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Review article

Different techniques utilized for characterization of metallic nanoparticles synthesized using biological agents: A review

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Abstract: Nanobiotechnology, an emerging stream, is an amalgamation of nanotechnology and biology. It involves synthesis of metallic nanoparticles mediated by biological materials of both plant and animal origins. The biological process of synthesis of nanoparticles is ecofriendly, requires less labor, and has many unique properties, derived from the biological matter used in their synthesis. The synthesis of metallic nanoparticles has to be followed by the characterization for different properties such as size, shape, capping materials, stability etc, which helps in clearly defining the synthesized nanoparticles on the basis of the observied properties. Keeping our past works, synthesis and characterization of nanoparticles in the background, we have reviewed different techniques utilized for characterization of metallic nanoparticles synthesized using biological agents. This review will serve as a comprehensive guide, assisting in better understanding techniques for characterization of metallic nanoparticles synthesized using biological agents.

Keywords: nanotechnology; nanoparticles; UV-Vis, FT-IR, SEM, TEM, DLS, Zeta Potential, characterization.

1. Introduction

A new area of nanotechnology, the nanobiotechnology, is the biology of nanoparticles made from plant extracts and other biological agents. A large field of research called nanobiotechnology is built on the idea of working with substances at the atomic and molecular scale **[1]**. Utilizing plant extracts and other biological agents to create metallic nanoparticles is a more environmentally friendly method of doing so than using other methods, which are more labor-intensive, complex, and likely to pose risks.

According to Kumar *et al.* (2017) the adjective 'green' employed in 'green-nanoparticles' has a dual connotation **[2, 3]**. On the one hand, the term "green" is employed in terms of technology, referring to an environmentally benign process for synthesis of metallic nanoparticles utilising plant extracts. The term 'green,' on the other hand, refers to the presence of the green pigment chlorophyll in plants. As a result, any nanoparticles generated from achlorophyllous extracts might be referred to as 'white-nanoparticles.' The green approach of nanoparticle manufacturing has various key applications in sectors such as biolabeling sensors, drug delivery systems, and so on. According to Ali and Ozan (2019) **[4]** nanoparticles can improve drug bioavailability.

Nanoparticles are gaining popularity due to their new physicochemical, magnetic, and optoelectronic capabilities, which are determined by their shape and size distribution **[5, 6]**.

Plant extract is not always used in the green production of nanoparticles. Green synthesis of metallic nanoparticles, on the other hand, can be defined as a bio-reduction method that requires relatively low energy to initiate the reaction; this method is also cost-effective when compared to other numerous physical and chemical synthesis method

which involves high radiation, high toxic reductants, and harmful stabilising agents; these are harmful to man and the environment **[7 – 10].**

Green synthesis (biological synthesis) of nanoparticles can be accomplished using several agents such as bacteria, fungi, yeast, and plants.

1.1. Bacteria

Numerous bacterial species have been widely used in commercial biotechnological applications such as bioremediation, genetic engineering, and bioleaching. Bacteria are commonly used in nanoparticle synthesis because of their capacity to decrease metallic ions, which leads in the generation of nanoparticles **[11, 12].**

1.2. Fungi

Fungus have always been utilised as medicine, and macro fungi have also been a wonderful source of nutrients **[13, 14].** Fungi possess mycochemicals such as tannins, alkaloids, flavonoids, phenolics, and others that give the fungi therapeutic capabilities **[15]**. Because of the presence of many intracellular enzymes, they are more effective biological agents for the synthesis of metal and metal oxide nanoparticles. It has been shown that competent fungi can produce more nanoparticles than bacteria. Fungi have an edge over other creatures because they have enzymes that reduce components on their cell surfaces **[16 – 19]**.

1.3. Yeast

Yeast are efficient extracellular enzyme secretors, capable of producing metallic nanoparticles both extracellularly and intracellularly **[20]**. Several researchers have demonstrated effective nanoparticle production using yeast **[21 – 24]**.

1.4. Plants

Plant extracts contain biomolecules that can convert metallic ions to nanoparticles in a single step. This procedure is rather quick and can be carried out at room temperature and pressure. The use of plant extracts in the manufacture of metallic nanoparticles is environmentally friendly. Various solvents can be used to prepare plant extracts (polar or non-polar). Plant extracts contain secondary metabolites such as alkaloids, phenolic components, terpenoids, tannins, saponins, and so on **[3, 25]**. Several research groups have demonstrated successful metallic nanoparticle production utilizing diverse plant extracts **[26 – 29]**.

The nanoparticles created by the methods and agents described above require additional investigation. The particles produced must have specified qualities. Before they can be classified as nanoparticles, their nano range size (less than 100 nm), stability, and a variety of other qualities must be determined. In this article we have reviewed the use of UV-visible spectrum analysis (UV-Vis), scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FT-IR), dynamic light scattering (DLS), X-ray Diffraction (XRD), and transmission electron microscopy (TEM) techniques for characterization of synthesized nanoparticles.

2. Characterization of nanoparticles

The synthesised nanoparticles must be characterised for a variety of features such as form, size, capping materials, stability, and so on. On the basis of availability and applicability of the techniques for the specific study, numerous methodologies for characterisation of nanoparticles are employed.

2.1. UV-visible spectrum analysis

UV-Visible spectroscopy is a technique for measuring the amount of light absorbed and dispersed by a sample (a quantity known as extinction, which is defined as the sum of absorbed and scattered light). In its most basic form, a sample is put between a light

source and a photodetector, and the intensity of a UV/Visible light beam before and after passing through the sample is measured.

These measurements are compared at each wavelength to determine the wavelength dependent extinction spectrum of the samples. Typically, the data is shown as extinction as a function of wavelength. Each spectrum is background corrected with a buffer blank to ensure that unusual features from the buffer are not included in the extinction-spectrum of the samples.

The optical properties of gold and silver nanoparticles are sensitive to size, shape, concentration, aggregation state, and refractive index near the nanoparticle surface, making UV-vis spectroscopy a useful technique for identifying, characterising, and researching nanomaterials **[30].**

The UV-vis spectrum analysis yields a graphical result, as illustrated in figure 1. During the synthesis (reduction of metallic ions) of nanoparticles by biological agents **[3, 26, 31]** a colour change is generally reported from pale yellow/pale turbid to dark colours (figure 2). Various studies indicate that a surface plasmon resonance (SPR) peak about 400 nm indicates that the particle in issue is in the nano range (below 100 nm). Smaller nanospheres absorb light and have peaks near 400 nm (figure 1), whereas larger particles scatter light and have peaks that expand and shift towards longer wavelengths (a phenomenon known as red shifting) **[32]**.

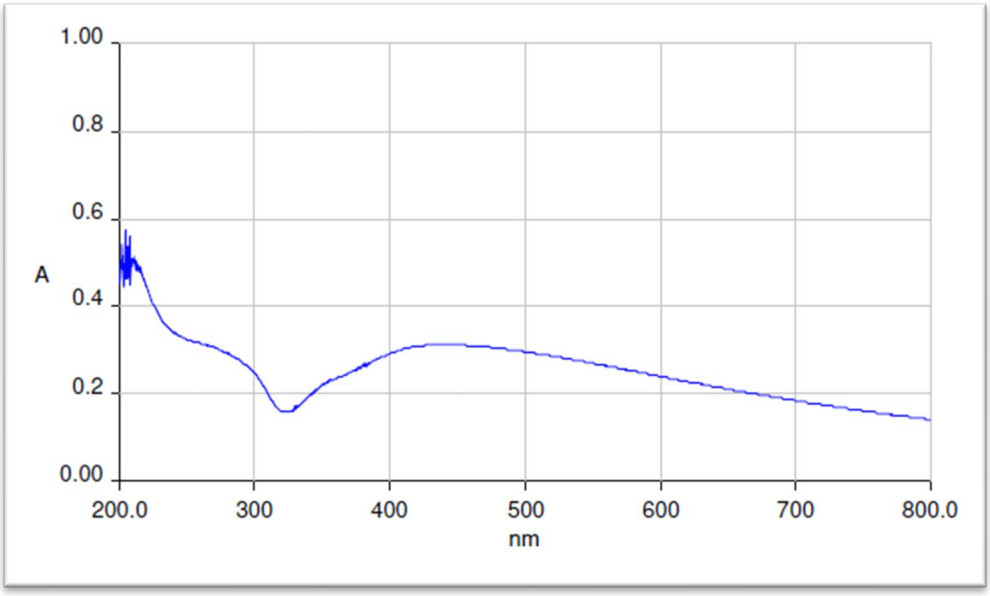

Figure 1. Result of UV-vis analysis of nanoparticles synthesized using *Swertia chirayita* aqueous leaf extract, showing SPR (surface plasmon resonance) peak at 150 nm (Kumar *et al.,* 2017) [3].

Several researchers have observed that when metallic particles are in the nano range, i.e., less than 100 nm, the SPR peak produced by UV-visible spectroscope is between 350 and 500 nm. Hamouda *et al.* (2019) **[33]** observed an SPR peak ranging from 320 to 580 for nanoparticles (particles smaller than 100 nm) produced with the cyanobacterium *Oscillatoria limnetica*. Anbazhagan *et al.* (2017) **[34]** found an SPR peak between 420 to 430 nm for fungi-derived nanoparticles. Elamawi *et al.* (2018) **[35]** identified a highest SPR peak at 385 nm for *Trichoderma longibrachiatum*-derived nanoparticles.

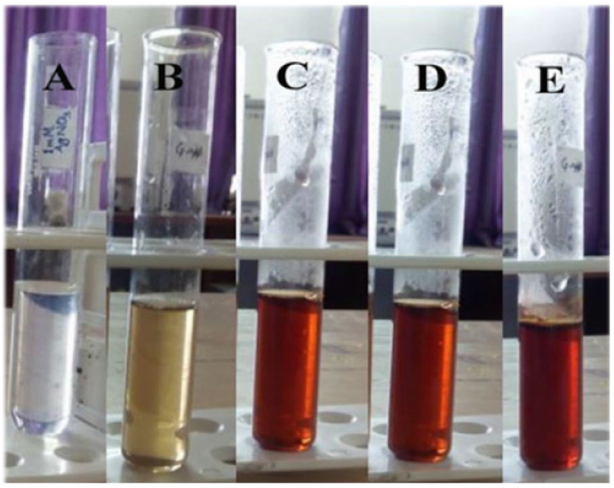

Figure 2. Change in colour of mixture of silver nitrate and aqueous extract of *Ganoderma applanatum* (Pres.) Pat. (A) $AgNO_3$ solution, (B) aqueous extract of *Ganoderma applanatum*, (C, D, E) showing gradual change in the colour after mixing of A & B (Dandapat *et al.*, 2019) [31].

2.2. Scanning Electron Microscope (SEM) analysis

Scanning electron microscope analysis can be used to examine the surface morphology of produced nanoparticles. To obtain SEM images, the nanoparticles' powder is first obtained and then coated with a heavy metal layer. The coated nanoparticles are then placed in the SEM stage, where they are attacked with electron beams, resulting in the release of secondary electrons, which are collected by the sensors to produce images. Since the wavelength of an electron beam is shorter than that of visible light, high resolution pictures of nanoparticles are obtained **[3, 27]**.

Some SEM equipment, such as the JEOL JSM-6390 LV (Jeol, Japan), include supplemental software that can quantify particle average size. Kumar *et al.* (2018) **[3]** used SEM to obtain electron micrograph and qunatify the size (using accessory software) and form of nanoparticles in their work on the synthesis of silver nanoparticles using aqueous leaf extract of *Punica granatum*.

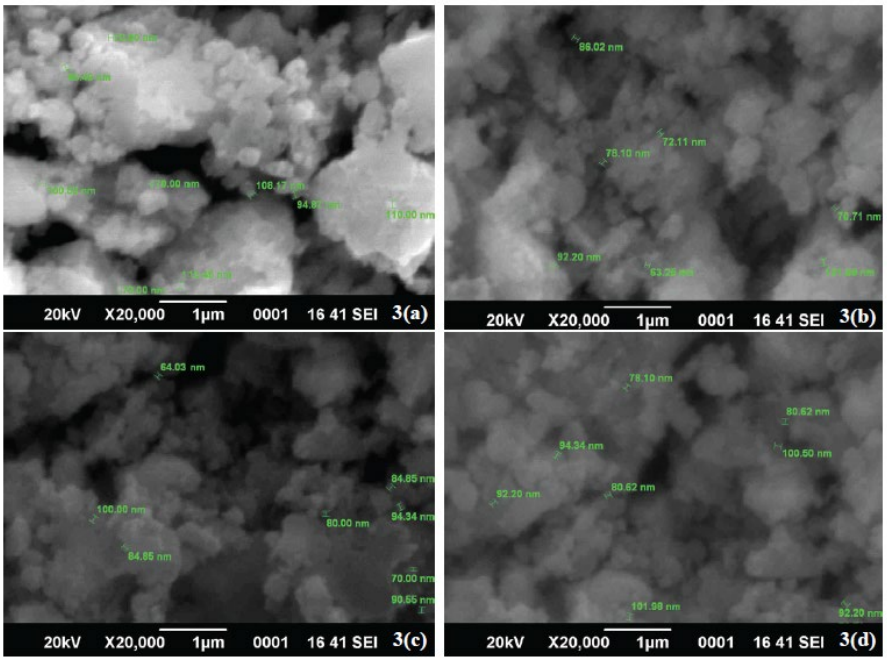

Figure 3. Scanning Electron Microscope images of nanoparticles, note the measurement of size fone using software (Kumar *et al.*, 2014) [26].

Khan *et al.* (2016) **[36]**, used *Ziziphus nummularia* leaves aqueous extract to produce silver nanoparticles, which they investigated using SEM and found to be spherical with diameters ranging from 30 nm to 85 nm. Amjad *et al.* (2021) **[37]** synthesized copper nanoparticles from *Fortunella margarita* leaves and subjected them to SEM analysis; they reported spherical nanoparticles with sizes ranging from 51.26 to 56.66 nm. Constantino-Alcazar *et al.* (2021) **[38]** produced green potassium nanoparticles from *Sideroxylon Capiri*, reporting particle sizes in the 200-360 nm range with spherical morphology.

2.3. Fourier transform infrared spectroscopy (FT-IR)

The chemical surface of nanoparticles is particularly sensitive to FT-IR. To detect functional groups present on the surface of nanoparticles, FT-IR spectra are obtained. The negatively charged molecules cap the metallic ions during the formation of metallic nanoparticles. The FT-IR analysis aids in the identification of (just the) functional groups present in compounds that cap the metallic ion.

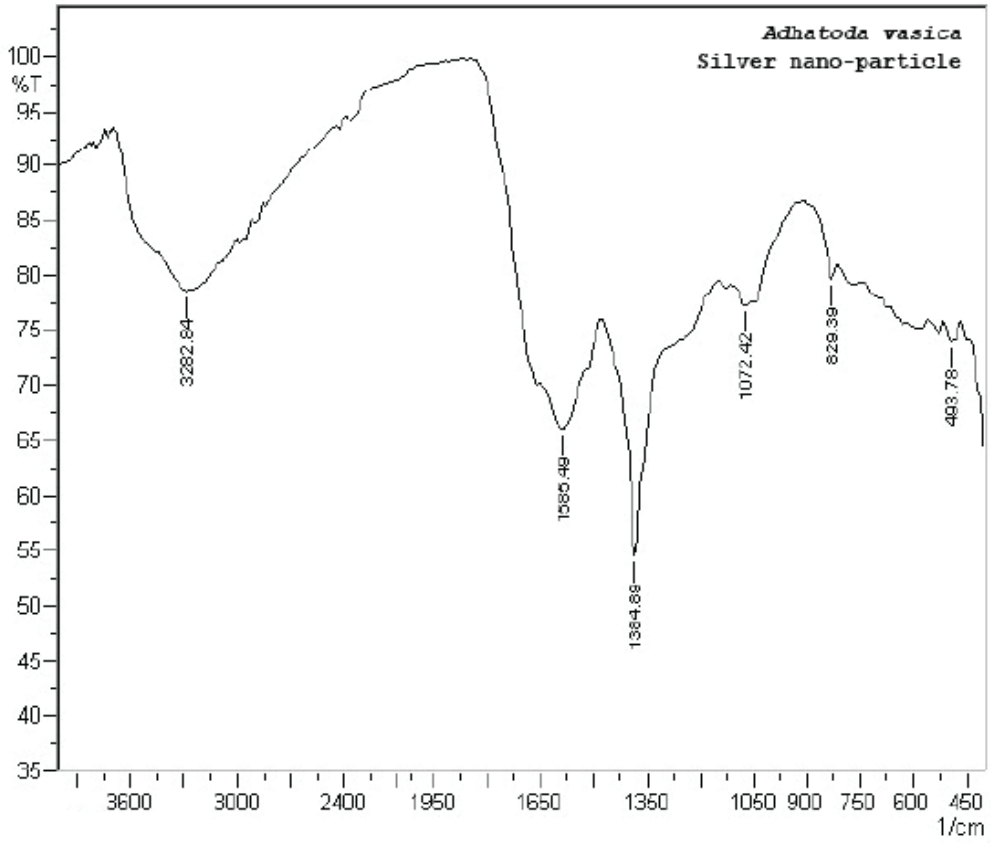

Figure 4. FT-IR spectrum of nanoparticles synthesized using aqueous extract of *Adhatoda vasica* (Kumar *et al.*, 2014) [26]

Figure 4 depicts the graphical findings of FT-IR study of nanoparticles generated using *Adhatoda vasica* aqueous extract **[26]**. The spectra shows broad transmission peak at 3282 cm^{-1}, which corresponds to hydrogen bonded hydroxyl group (O-H and H stretch) of alcohols and phenols. Another broad stretch of 1585 cm^{-1} corresponds to C=C stretch as aliphatic nitro compounds; 1384 cm^{-1} corresponds to N=O bend as aliphatic nitro compounds; 1072 cm^{-1} corresponds to C=N stretch as aliphatic amines; 829 cm^{-1} corresponds to aliphatic phosphate symmetric P-O-C and 493 cm^{-1} corresponds to S-S stretch as polysulfide **[26, 39]**

Coates (2006) **[39]**, presented an entire list of group frequencies (cm^{-1}) and their matching functional groups that can be used to determine functional groups based on FT-IR group frequencies. Rautela *et al.* (2019) **[40]** discovered multiple peaks demonstrating

the complex nature of *Tectona grandis* seed extracts. They attributed the bands at 1745, 1643, 1508 and 1038 cm^{-1} to stretching vibrations of carboxylic acid or ester C=O bonds, N-C=O amide bonds of proteins, nitro compounds, and C-N amine bonds, respectively. They observed a shift in the peaks of synthesised silver nanoparticles and suggested that functional groups of seed extract participated in the formation of AgNPs in their study.

Another breakthrough in the FTIR approach has been made, known as attenuated total reflection - fourier transform infrared (ATR-FTIR) spectroscopy. The chemical characteristics of the capping components of green nanoparticles can be determined by ATR-FTIR **[41, 42]**

2.4. Dynamic light Scattering (DLS) and Zeta-potential analysis (ZPA)

Dynamic light scattering (also known as static, Rayleigh, or multi-angle light scattering) measures particle size directly **[43].** Light scattering occurs when light collides with a small object (a particle or a molecule) and changes its direction. A DLS apparatus works by illuminating a sample with a laser beam. A rapid photon detector detects variations in scattered light from a sample, and the known scattering angles can calculate the whole particle size distribution within a sample. DLS instruments are employed in a variety of applications, including emulsions, micelles, polymers, proteins, colloids, and nanoparticles **[3, 26, 31].**

The light scattering analysis of nanoparticles is an important approach for determining the size of nanoparticles in solution. It analyses the modulation of scattered light intensity as a function of time to quantify the light scattered from a laser passing through the colloidal solution. The findings of light scattering analysis demonstrate a sized distribution by number, intensity, and volume **[3]**, as illustrated in figure 5. Kumar *et al.* (2018) **[3]** observed two peaks at 96.5 nm and 141.6 nm, with intensities of 91.0 percent and 9.0 percent, respectively. They concluded that 91.0 percent of the light was dispersed by particles with a size of 96.5 ± 4.843 nm.

The intensity distribution graph (figure 5) revealed two peaks at 96.5 nm and 141.6 nm with intensity of 91.0% and 9.0% respectively. The intensity data shows how light is scattered by particular particle size bin, here the data shows that particles whose size were 96.5 ± 4.843 nm; 9.0% of light was scattered by particles whose size were 141.6 ± 32.40 nm. The graph thus confirms the formation of silver particles in nano range **[3]**.

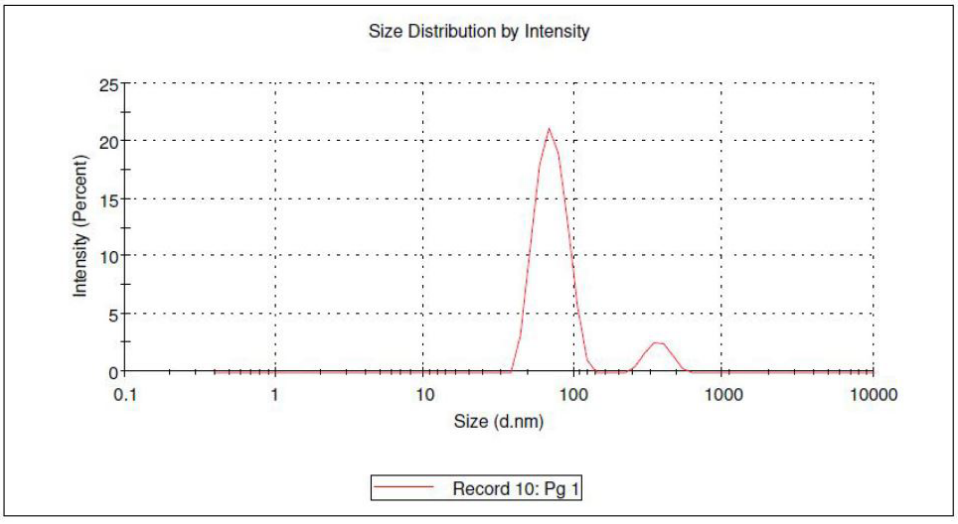

Figure 5. Results of light scattering analysis of nanoparticles synthesized using aqueous leaf extract of *Punica granatum* showing size distribution by intensity (Kumar *et al.,* 2018) [3]

The volume distribution graph (figure 6) revealed two peaks at 85.6nm and 24.76 nm, indicating particle volume distribution in distinct size bins. They concluded that 100% of

the particles generated were in the nano size range, with 84.2 percent of particles measuring 85.6 ± 14.60 nm and 15.8 percent measuring 24.76 ± 2.114 nm. As a result, the production of silver particles in the nano range was confirmed **[3]**.

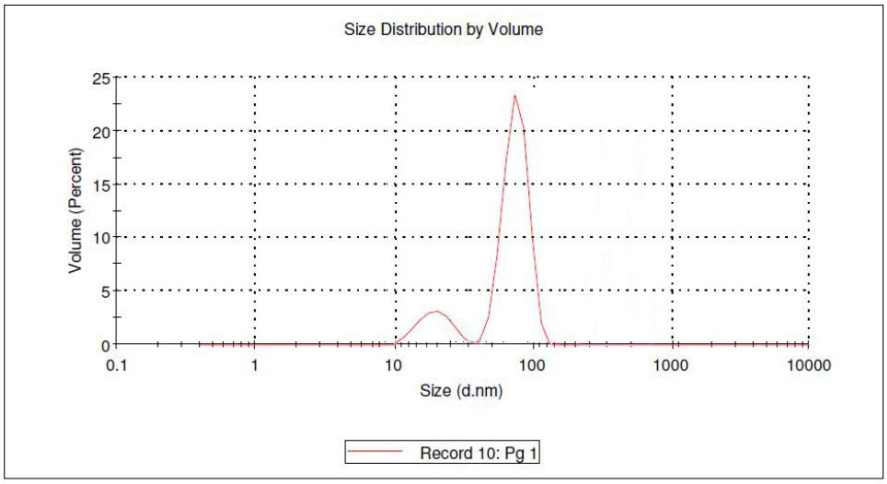

Figure 6. Results of light scattering analysis of nanoparticles synthesized using aqueous leaf extract of *Punica granatum* showing size distribution by volume (percent) (Kumar *et al.,* 2018) [3]

The number distribution graph (figure 7) shows one major peak at 40.32 nm with number distribution of 30%. This indicates that at least 30% of synthesized nanoparticles had size of 40.32 ± 4.413 nm [3].

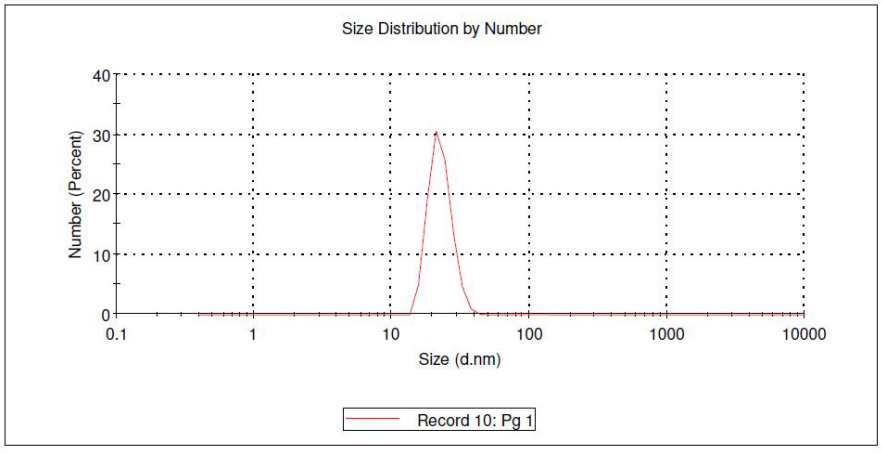

Figure 7. Results of light scattering analysis of nanoparticles synthesized using aqueous leaf extract of *Punica granatum* showing size distribution by number (Kumar *et al.,* 2018) [3]

Kumar *et al.* (2017) **[2]** investigated the Zeta potential of nanoparticles produced from *Swertia chirayita* aqueous leaf extract. They discovered a peak of -15.2 mV with a 100% area distribution (figure 8). Nanoparticles having Zeta potential values more than +25 mV and less than -25 mV are often quite stable **[30]**. They concluded that the silver nanoparticles generated using *Swertia chirayita* aqueous leaf extract were stable in aqueous solution.

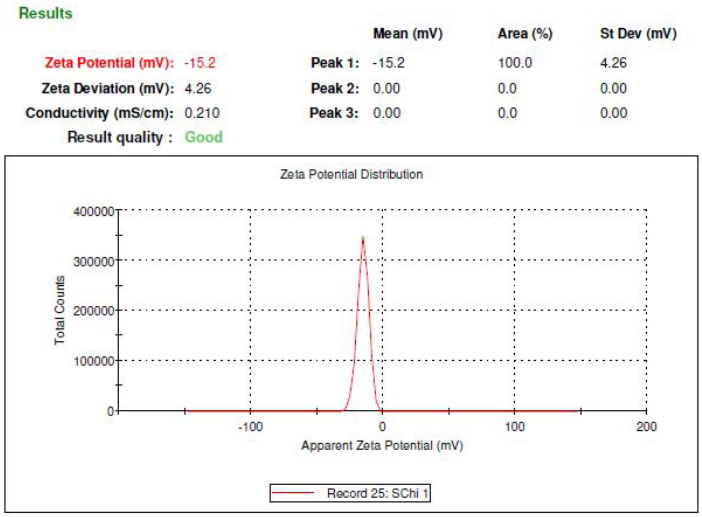

Figure 8. Result of zeta potential analysis of nanoparticles synthesized using aqueous leaf extract of *Swerti chirayita* (Kumar *et al.*, 2017) [2].

2.5. XRD analysis

The significant interest in nanoparticles that has existed in the last few decades is, to a large extent, due to the fact that when particle size decreases, numerous new traits and characteristics are represented by the particles. The use of the X-ray Diffraction (XRD) technology to assess particle size is not novel, but its application in determining the size of silver nanoparticles generated without any biological agent is a novel approach that researchers are pursuing.

XRD has a high potential for analysing nanostructures since the width and shape of reflections reveal information about the material's substructure (sizes of microcrystallites, lattice structure). The Scherrer, Williamson-hall, and Warren-Averbach methods are the most commonly employed for analysing X-ray diffraction line profiles **[44 – 47]**.

The XRD analysis produces an array of data that may be analysed using various software packages such as Match! and Origin 9 (figure 9). Match! software provides a platform for comparing collected data to the Joint Committee on Powder Diffraction Standards database (JCPDS). JCPDS has been renamed as the International Centre for Diffraction DATA (ICDD) **[44]**.

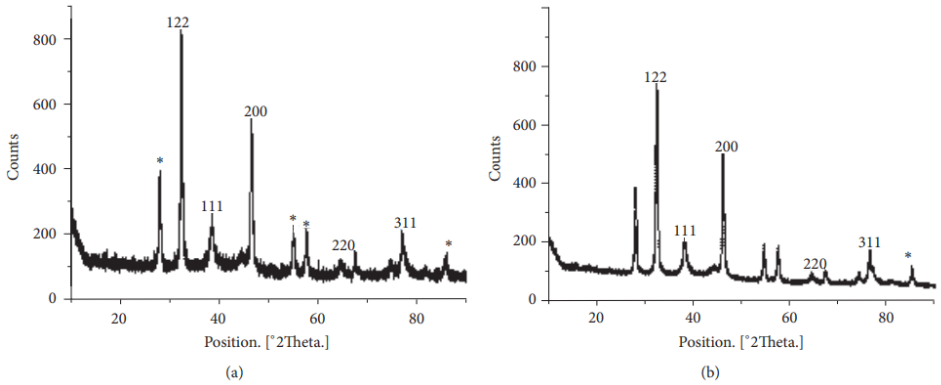

Figure 9. Results of XRD analysis of silver nanoparticles synthesized using (a) aqueous leaf extract of *Allophylus serratus* and (b) aqueous leaf-derived callus extract (Jemal *et al.*, 2017) [45]

2.6. Transmission electron microscope (TEM) analysis

Transmission electron microscopy (TEM) is a method that is widely used to characterise nanomaterials. It is used to estimate quantitative particle and/or grain size, size distribution, and particle morphology **[48 – 50]**. TEM has two major advantages over SEM: first, it can give superior spatial resolution; second, it can do more analytical measurements **[48, 50 – 51].** The most critical part of TEM is that sample preparation takes time; thus, sample preparation is critical in order to produce the highest-quality images possible.

Kumar *et al.* (2017) **[52]** performed TEM examination of nanoparticles generated using Andean blackberry fruit extract, and TEM analysis of AgNPs revealed the creation of a crystalline spherical shape with a size range of 12-50 nm (figure 10).

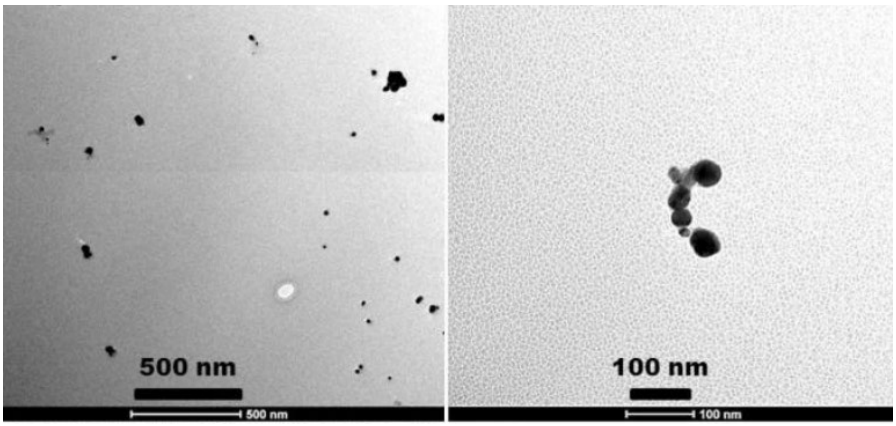

Figure 10. Transmission electron images of silver nanoparticles synthesized using Andean blackberry fruit extract (Kumar *et al.,* 2017) [52]

Rautela *et al.* (2019) **[40]** characterised silver nanoparticles produced from *Tectona grandis* seeds extract. They observed spherical silver nanoparticles with sizes ranging from 10 to 30 nm. Ashraf *et al.* (2016) **[53]** performed TEM examination of silver nanoparticles generated using *Aloe vera* extract and observed spherical silver nanoparticles in the size range of 5-85 nm.

3. Properties of nanoparticles

Nanoparticle characteristics are generally classified as physical or chemical. Table 1 shows the properties of a few popular metallic nanoparticles **[54].** Color, light penetration, absorption and reflection capabilities, UV absorption and reflection capabilities, magnetic and electric properties, etc. are examples of physical properties of nanoparticles. Chemical properties of nanoparticles include qualities such as nanoparticle reactivity with the target (stability), antibacterial and antifungal activities, toxicity, etc.

Table 1. Physical and chemical properties of few common metallic nanoparticles.

Nanoparticles	Properties	References
Aluminium	High reactivity, sensitive to moisture, heat and light, large surface area	[55]
Iron	Reactive and unstable; sensitive to air and water	[56]
Silver	Absorbs and scatters light, stable, antibacterial, known to possess alleviating effect on mammalian models	[14, 27, 57, 58, 59]
Gold	Interactive with visible light, reactive	[60]

Copper	Ductile, very high thermal and electrical conductivity, highly flammable solids	[61]
Zinc	Antibacterial, anti-corrosive, antifungal, UV filtering	[62]

4. Applications of nanoparticles

Various methods for preparation of nanoparticles have been demonstrated such as physical **[63 – 66]**, chemical **[66 – 69}** and green synthesis **[2 – 4, 19, 66]**. Since nanoparticles synthesized from plants, microbes and biomolecules are cost effective, biodegradable, biocompatible, easily modifiable, less toxic, they are recognized for their application in various fields like biomedicine, electronics, agriculture, cosmetics, textiles, etc. **[70 – 75]**

5. Conclusion

This review covers the relevance of several strategies in the characterization of metallic nanoparticles generated utilising biological (animal and plant components). Typically, more than one technique is necessary to create a complete characteristic image of nanoparticles. This review will serve as a thorough guide, assisting the scientific and academic community in better understanding the characterization of nanoparticles by addressing the role of the approaches presented in the preceding text. Of course, there are scientific potential for newer ways to emerge throughout time.

Both academic and industrial researchers are increasingly interested in investigating the possibilities of employing metallic nanoparticles as next-generation medicinal agents.

Although green nanoparticles play an essential role in clinical research, numerous variables must be considered, including raw material source, manufacturing process, stability, bio-distribution, controlled release, accumulation, and so on.

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