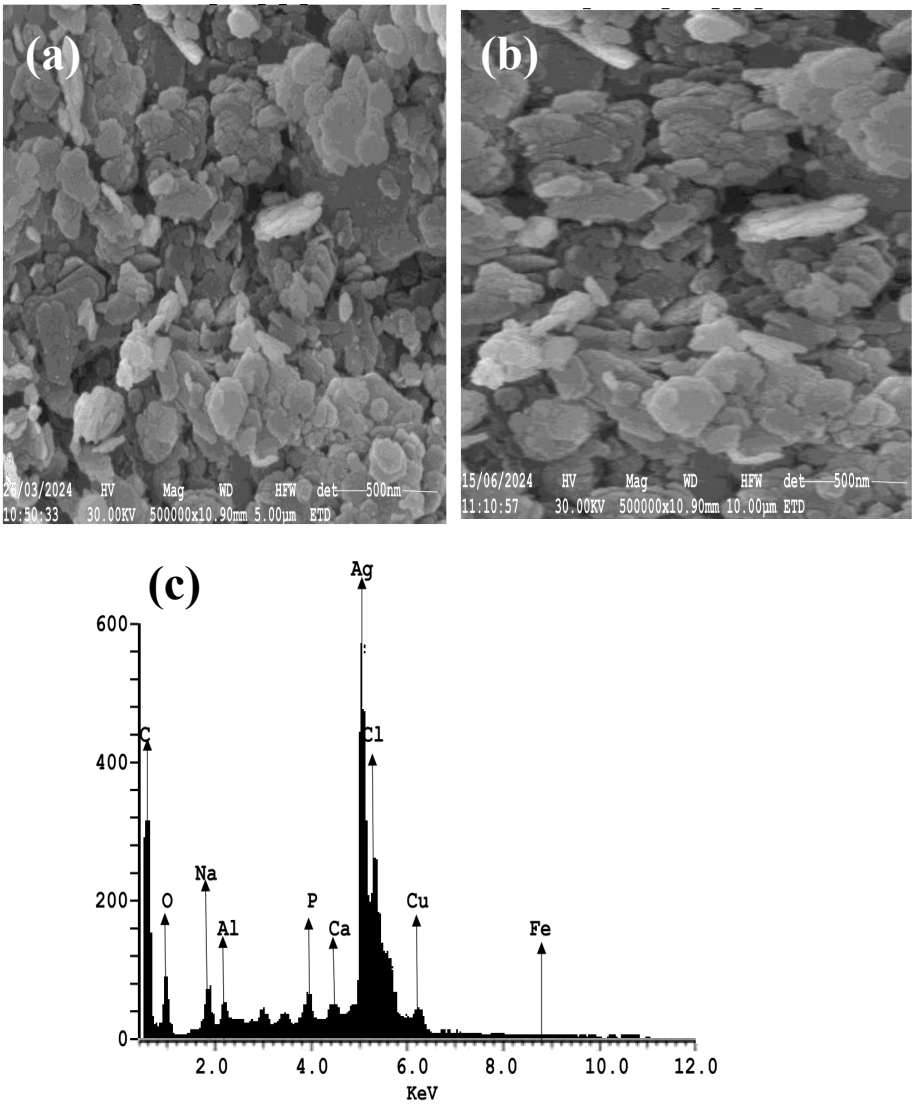

Figure 4. SEM/EDX characterization of AgNPs synthesized by ethanol extracts of *HU* (a and b: SEM angles of capture; c: EDX)

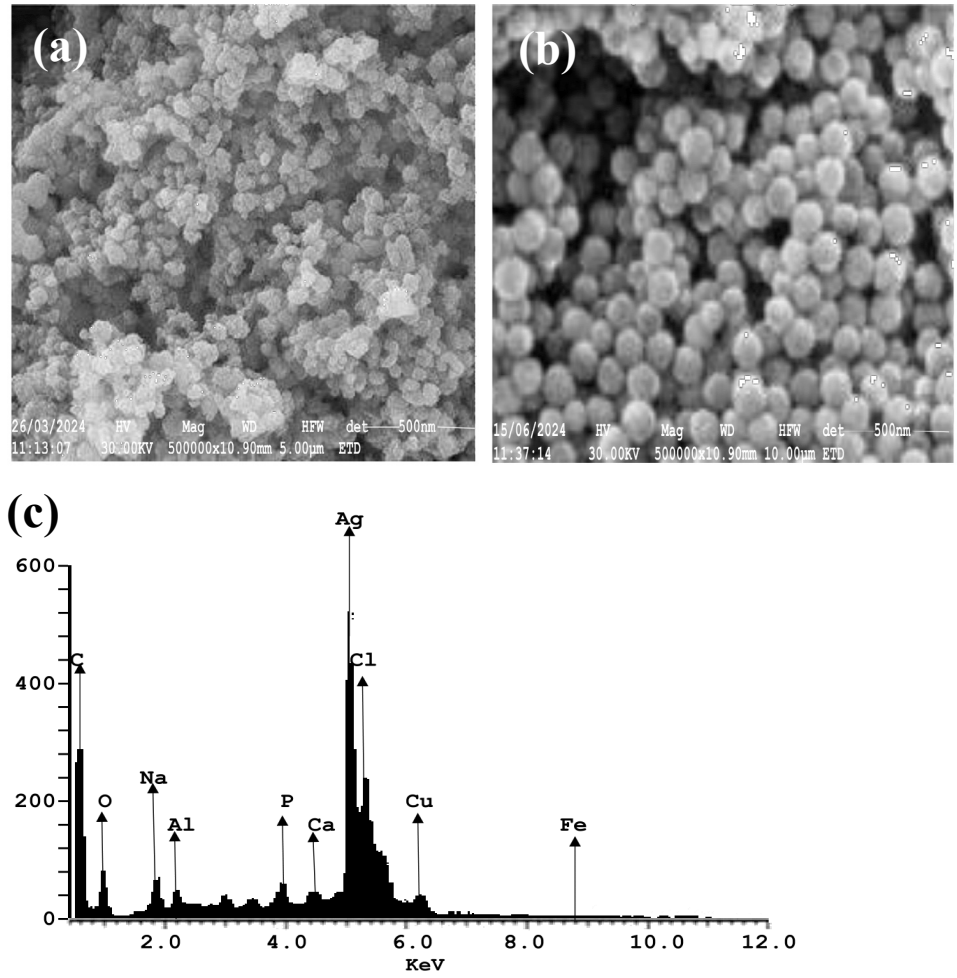

Figure 5. SEM/EDX characterization of AgNPs synthesized by aqueous extracts of *HU* (a and b: SEM angles of capture; c: EDX)

DPPH free radical scavenging activities of AgNPs-HU complex

The DPPH activity of AgNPs-*HU* complex synthesized by ethanol and aqueous extracts of *HU* showed radical scavenging activities compared to the standard (ascorbic acid). The result in Figure 6 is the linear regression equation comparing the DPPH activity at 20 to 100 µ/ml concentration. Table 1 and

Figure 7 show the ANOVA summary Table to compare the radical scavenging activity of AgNPs synthesized by ethanol and aqueous extracts of *HU*/standard and Half-maximal inhibitory concentration of radical scavenging activity of AgNPs-*HU* complex synthesized by *HU* extracts respectively.

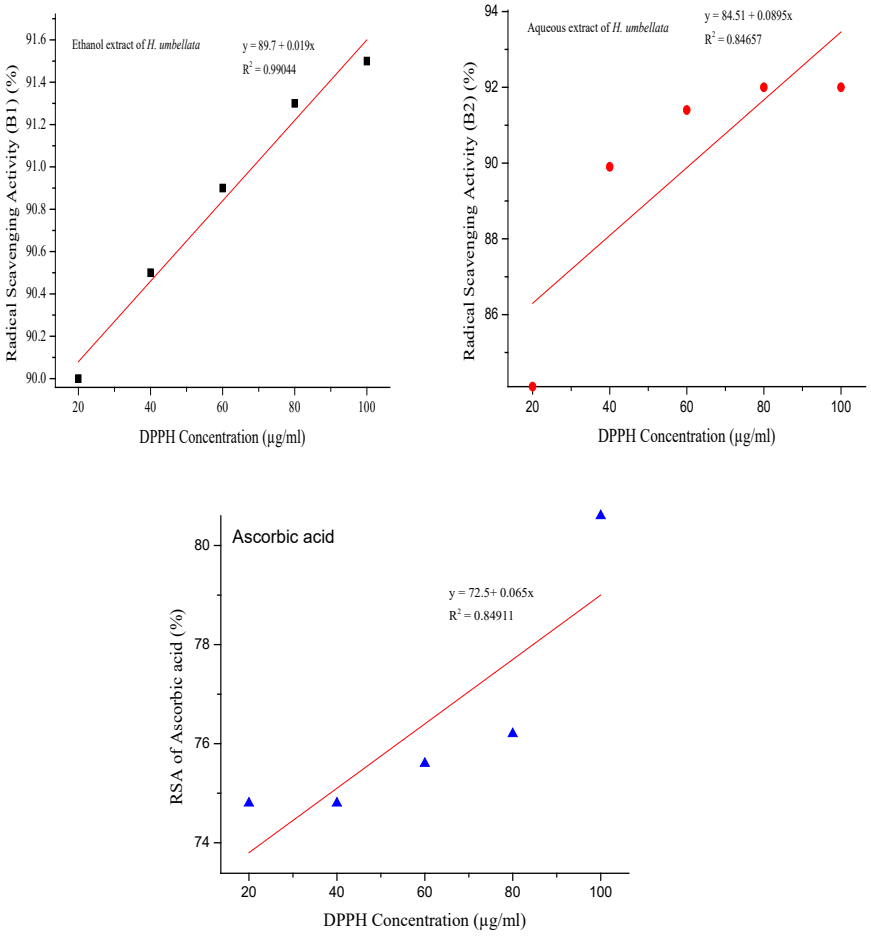

Figure 6. Radical scavenging activity of AgNPs synthesized by ethanol and aqueous extracts of *HU* and standard (ascorbic acid).

Table 1: ANOVA summary Table to compare the radical scavenging activity of AgNPs synthesized by ethanol and aqueous extracts of *HU* and standard (Ascorbic acid)

Sample		DF	Sum of squares	Mean square	F Value	Prob>F
AgNPs by ethanol extract of *H. umbellata*	Model	1	1.444	1.444	154.7	0.001
	Error	3	0.028	0.009		
	Total	4	1.472			
AgNPs by aqueous extract of *H. umbellata*	Model	1	32.04	32.04	7.588	0.07
	Error	3	12.67	4.222		
	Total	4	44.71			
RSA of Ascorbic acid	Model	1	16.9	16.9	7.752	0.069
	Error	3	6.54	2.18		
	Total	4	23.44			

Significant at $p<0.05$

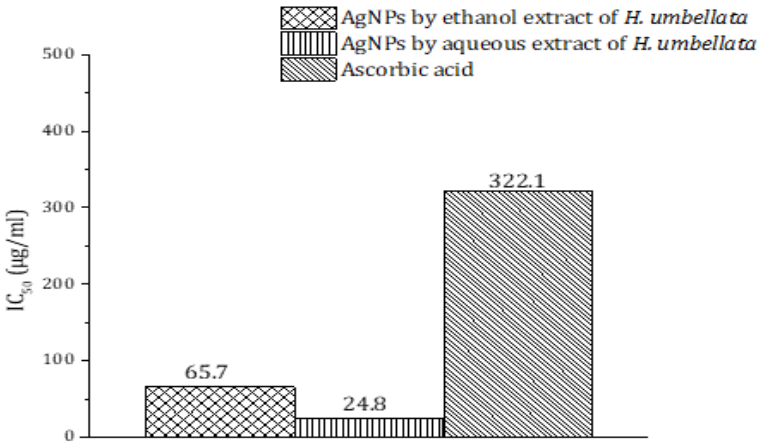

Figure 7. The half-maximal inhibitory concentration of Radical scavenging activity of AgNPs synthesized by ethanol and aqueous (B2) extracts of *HU* and standard (ascorbic acid).

Antimicrobial application

The result of the antimicrobial inhibition of MRSA by AgNPs-*HU* complex synthesized by ethanol and aqueous extracts of *HU* is presented in Figure 8 below. The result indicated that aqueous extracts of AgNPs-*HU* complex had a

percentage bacteria inhibition above 60% at a concentration of 800 mg/L whereas ethanol/aqueous extracts of HU-AgNPs complex at a concentration of 200 to 600 mg/L showed bacteria inhibition below 40% threshold. The result of Half maximal effective concentration of AgNPs-*HU* complex synthesized by ethanol and aqueous of *HU* against Methicillin-Resistant *Staphylococcus aureus* (MRSA) is also presented in Figure 9.

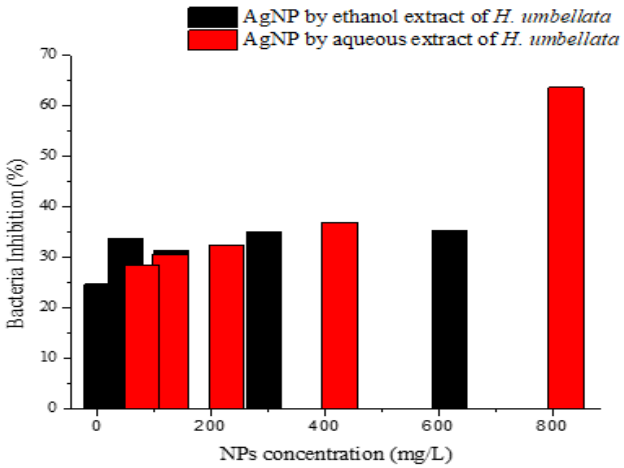

Figure 8. Antimicrobial inhibition of Methicillin-resistant *Staphylococcus aureus* (MRSA) by Ag NPs synthesized from ethanol (B1) and aqueous (B2) extracts of *HU.*

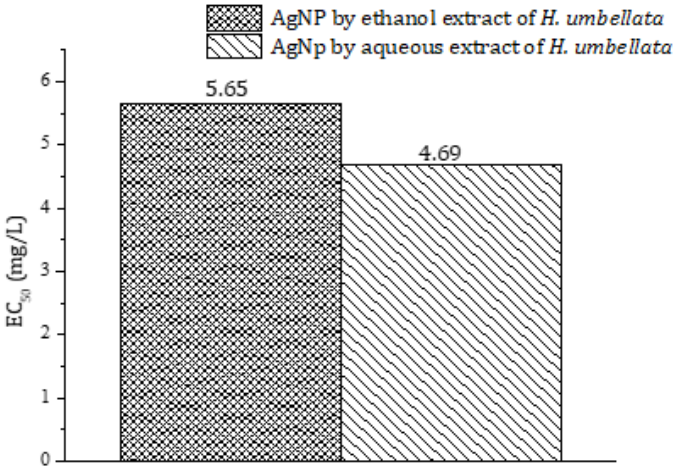

Figure 9. The half-maximal effective concentration of AgNPs synthesized by ethanol and aqueous (B2) extracts of *HU* against Methicillin-Resistant *Staphylococcus aureus* (MRSA).

Discussion

The UV-Vis characterization slightly agreed with the report of Asif *et al.* (2022) who averred that green synthesized AgNPs formed intense surface plasmon resonance (SPR) peaks at 370 – 470 nm. The SPR is a function of the morphology, shape, size, and chemical composition of AgNPs in a liquid medium. These absorption values at wavelength 370 to 470 showed how efficiently the nanoparticles were produced and how stable they were over time. The UV-Vis result revealed that ethanol extract was more stable at 24/72 hrs compared to aqueous extract. The functional groups present in the AgNPs by *HU* were -OH-stretch. A strong absorption IR band between 3200 to 3600 is regarded as OH-stretching indicating that the compound is an alcohol group (Liu, 2021). The strong OH stretching bands may indicate the presence of phenols and flavonoids (Sharma *et al.*, 2020) present in the ethanol and aqueous extracts of *HU*.

The specific crystal planes, signifying the formation of well-defined nanoparticles. Although there were no reports on the XRD characterization of AgNPs by *HU,* however the hkl crystalline planes for *Olea europaea* leaf extract (Sharma *et al.*, 2020), *Annona squamosa* leaf extract (Vivek *et al.*, 2012), *Salvia verticillata* and *Filipendula ulmaria* extracts (Mihailović *et al.*, 2023) were similar with that of *HU* in this study. It is therefore safe to state that green synthesis of AgNPs using plant extracts under XRD characterization forms similar corresponding Miller indices (hkl). The SEM micrograph of AgNPs synthesized by ethanol extracts of *HU* formed an irregular shape distribution compared to AgNPs synthesized by aqueous extracts of *HU* where the shape distribution was well defined. The shape morphology of the AgNPs by ethanol extract of *HU* may be attributed to the alcohol used for the extraction of the plant. The SEM micrograph revealed that AgNPs synthesized by aqueous extracts of *HU* are more stable however, formed agglomeration owing to the weak force binding them together. The strong EDX signal at 5.0 Kev recorded by the AgNPs synthesized by *HU* depicts strong surface plasmon resonance (SPR). SPR is an electronic technique used to monitor responses from cellular activities (Nguyen *et al*., 2015).

This study revealed near-perfect linear regression line for ethanol extracts (R^2=0.99044) and ascorbic acid (R^2= 0.84911), while AgNPs synthesized by aqueous extract of *HU* recorded a scatter graph (R^2=0.84657). The R^2 values equal to or close to 1 are regarded as a perfect linear regression relationship. The result of the comparison of the scavenging activity of AgNPs-*HU* complex affirmed that DPPH activity of AgNPs-*HU* complex synthesized by ethanol extract of *HU* ($p<0.001$) had a better scavenging activity compared to ascorbic acid ($p<0.069$) and AgNPs-*HU* complex synthesized by aqueous extract of *HU* ($p<0.07$). The IC_{50} values were lowest for AgNPs synthesized by ethanol

(IC_{50} = 65.7) and aqueous (IC_{50} = 24.5) extracts of *HU* compared to ascorbic acid (IC_{50} = 322.1). The report by Adeneye *et al.* (2011) and Abubakar *et al.* (2019) on the antioxidant properties of *HU* recorded IC_{50} values > 150 compared to the combined antioxidant properties of AgNPs-*HU* complex. We can confidently infer that the antioxidant activities of AgNPs-*HU* complex synthesized by ethanol and aqueous extracts of *HU* will be far better than the antioxidant activities of *HU* extracts alone.

The antibacterial properties of AgNPs synthesized by ethanol and aqueous extracts of *HU* against Methicillin-Resistant *Staphylococcus aureus* (MRSA) showed the same level of bacteria inhibition (<35% inhibition) for ethanol and aqueous extracts of *HU*, however, at highest concentration (>80 mg/L), the bacteria activity showed 60% bacteria inhibition. The EC_{50} values for AgNPs synthesized by ethanol and aqueous extracts of *HU* were 5.65 and 4.69 respectively. The EC50 values may replace the minimum inhibitory concentration (MIC) levels of AgNPs synthesized by ethanol and aqueous extracts of *HU.* The broth dilution method of antibacterial studies remains the best option owing to the precision of the result and accuracy of measurements. Small variations in MIC values for plant-related extracts may be attributed to the stability and microtechnique limitations (Van de Vel *et al.*, 2019).

Conclusion

The process of synthesis and characterization of AgNPs encompasses a variety of approaches, each with its own merits and drawbacks. Green synthesis using ethanol and aqueous extracts of *HU* seeds was explored in this study owing to its reducing properties and medicinal applications. Understanding the characteristics and actions of these nanoparticles is important in fully exploiting their capabilities across different domains, ranging from healthcare to environmental science. The detailed characterization (UV-VIS spectroscopy, FTIR analysis, XRD patterns, and SEM imaging) employed in this study validates the successful creation of stable and biologically effective AgNPs utilizing ethanol and aqueous extracts of *HU*. These nanoparticles exhibited promising diverse applications, particularly as an antibacterial on MRSA and as an antioxidant. We recommend further studies on *HU*-AgNPs complex as alternative treatment regimen for combating new clones of MRSA infections.

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