



Síntese Verde de Nanopartículas de Prata Utilizando Cascas de Laranja (Citrus sinensis)

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**Green Synthesis of Silver Nanoparticles Using
Orange (*Citrus sinensis*) Peels**

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Abstract

Recently, silver nanoparticles (Ag-NPs) derived from biological sources act as an alternative route to reduce heavy chemicals and high costs of energy of physical and chemical approaches. In pursuit of a greener route, this thesis aimed to synthesize Ag-NPs from a green solvent-mediated orange peel (OP) extract.

A screening of four types of natural deep eutectic solvents (NADES) and water OP extracts was reported by evaluating antioxidant activity and Total Phenolic Content (TPC). Water-assisted OP extract was ultimately chosen for Ag-NPs by presenting high TPC (90.7 mg_{GAE}/g_{OP}) and Trolox Equivalent Antioxidant Capacity (TEAC) (3.2 mg_{TE}/g_{OP}); besides, production of NADES was time-consuming and exhibited high viscosity and acidic properties.

Ag-NPs were synthesized at 25 °C in alkaline medium and monitored for 24 hours. Different ratios of Ag⁺:TPC content were tested (varying from 0.75-12). Accordingly, Ag⁺ concentration was kept constant and OP extract volume decreased with an increase in ratio.

A gradually colorless to brownish tone appeared after 15 minutes, indicating formation of Ag-NPs. UV-VIS Spectroscopy (UV-VIS) revealed surface plasmon resonance (SPR) peaks in the region 407-412 nm, related to Ag-NPs absorption band. In addition, a peak at 280 nm associated with UV absorption of polyphenols was reported in all samples, suggesting OP extracts were not capable of completely reducing Ag⁺ to Ag⁰. Dynamic Light Scattering (DLS) data confirmed Ag-NPs with an average hydrodynamic diameter of 99-112 nm and a polydisperse index of 0.210-0.263, revealing monodisperse nature of Ag-NPs. Moreover, Ag-NPs confirmed high stability due to a zeta potential value of -21.0 mV.

Studied ratios did not show significant differences in particle size characterization but a trend in antioxidant activities was established. TPC and TEAC of Ag-NPs was lower in comparison to H₂O-assisted OP extract due to interaction of the reducing agents in the bioreduction; it is also noted that bigger ratios present higher TPC and TEAC content, and vice-versa.

Overall results suggested the great potential of OP as a biological source of phenolics for green Ag-NPs synthesis.

Keywords: silver nanoparticles; agro-industrial waste; biosynthesis; green solvents.

Resumo

Recentemente, as nanopartículas de prata (Ag-NPs) derivadas de fontes biológicas atuam como uma alternativa para reduzir o uso de químicos e os altos custos de energia das abordagens de síntese física e química. Em busca de uma rota mais verde, o principal objetivo desta tese foi sintetizar Ag-NPs a partir de um extrato de casca de laranja (OP) mediado por um solvente verde.

Fez-se uma triagem de extratos obtidos a partir de quatro tipos de solventes eutéticos naturais profundos (NADES) e água, relatando-se a atividade antioxidante e fenóis totais (TPC). Escolheu-se o extrato obtido pela utilização de água como solvente para posterior síntese de Ag-NPs por apresentar um elevado valor de TPC (90,7 mg_{GAE}/g_{OP}) e atividade antioxidante equivalente ao trolox (TEAC) (3,2 mg_{TE}/g_{OP}); além disso, a produção de NADES era demorada e estes exibiram alta viscosidade e propriedades ácidas.

Sintetizaram-se Ag-NPs a 25 °C em meio alcalino e monitorizou-se a reação durante 24 horas. Testaram-se diferentes razões de Ag⁺:TPC (variando de 0.75-12). Desta forma, a concentração de Ag⁺ manteve-se constante e diminuiu-se volume de extrato de OP com o aumento da razão.

Visualizou-se a mudança gradual de um tom incolor a acastanhado após 15 minutos de reação, indicando a formação de Ag-NPs. A análise por espectroscopia UV-VIS revelou picos de ressonância de plasmão de superfície (SPR) na região 407-412 nm, relacionados com a banda de absorção de Ag-NPs. Além disso, detetou-se um pico a 280 nm em todas as amostras, associado à absorção UV de compostos fenólicos, o que sugere que os extratos não foram capazes de reduzir completamente Ag⁺ a Ag⁰. Confirmou-se um diâmetro hidrodinâmico médio de 99-112 nm a partir de dados do Espalhamento Dinâmico de Luz (DLS) e um índice de polidispersidade de 0,210-0,263, revelando a natureza monodispersa das partículas. Além disso, confirmou-se a sua alta estabilidade devido ao valor potencial zeta de -21,0 mV.

As razões estudadas não mostraram diferenças significativas na caracterização do tamanho das partículas, no entanto, estabeleceu-se uma tendência relativamente à atividade antioxidante. Quantificaram-se valores menores de TPC e TEAC de Ag-NPs em comparação ao extrato de OP obtido por água, devido à interação dos agentes redutores na biorredução; além disso, observou-se que razões maiores apresentam maior conteúdo de TPC e TEAC, e vice-versa.

Em suma, os resultados sugeriram o potencial do uso da casca de laranja como fonte natural de compostos fenólicos para a síntese verde de Ag-NPs.

Palavras-chave: nanopartículas de prata; resíduos agroindustriais; síntese verde; solventes verdes.

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Abbreviations and Acronyms

Ag	Silver
ChChl:Fruc	Choline Chloride:Fructose
ChChl:Glu	Choline Chloride:Glucose
COD	Chemical Oxygen Demand
DLS	Dynamic Light Scattering
FTIR	Fourier Transform Infrared
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
LP:MA	L-Proline:DL-Malic Acid
MA:Gluc	DL-Malic Acid:Glucose
NADES	Natural Deep Eutectic Solvents
NP	Nanoparticles
OP	Orange Peel
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
SPR	Surface Plasmon Resonance
TEAC	Trolox Equivalent Antioxidant Capacity
TEM	Transmission Electron Microscopy
TPC	Total Phenolic Content
UV-VIS	UV-Visible Spectroscopy
XRD	X-Ray Diffraction

Chapter 1. Introduction

1.1. Framework

On December 29, 1959, Richard Feynman, awarded the Nobel Prize in Physics, gave the lecture There's Plenty of Room at the Bottom, at Caltech University, where he discussed, for the first time, the possibility of manipulating atoms or molecules to the production of new materials, defining nanotechnology as a new multidisciplinary area (Gonçalves, 2015).

The core purpose of nanotechnology entails the usage and development of nanoparticles (NPs); which are nanometer-sized (<100 nm) particles with different physico-chemical properties compared to their bulk counterpart (Okpara *et al.*, 2020). Ag-NPs are among the most interesting and applied metallic NPs in the fields of medicine, agriculture, environment and food (Paiva-Santos *et al.*, 2021) and can be synthesized by physical, chemical and biological methods.

From physical and chemical methods arise environmental issues, namely the requirement of high energy and pressure conditions and equipment and the use of heavy chemicals. This has led to explorations of eco-friendly synthesis based on diverse biological and renewable entities; these can be plants, microorganisms, household and industrial waste (Lieu *et al.*, 2018). It complies with green chemistry; by opening a larger scope of different naturally derived sources, it is less costly, energy efficient and non-toxic (Hasan *et al.*, 2022).

Mechanism of green synthesis is not yet fully understood, as reported by literature (Hasan *et al.*, 2022); however, it is assumed the reduction of Ag^+ to Ag^0 and consequent nucleation and growth to Ag-NPs. Bioactive compounds present in the biological sources (i.e., phenolics present in orange peels (OP)) are responsible for the reduction (Dikshit *et al.*, 2021).

In pursuit of a greener route, the aim of this project was to synthesize Ag-NPs from a green solvent-mediated OP (*Citrus sinensis*) extract and investigate the effect of ratio on particle size and antioxidant activity. More specifically, some goals were drawn:

- Quantify the Total Phenolic Content (TPC) and antioxidant activity and study the feasibility of OP as a source of reducing agents for the production of biogenic Ag-NPs;
- Screen the optimal green solvent (Natural Deep Eutectic Solvents (NADES) and water) for the extraction of OP bioactive compounds and subsequent production of biogenic Ag-NPs;
- Study the influence of ratio of Ag^+ source and polyphenol content on the production and characterization of biogenic Ag-NPs by UV-VIS Spectroscopy, Dynamic Light Scattering (DLS), TPC and antioxidant activity;
- Contribute to the three pillars of sustainability (environmental, economic and social aspects).

This purpose aligns with the green principles and work developed by GreenSapiens, a research group based in Rome, Italy, and whose facilities are located at Sapienza University, where this project was conducted. GreenSapiens collaborates internationally with various universities and research groups to carry out innovative processes and bridge the gap between laboratory research and development and commercial manufacture. Some key areas of expertise and previous projects of the group involve greener and cost-effective routes for the biogenic synthesis of metallic NPs from agro-industrial waste, recovery of value-added compounds and production of NADES. Thus, this thesis was a combination of these lines of investigation.

1.2. Organization

This thesis presents a green approach for the synthesis of Ag-NPs and is structured chapter-wise. Chapter 1 presents a brief overview of the reported research and introduces the strategy and outline of the thesis. Chapter 2 reports the state of art regarding Ag-NPs properties, applications, characterization, toxicity and traditional and green synthesis. Chapter 3 describes the used experimental methods and Chapter 4 discusses the results. Finally, Chapter 5 summarizes the conclusions of the thesis as well as future perspectives and proposal for future studies in the field of the Ag-NPs green synthesis.

Chapter 2. State of Art

2.1. Silver Nanoparticles (Ag-NPs)

Nanotechnology focuses on the study and development of functional materials, devices and systems at a nanoscale level. Recent advances in this field allow the synthesis of various types of novel nanomaterials; NPs are naturally occurring or engineered particles in the range of 1 to 100 nm (Huston *et al.*, 2021; Sampath *et al.*, 2022). NPs are typically classified into organic and inorganic. Organic NPs incorporate carbon, whereas inorganic NPs comprise metallic (Ag, Au, Cu, Al), magnetic (Co, Fe, Ni) and semi-conductor components (ZnO, ZnS, CdS) (Zorraquín-Peña *et al.*, 2020).

In fact, metallic NPs were introduced to the world ancient times. An example is the Lycurgus Cup; it is a glass Roman artefact that shows dichroism. This property is due to silver (Ag) and gold (Au) NPs that illuminate externally (green), or internally (red), respectively (Shnoudeh *et al.*, 2019). Besides, Ancient Romans treated their water with Ag coins and even more recently space programs employed it for purifying water aboard the Apollo spacecraft, MIR space station and the Space shuttle (Jain *et al.*, 2009).

Ag-NPs are the most widely employed NPs. Total estimated production of Ag-NPs was about 500 tonnes in 2009, expecting to be increased to 900 tonnes by 2025 (Calderón-Jiménez *et al.*, 2017; Islam *et al.*, 2021). Novel and tunable properties of Ag-NPs lead to the enhancement of mechanical, optical, electrical, magnetic, catalytic and photochemical attributes, boosting performance over bulk materials (Calderón-Jiménez *et al.*, 2017). In addition, Ag-NPs include heightened antibacterial, antifungal, antiviral, antioxidant activity and biocompatibility. These properties endow Ag-NPs with innumerable advantages for applications in bioengineering and biotechnology, mainly in biomedical and environmental remediation fields (Paiva-Santos *et al.*, 2021; Akhil *et al.*, 2023).

2.2. Properties and Applications of Ag-NPs

2.2.1. Physicochemical Properties of Ag-NPs

Major physicochemical properties of Ag-NPs include size, shape, surface chemistry and agglomeration tendency. Size is a critical factor in mediating biological effects; for example, Rodríguez-Félix *et al.* (2022) mention a higher antioxidant activity in particles between 1-10 nm. In fact, smaller Ag-NPs have an increased surface-to-volume ratio, resulting in a higher reactivity and improved activities, due to a large proportion of atoms in the surface (Farkas *et al.*, 2011).

Since catalytic activity is enhanced, Ag-NPs are heavily utilized as colorimetric probes for chemical and biosensing of metal ions, pesticides, drugs or toxins (Shaikh *et al.*, 2021). An example is proposed by Saha *et al.* (2017); Ag-NPs prepared with *Gmelina arborea* fruit extract successfully degraded methylene blue dye within 10 minutes. Talebian *et al.* (2020) also reported Ag-NPs as a photocatalytic coating layer of respiratory face masks for COVID-19 and test kits. Optical properties of Ag-NPs depend primarily on surface plasmon resonance (SPR); plasmon refers to the collective coherent oscillation of metal-free electrons (Wei *et al.*, 2015; Tarannum *et al.*, 2019).

Shape is related to the high production of Ag⁺ ions and consequently impacts the degree of particle toxicity. Ag-NPs are commonly spherical, containing about 8.43×10^{10} mL⁻¹ particle

concentration and presenting the highest antimicrobial activity; irregular, oval, triangular or cubic shapes are also synthesized (Rodríguez-Félix *et al.*, 2022).

Surface charge determines Ag-NPs interaction or attachment with other biological or living systems; it can be changed by coating or capping with other materials. Ultimately, it is a high indicator of toxicity since it affects various biological transport processes (i.e., surface adsorption, cellular uptake and elimination) (Zhang *et al.*, 2020).

2.2.2. Antimicrobial Activity of Ag-NPs

It is well known that Ag and its compounds possess a strong antibacterial activity against bacteria, viruses and fungi. In fact, Ag-NPs have been proved as antimicrobial agents against over 650 microorganisms (Dakal *et al.*, 2016) and are exploited in cosmetic and chemicals (i.e., skin cleansers, fluids, lotions, toothpastes, paints), textile coatings, food storage and water purification (Tarannum *et al.* 2019; Shaikh *et al.*, 2021; Ong & Nyam, 2022).

Antimicrobial activity of Ag-NPs is influenced by shape, size, surface and charge. Charge of the Ag-NPs modulates antibacterial activity due to their interaction with biological environments and cellular uptake (Zorraquín-Peña *et al.*, 2020). For example, positively charged Ag⁺ ions released from Ag-NPs confer electrostatic attraction to cell membranes who carry negative charge, due to the presence of carboxyl, phosphate and amino groups, thereby facilitating Ag-NPs attachment (Qing *et al.*, 2018). Particle size and shape also influence the release of Ag⁺ ions; accordingly, small size and spherical and triangular forms are more prone to higher antimicrobial activity due to their larger surface area (Ahmad *et al.*, 2020; Zorraquín-Peña *et al.*, 2020).

Hence, antimicrobial activity is likely linked to various mechanisms: (1) adhesion onto microbial cell surface and membrane damage, (2) penetration into cell and nucleus and interaction with cellular organelles, (3) formation of reactive oxygen species (ROS) and consequent increase in oxidative stress, (4) modulation of signal transduction pathways. As a result, cell viability is disrupted (Dakal *et al.*, 2016).

2.2.3. Antioxidant Activity of Ag-NPs

Antioxidant activity of Ag-NPs is related to the presence of various biomolecules at surface level and electron donating ability (Paiva-Santos *et al.*, 2021).

Mostafa *et al.* (2019) subjected *Centaurea pumilio* L. as a reducing agent in the green synthesis of Ag-NPs, forming spherical 6-8 nm particles. A previously phytochemical study of the species revealed twenty-nine constituents and eight phenolic compounds, showing high antioxidant activity. The resulting Ag-NPs were tested on skin cells and potently inhibited collagenase, elastase and tyrosinase, showing promising abilities in cosmetic and anti-aging activities. Therefore, Ag-NPs can be used as radical scavengers against damage caused by free radicals.

2.2.4. Anticancer Activity of Ag-NPs

Ag-NPs exhibit a wide spectrum anticancer activity in size-, dose- and time-dependent manners and can be used as antiseptic, therapeutic agents or drug carriers. Increased dose and prolonged time can cause more tumor cell apoptosis and necrosis, inducing ROS formation and DNA damage. Moreover, it is dependent on size, coating and surface charge (Xu *et al.*, 2020).

According to Kovács *et al.* (2022), mechanisms of anticancer activity of Ag-NPs are endocytosis-related. Ag-NPs are collected in endosomes and organelles are directed to an acidic lysosomal environment, increasing Ag⁺ release and unbalancing cellular homeostasis; this increases ROS formation and ATP synthesis arrest, and consequently DNA damage. Breast cancer

cells (MCF-7), colon cancer cells (HCT116), HeLa or lung carcinoma A549 cells are some examples of cell lines that Ag-NPs can easily interact with. For example, Oh *et al.* (2018) reported anticancer properties of Ag-NPs synthesized using *Chaenomeles sinensis* extract against MCF-7 cells.

In addition, Ag-NPs can also promote wound, bone and infections healing; for this, they are used as an additive in plastic catheters, dental materials, vaccines or gels (Jain *et al.*, 2009).

2.3. Synthesis of Ag-NPs

Abiotic and biotic mechanisms, such as precipitation, erosion, combustion or volcanic activities, can naturally form NPs. However, two approaches for the synthesis of NPs are mainly explored in the literature (i.e., top-down and bottom-up) (Maátková *et al.*, 2022). Figure 1 shows a scheme of top-down and bottom-up synthesis of NPs.

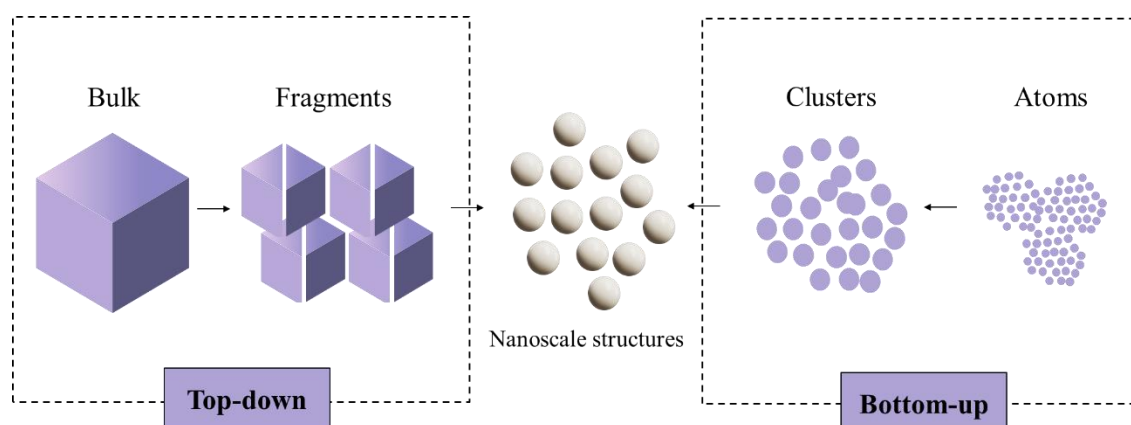


Figure 1 - Scheme of top-down and bottom-up approaches (adapted from Lu *et al.*, 2020).

Systems based on top-down methods comminute bulk materials into nanoscales through physical methods; the existence of ligands prevents the inverse assembling into large scales. Bottom-up routes exploit different chemical and biological techniques to assemble atoms to clusters to nanoscale structures (Lu *et al.*, 2020). Physical and chemical methods are effective and preferred among industries; however, environmental implications are the main hinderance. A review of the most frequent physical and chemical methods for the synthesis of Ag-NPs is described in Table 1.

Table 1 - Summary of background information for chemical and physical routes on Ag-NPs synthesis.

Route	Method	Action	Reference
Chemical	Photochemical	Reduction of an Ag ⁺ source (with or without a light source)	Ying <i>et al.</i> (2022)
	Chemical reduction		
Physical	Laser ablation	Laser irradiation	Shaikh <i>et al.</i> (2021)
	Ball milling	Subjection to high energy collision	Shnoudeh <i>et al.</i> (2019)
	Ion sputtering	Sputtering through inert gas ion beams (He, Ne, Ar, Kr or Xe)	Shaikh <i>et al.</i> (2021)

Chemical route involves the use of solvents or liquid reagents (i.e., sodium borohydride (NaBH_4), sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$), ethylene glycol ($\text{C}_2\text{H}_6\text{O}_2$), Tollens' reagent) to reduce Ag^+ to Ag^0 , occurring agglomeration and formation of Ag-NPs in the oligomeric clusters. In fact, the use of highly hazardous reagents and, consequently, the production of noxious by-products and derivatives, causes a severe ecotoxicological concern. Moreover, physical methods consume a sizable quantity of energy in Ag-NPs synthesis due to furnaces and tubes with high temperature and pressure (Hasan *et al.*, 2022; Ying *et al.*, 2022; Singh *et al.*, 2023). As such, there is an increased demand for greener approaches in view of more safety, rapidness, economic viability and availability (Dikshit *et al.*, 2021). For instance, Baláz *et al.* (2017) reported the novel combination of green and mechanochemical synthesis of Ag-NPs. Ball milling of *Origanum vulgare* L, or eggshell membrane extract and AgNO_3 was applied; it resulted in Ag-NPs with 4 nm and 3 nm, respectively.

However, biological-based synthesis is cost-effective as less resource and energy-intensive; it applies a bottom-up approach in which substances with a biological origin are employed as biofactories for the development of green-synthesized Ag-NPs. These reducing agents can include complex mixtures or individual compounds derived from plants, microorganisms, templates or agro-industrial waste (Ying *et al.*, 2022). This acts as key distinction between biological synthesis and chemical-based protocols (Hasan *et al.*, 2022).

2.3.1. Characterization of Ag-NPs

Several techniques are used to characterize and study morphological, chemical and structural features of Ag-NPs; such analysis is vital to subsequently determine functional aspects and end-use applications. Table 2 describes the most commonly used techniques for characterization.

Table 2 - Methods used for Ag-NPs characterization.

Characterization Method	Application	Reference
UV-Visible Spectroscopy (UV-VIS)	Size, shape, stability	
Fourier Transform Infrared (FTIR)	Identification of surface residues and functional groups	Khan <i>et al.</i> (2017) Kovács <i>et al.</i> (2022) Jassal <i>et al.</i> (2022)
Scanning Electron Microscopy (SEM)	Size, shape, surface properties	
Transmission Electron Microscopy (TEM)	Size distribution, shape, dispersity	
X-Ray Diffraction (XRD)	Morphology, crystal structure and crystallite size	
Dynamic Light Scattering (DLS)	Particle size distribution, hydrodynamic diameter	

As mentioned before, biological compounds act as reducing agents and cause a reduction in Ag^+ ions. A change of color occurs, ensuring the optimal growth of the crystal; thus, color change of the solution is the simplest way to analyze the formation of Ag-NPs (Sunny *et al.*, 2022). As exemplified by Tarannum *et al.* (2019), literature reports the appearance of colorless or pale yellow solution to dark brown as a primary indication of Ag-NPs synthesis.

Spectroscopy is the study of the emission and absorption of light and radiation by substances, as a function of the wavelength of the electromagnetic radiation. Conduction and valence bands

in Ag-NPs lie very close to each other and electrons move freely, instigating a SPR band (Shnoudeh *et al.*, 2019). UV-VIS data shows the maximum absorbance wavelength for synthesized Ag-NPs is in the range of 400-460 nm and a peak confirms its presence (Tarannum *et al.*, 2019).

Size and hydrodynamic diameter of particles ranging in size from 10 to 1000 nm are estimated by DLS, providing an intensity-based distribution. It is considered a noninvasive, reproducible, easy and short duration technique. However, it is easily affected by the presence of larger particles, and so, the measured size is somewhat larger than the size measured from XRD and TEM (Dawadi *et al.*, 2021). The principle of DLS relies on the Brownian movement of particles in a solution or suspension, resulting in the changeability of the scattering intensity of light, that is, Rayleigh scattering, at various intensities (Dawadi *et al.*, 2021). Relative discrepancies in intensity will yield the velocity of the Brownian motion and particle size can be calculated using the Stokes-Einstein relationship (Equation 1) (Shnoudeh *et al.*, 2019).

$$D_h = \frac{K_B T}{3\pi\eta_0 D_t} \quad (1)$$

where D_h is the hydrodynamic meter, K_B the Boltzmann constant, T the temperature in K, η_0 the solvent viscosity, and D_t the translational diffusion coefficient.

2.4. Formation Mechanism of Biogenic Ag-NPs

Biogenic synthesis of Ag-NPs consists of reduction of Ag^+ to Ag^0 and successive nucleation and growth of Ag^0 into Ag-NPs (Dikshit *et al.*, 2021; Islam & Sun, 2022). An illustration of the proposed mechanism of synthesis is shown in Figure 2.

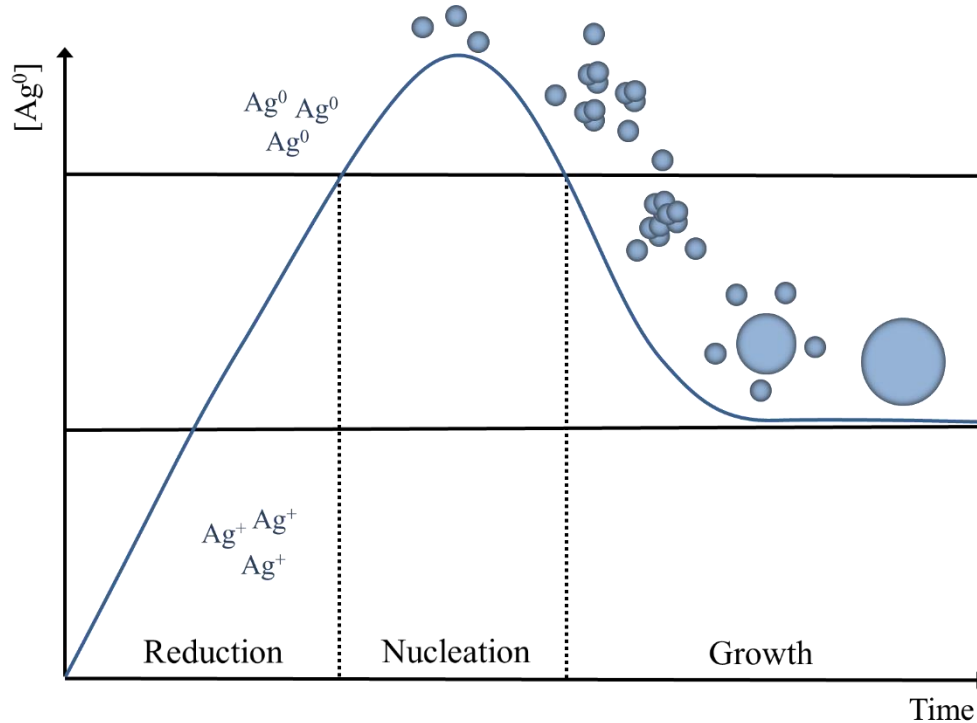


Figure 2 - Mechanism scheme of biogenic synthesis of Ag-NPs (adapted from Liu *et al.*, 2015).

Reduction of Ag^+ of a silver source to Ag^0 occurs with the formation of a chelate between the metal and the functional groups present in numerous biomolecules (i.e., phenolics, flavonoids, flavanones, terpenoids, proteins, polysaccharides, vitamins, carbohydrates, saponins, tannins,

sugars, amino acids, phytosterols, carboxylic acids and alkaloids). It is assumed that functional groups such as -CHO, -C=O, -COOH, -OH, -C-O-C and -C=C bind to metals; thus, surface chemistry of biomolecules affects reduction and capping of metal ions. Capping refers to their incorporation on the surface of Ag-NPs, contributing to the avoidance of agglomeration (Makarov *et al.*, 2014). Silver nitrate (AgNO₃) is frequently used as a precursor for being stable, easily available and less expensive; however, silver tetrafluoroborate (AgBF₄) and silver perchlorate (AgClO₄) were also reported in papers (Szczyglewska *et al.*, 2023).

Nucleation involves migration between metal plates and particles, forming agglomerates called clusters. Clusters tend to form small Ag-NPs that spontaneously coalesce into larger particles via non-covalent interactions, occurring growth phase and an increase in the thermodynamic stability; this is known as Ostwald ripening (Ramrakhiani & Ghosh, 2018; Rodríguez-Félix *et al.*, 2022). The ability of stabilization of the biological reducing agent is shown in the termination phase, where Ag-NPs acquire the most energetically favorable conformation (Makarov *et al.*, 2014). Through adjusting variable parameters like reaction time, temperature, pH, precursor and extract concentration, biogenic Ag-NPs can be synthesized with adequate size and shape (Hasan *et al.*, 2022).

2.4.1. Critical Factors

Various experimental parameters are key to optimization of Ag-NPs biosynthesis, affecting the yield, size, shape, stability, charge and agglomeration. It is recognized that control of shape and size of Ag-NPs further enhances their functionality for future applications (Dikshit *et al.*, 2021).

Critical factors involved in the optimal synthesis include composition and concentration of the reducing extract, Ag⁺ source and concentration, as well as the stoichiometric ratio of reducing agent and metal solution. Additionally, external factors like incubation temperature, reaction mixture pH, reaction time and exposure to light must be considered (Makarov *et al.*, 2014; Paiva-Santos *et al.*, 2021).

According to Makarov *et al.* (2014), alteration of pH results in a charge change of the natural phytochemicals, affecting their reducing ability; thus, this factor exerts great influence in the shape and size of Ag-NPs. Functional groups are present in protonated form in acidic conditions, therefore, less accessible and result in poor stability and aggregation of NPs; conversely, basic pH allows hydrogen bonding dissociation, optimizing the synthesis at pH range of 7-11 (Tarannum *et al.*, 2019).

It seems most studies agree that smaller-sized and more uniformly distributed Ag-NPs are generated at an optimal room temperature (25-27 °C). A decrease in temperature leads to low production rates; an increase can result in denaturation of phytochemicals and agglomeration of larger-sized Ag-NPs (Tarannum *et al.*, 2019; Shaikh *et al.*, 2021). However, temperature elevation can also speed up reaction time and consequently form smaller-sized Ag-NPs with bigger peak intensity (Paiva-Santos *et al.*, 2021).

Indeed, literature claims biogenic synthesis of Ag-NPs is more eco-friendly than chemical and physical routes for perceived ecological and economic impacts. However, it is recognized that there is a lack of identification of specific bioactive compounds involved in the process. For example, nearly 4000 phytochemicals have been found in plants to date; therefore, it is considered an immutable parameter and hinders to ascertain the full mechanism involved in Ag-NPs synthesis (Hasan *et al.*, 2022).

2.5. Biogenic Synthesis of Ag-NPs

2.5.1. Synthesis of Plant-Assisted Ag-NPs

Plant-assisted biosynthesis is the most preferred to circumvent the constraints of physical and chemical approaches, among green routes. It is a straightforward method; often, plant extracts are mixed with a metal salt solution at room temperature until a change of the solution color primarily indicates the formation of NPs (Shnoudeh *et al.*, 2019).

Plant extracts derive from various parts of plants, including leaves, roots, fruits, seeds, stems and flowers. These fragments contain a range of phytochemicals and bioactive compounds (i.e., organic acids, proteins, vitamins and secondary metabolites, namely alkaloids, phenolics, flavonoids, terpenoids and polysaccharides) that act as capping and reducing agents in the reduction and stabilization of Ag⁺ ions (Dikshit *et al.*, 2021; Huston *et al.*, 2021; Patil *et al.*, 2021). Common features of these biological compounds are their reducing and chelating abilities to interact with silver ions (Islam & Sun, 2022).

Table 3 presents a collection of studies reporting on the biosynthesis and applications of biogenic Ag-NPs using various plants.

Table 3 - Plant-assisted Ag-NPs biosynthesis and applications.

	Extract/Plant	Ag Precursor	Morphology/Size (nm)	Application	Ref.
Flowers	<i>Allium sativum</i>	AgNO ₃	Cubic/26	Detection of heavy metals	Aravind <i>et al.</i> (2018)
	<i>Syzygium aromaticum</i>	AgNO ₃	Spherical/30-60	Wound healing	Parveen <i>et al.</i> (2018)
	<i>Fraxinus excelsior</i>	AgNO ₃	Spherical	Coloration of woven glass	Hasan <i>et al.</i> (2021)
Leaf	<i>Ziziphus jujuba</i>	AgNO ₃	Cubic/20-30	Environmental protection	Gavade <i>et al.</i> (2015)
	<i>Camellia sinensis</i>	AgNO ₃	Spherical/10-60	Colorimetric sensing of Hg ²⁺	Chandra <i>et al.</i> (2000)
	<i>Symphytum officinale</i>	AgNO ₃	Irregular/87	Photoaging protection	Singh <i>et al.</i> (2018)
	<i>Azadirachta indica</i>	AgNO ₃	22-30	Plant growth	Ansari <i>et al.</i> (2023)
Seed	<i>Persea americana</i>	AgNO ₃	Spherical/20-40	Dye sensing	Girón-Vázquez <i>et al.</i> (2019)
	<i>Salvia hispanica</i>	AgNO ₃	Spherical/1-27	Environmental protection	Hernández-Morales <i>et al.</i> (2019)

A study of the effect of pH on the synthesis of Ag-NPs was made by Gavade *et al.* (2015). Synthesized 20-30 nm cubic Ag-NPs using leaf extract of *Z. jujuba* were submitted to different pH (4-9). Phenolic groups present in leaf extract ionized and formed Ag-NPs rapidly in neutral and basic pH, displaying narrower bands due to particle size decrease. Meanwhile, pH 4 to 6 presented wider bands and an increase in particle size since formation and aggregation of Ag-NPs was slower.

On the other hand, Girón-Vázquez *et al.* (2019) used different concentrations of *P. americana* seed extract (0.25, 0.5 and 2.0 g) to form spherical Ag-NPs. Results showed that the size of Ag-NPs increased with the increase in extract content; in addition, antimicrobial activity against *E. coli* was superior, being suitable for dye sensing. Similarly, Hasan *et al.* (2021) treated 2 glass woven fabric samples (with 0.01 M and 0.02 M AgNO₃, respectively) *in situ* with spherical Ag-NPs produced from *F. excelsior*. Overall, both samples provided stable and durable colors; however, the sample with a bigger quantity of precursor exhibited a darker tone.

A. sativum flower bud extract was explored by Aravind *et al.* (2018) as a reducing agent for Ag-NPs for selective detection of Cd²⁺ ions in water samples collected from pigment, cosmetics and fertilizer industries. The resulting cubic NPs exhibited antibacterial property against *E. coli* and *S. aureus* from a lake water sample collected from Kerala. Likewise, Chandra *et al.* (2000) developed Ag-NPs prepared from *C. sinensis* for colorimetric sensing of Hg²⁺ ions. Tea leaves were collected from three different elevations (86, 1700 and 2000 m) above sea level in eastern Nepal to understand the effect of elevational gradient. Ag-NPs prepared from hilly region (1700 nm) presented the highest Ag₂O nanocrystallites content, which helped to shape smaller and narrow-sized particles; on the contrary, larger and broader-sized particles were produced from mountainous region (2000), being less suitable for Hg²⁺ sensing.

Photoaging occurs by overexpression of matrix metalloproteinase-1 (MMP-1) and degradation of type I procollagen. Singh *et al.* (2018) synthesized irregular Ag-NPs from *S. officinale* leaf extract and investigated their anti-aging effect in human keratinocyte cells (HaCaT). In the presence of 100 µg/mL Ag-NPs, MMP-1 expression was decreased to 59% of the normal value and expression of type I procollagen was enhanced, making Ag-NPs suitable for cosmetic. Another application related to biomedical sciences was tested by Parveen *et al.* (2018). The authors studied the wound healing property of Ag-NPs prepared from *S. aromaticum* flower bud extract. The spherical NPs were added to a sample cream base and tested in excision and incision wounds in albino rats; silver sulfadiazine was used as reference standard. Wounds treated with 5% Ag-NPs required 28 days for healing whereas it took 38 days for the reference cream.

2.5.2. Synthesis of Microbe-Assisted Ag-NPs

Several microorganisms such as bacteria, fungi, algae and viruses are also reported for Ag-NPs synthesis. In comparison to plant-assisted biosynthesis, microbial systems may involve an additional cultivation and purification step aimed to obtain the active agents. Cell lysates, whole cells or extracts through intracellular and extracellular approaches are combined with the metal solution (Maátková *et al.*, 2022). Intracellular mechanism is probably linked to enzymes and electrostatic interactions; Ag⁺ is reduced to Ag⁰ and silver ions are synthesized within the protoplasm. Extracellular synthesis involves the formation of Ag-NPs in the secretions of the microorganism (Akhil *et al.*, 2023). Thus, there is a need for more maintenance and control, as biosafety issues might arise with microbial strains (Maátková *et al.*, 2022).

More than 120 species of fungi from different taxa are reported to produce Ag-NPs (Loshchinina *et al.*, 2022). Presence of mycelia provides an increased and resilient surface area, making fungal isolates a more prolific source of Ag-NPs than bacteria. Intercellular enzymes and proteins promote metal bioaccumulation and chelation; besides, downstream handling and processing is simpler (Ahmed *et al.*, 2022).

Table 4 shows a collection of studies reporting on the biosynthesis and applications of biogenic Ag-NPs using microorganisms.

Table 4 - Microbe-assisted Ag-NPs biosynthesis and applications.

	Microorganism	Morphology/Size (nm)	Application	Reference
Fungi	<i>Fusarium keratoplasticum</i>	Spherical/6-36	Paper conservation	Fouda <i>et al.</i> (2019)
	<i>Fusarium oxysporum</i>	Spherical/28	Antiseptic clothing	Ballotin <i>et al.</i> (2017)
	<i>Penicillium aculeatum</i>	Spherical/4-55	Drug delivery	Liang <i>et al.</i> (2017)
Algae	<i>Chlorella pyrenoidosa</i>	Cubic/2-15	Wastewater treatment	Aziz <i>et al.</i> (2015)
	<i>Portieria hornemannii</i>	Spherical/35-50	Fish diseases treatment	Fatima <i>et al.</i> (2020)
	<i>Chlorella vulgaris</i>	Spherical/55	Dye degradation	Rajkumar <i>et al.</i> (2021)
Bacteria	<i>Bacillus licheniformi</i>	Spherical/19-63	Biofilm control	Shanthi <i>et al.</i> (2016)
	<i>Bacillus subtilis</i>	Spherical/2-26	Antibiotics	Yu <i>et al.</i> (2021)
	<i>Pseudomonas proteolytica</i>	Spherical/7	Bactericidal activity	Shivaji <i>et al.</i> (2011)
	<i>Leptolyngbya sp.</i> WUC 59	Spherical/20-35	Seed germination	Singh <i>et al.</i> (2020)

An interesting application of a fungal isolate for Ag-NPs synthesis is given by Fouda *et al.* (2019). The authors utilized biogenic Ag-NPs synthesized by *F. keratoplasticum* to inhibit the growth of bacteria and fungi isolated from an Egyptian archaeological manuscript. *B. subtilis* and *P. chrysogenum* showed the highest cellulase activity in the manuscript; therefore, these strains were inoculated in a filter paper to simulate the heritage document biodeterioration and treated with Ag-NPs. The effect of NPs was found to be concentration dependent; after 21 days of aging, 1 mM of Ag-NPs showed a total inhibition against *B. subtilis* strain, while 2 mM Ag-NPs completely inhibited *P. chrysogenum*. On the other hand, untreated papers formed mucous layers and growth of *B. subtilis* in the same period. Thus, biogenic Ag-NPs can be applied by spraying to protect paper from biodeterioration.

Also, Ballotin *et al.* (2017) reported the synthesis of spherical Ag-NPs from a *F. oxysporum* filtrate solution. Antimicrobial activity against *Candida* sp. (*C. parapsilosis* and *C. albicans*) and *Xanthomonas axonopodis* pv. *citri* was monitored. A 1 cm² cotton fabric impregnated with Ag-NPs was washed 10 times; 17.5% of silver remained in the fabric after the first wash, 5% after the second and 3% for the remaining washing cycles. In addition, *C. parapsilosis* presented an inhibition halo of 16 mm while *C. albicans* and *X. axonopodis* exhibited an halo of 11 mm; thus, the textile impregnation of Ag-NPs revealed an efficient inhibition of microbial growth. These results point biogenic Ag-NPs as potential antiseptic agents in textiles for medical and agronomic applications.

Red algae *P. hornemannii* was employed for the first time for the synthesis of Ag-NPs. Fatima *et al.* (2020) obtained spherical and Ag-NPs in the range 35-50 nm and studied the antimicrobial activity against pathogenic *Vibrio* species (*V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus* and

V. anguillarum). Zone of inhibition of ranged between 16-28 mm, yielding promising results against fish pathogens responsible for severe mortalities in aquaculture.

An example of synthesis of Ag-NPs by using aqueous extract of the cyanobacterium *Leptolyngbya* sp. WUC 59 taken from polluted water is given by Singh *et al.* (2020). The resulting spherical NPs showed a significant potential as biocontrol agents in enhancing seed germination and early seedling growth of wheat (*Triticum aestivum* L.) at low concentration (25 mg/L).

2.5.4. Toxicity and Sustainability of Biogenic Ag-NPs

Toxicity of physio-chemically synthesized Ag-NPs is attributable to particle size, dosage, coating agents, release of Ag⁺ ions, ROS (i.e., superoxide radical (O₂⁻), hydroxyl radical (OH⁻), hydrogen peroxide (H₂O₂)) and DNA binding (Khoshnamvand *et al.*, 2020; Ahmed *et al.*, 2022). For example, Farkas *et al.* (2011) identified the release of an average concentration of 11 µg/L of 10 nm Ag-NPs in a nano-washing machine effluent. Specifically, coating agents of these Ag-NPs consist of chemicals or synthetic polymers. It is known that intrinsic activities of bioactive compounds attached to biogenic Ag-NPs surface reduces toxicity; still, studies of impact and release into the environment are scarce and should be thoroughly developed (Paiva-Santos *et al.*, 2021).

Exposure and deposition of Ag-NPs via the air, soil and water to human population and aquatic, atmospheric and lithospheric systems is inevitable (Akhil *et al.*, 2023). Xu *et al.* (2020) refer absorbed Ag-NPs are distributed in the respiratory, digestive, urinary, nervous and reproductive system, mainly in the liver, kidney and lungs. Plants are in direct contact with all environmental matrices; NPs present in the air, soil and water are absorbed into plant body through the stomatal openings on leaves or selectively up taken by plant roots, hampering their growth and development (Dikshit *et al.*, 2021). An example regarding aquatic chains is given by Khoshnamvand *et al.* (2020), that adopted aquatic organisms, namely *Chlorella vulgaris*, *Daphnia magna* and *Danio rerio*, as different trophic level models to evaluate the toxicity of biogenic Ag-NPs from leaf extract of *Alcea rosea*. Mortality of *D. magna* and *D. rerio* was observed with increased dose of Ag-NPs, indicating possible exerted toxic effects.

2.6. Synthesis of Agri-Food Waste-Assisted Ag-NPs

Management of food and agriculture waste has emerged as a major concern in recent years; these can be generated in pre- and post-consumer stages, including harvesting, transportation, storage and marketing (Rodríguez-Félix *et al.*, 2022). Accordingly, synthesis of Ag-NPs using agro-industrial waste broadens the panorama of newer precursor agents, conveniently exploiting and assessing such materials as resources in the burgeoning field of nanotechnology (Omran *et al.*, 2017).

It has been reported the synthesis of Ag-NPs using cereals of low-quality crops (Lieu *et al.*, 2018), alcoholic beverages, bagasse (Shen *et al.*, 2016; Arroyo *et al.*, 2020) and torn and bruised vegetable waste and fruit peels (Mythili *et al.*, 2018). Examples of fruit waste include bananas, apples, pears and citrus crop (oranges, grapefruits, lemons and mandarins); which is the most abundant in the world (Rodríguez-Félix *et al.*, 2022).

2.6.1. *Citrus sinensis*

Orange (*Citrus sinensis*) production and processing generates more than 3 million tonnes of waste annually; by-products include peel (flavedo and albedo), pulp and seeds. Such waste is bulky, heterogenous, highly biodegradable and chemically complex, containing a wide range of bioactive compounds (Panić *et al.*, 2021; Viñas-Ospino *et al.*, 2021).

Specifically, orange peels (OP) represent nearly 50% of the wet-fruit mass and are an excellent source of bioactive compounds: essential oils, polyphenols, fibers, minerals, carotenoids, pectin and monosaccharides; all of which have potential applications in nanobiotechnology, such as in the synthesis of Ag-NPs (Brezo-Borjan *et al.*, 2023). Moreover, it presents a chemical oxygen demand (COD) of 1085 mg O₂/g, has a low pH (3-4), a high water content (80-90%) and organic matter (95% of total solids) (Negro *et al.*, 2016; Mackenzie *et al.*, 2019). Still, it is often used as low-value animal feed additive, incinerated or dumped in landfills; this exerts environmental issues and financial costs (Omran *et al.*, 2017).

Besides, extraction of bioactive compounds is mostly performed by costly and toxic aqueous-organic systems, such as *n*-hexane, toluene, *n*-butanol, ethanol and methanol in conventional (i.e., percolation, maceration, Soxhlet extraction) or non-conventional (i.e., microwave-assisted extraction, ultrasound-assisted extraction and enzyme-assisted extraction) techniques (Gómez-Urios *et al.*, 2022). Water is also commonly used; however, it is only effective against polar compounds and has less effect on non-polar compounds (Dheyab *et al.*, 2021). In this regard, it is urgent to replace petrochemical solvents and opt for green solvents who could overcome such limitations. Alternatively, Pires *et al.* (2022) mention that the use of NADES in extraction processes of bioactive compounds in different plant matrices has shown similar results to conventional organic solvents. On the other hand, Panić *et al.* (2021) mention that NADES-assisted valorization of orange peel has been rarely investigated.

In fact, ideal solvents should be low cost, non-volatile, non-flammable, stable and based on renewable sources; NADES are able to address all these requirements. NADES are obtained by the complexation of a hydrogen bond acceptor (HBA) (i.e., non-toxic quaternary ammonium salts) and a hydrogen bond donor (HBD) (i.e., amines, sugars, carboxylic acids, polyalcohols) (Viñas-Ospino *et al.*, 2021). In addition, Radošević *et al.* (2016) demonstrated low cytotoxicity of NADES by testing two human cell lines (MCF-7 and HeLa) *in vitro*; thus, NADES can be directly used in products for human consumption without downstream purification steps. However, the major disadvantage of NADES, comparingly to conventional solvents, is their inherent high viscosity, that is affected by hydrogen bonding and van der Waals interactions. Consequently, the efficiency of NADES as extraction solvents is hindered, since diffusion coefficients of analytes are reduced, leading to a slow mass transfer. Yet, this condition is adjustable by adding water or increasing the temperature (Dai *et al.*, 2013; Hikmawanti *et al.*, 2021). As a result, physicochemical characteristics of NADES can be modulated to meet specific demands of the process. Hence, they are confirmed as excellent solvents for performing chemical and enzymatic reactions and were tested in this work.

Table 5 presents a collection of studies from literature that used *C. sinensis* as a reducing agent for the synthesis of green Ag-NPs. All experiments used water as solvent.

Table 5 - Collection of reported studies using *Citrus sinensis* as a reducing agent for Ag-NPs synthesis.

Agents	Conditions	Morphology/Size (nm)	Peak (nm)	Activity	Ref.
Peel/AgNO ₃	25/60 °C pH 11 4 h	Spherical/17-32	442	Antibacterial	Nahar <i>et al.</i> (2020)
Peel/AgNO ₃	25 °C 5 h	Spherical/10-50	440	Antimicrobial	Niluxsshun <i>et al.</i> (2021)
Peel/AgNO ₃	40 °C pH 9 24 h	Spherical/3-14	422		Omran <i>et al.</i> (2017)
Peel/AgNO ₃	25 °C pH 10 2 h	Spherical/5-25	420	Antioxidant and antibacterial	Mogole <i>et al.</i> (2021)
Peel/AgNO ₃	37 °C	Cubic/41	420	Antibacterial	Logeswari <i>et al.</i> (2013)
Peel/AgNO ₃	50 °C pH 10 4 min	Irregular/55	420	Optical	Aminu & Oladepo (2021)
Leaf/AgNO ₃	25 °C 12 h	Spherical/78	446	Antioxidant and anticancer	Chen <i>et al.</i> (2022)
Peel/AgNO ₃	25/60 °C	Spherical/10-35	424/445	Antibacterial	Kaviya <i>et al.</i> (2011)

Kaviya *et al.* (2011) prepared spherical OP-Ag-NPs at room temperature (25 °C) and 60 °C, using peel extract as a reducing and capping agent. Size of the OP-Ag-NPs was determined to be 35 nm and 10 nm for 25 °C and 60 °C, respectively; temperature increment may lead to the formation of smaller NPs due to a faster consume of reactants. Moreover, OP-Ag-NPs exhibited higher antibacterial activity against *E. coli* (16 mm) and *P. aeruginosa* (13 mm) (Gram-negative) than *S. aureus* (9 mm) (Gram-positive) due to differences in the structure of the cell wall. Logeswari *et al.* (2013) also synthesized OP-Ag-NPs similarly, but at a mild temperature (37 °C); contrarily, highest antibacterial activity was higher against *P. aeruginosa* (16 mm), followed by *S. aureus* (12 mm) and *E. coli* (12 mm).

Antioxidant and anticancer assays were explored on OP-Ag-NPs synthesized by Chen *et al.* (2022). Resulting OP-Ag-NPs presented an IC₅₀ of 21 µg/mL compared to 15 µg/mL of vitamin E. In addition, viability of human lung cancer cells decreased dose-dependently in the presence of OP-Ag-NPs; for example, cell viability was less than 10% when exposed to 500 µg/mL OP-Ag-NPs. In conclusion, antioxidant activity is likely linked to anti-human lung cancer effects.

Aminu & Oladepo (2021) utilized OP-Ag-NPs for visual colorimetric detection of Hg²⁺ ions in water, reporting sensitivity and selectiveness ten times faster than nanocomposite sensors.

The above reports suggest that *C. sinensis* peels may act as a promising source of reducing agents for the synthesis of biogenic Ag-NPs. Accordingly, the present work aims to study this purpose.

Chapter 3. Materials and Methods

3.1. Chemicals and Reagents

DL-Malic acid (MA) (>99%, CAS no. 6915-15-7) and L-proline (LP) (>99%, CAS no. 147-85-3) were purchased from Thermo Scientific (China). D(-)-fructose (Fru) (>99%, CAS no. 57-48-7), D(+)-glucose (Glu) (>99.5%, CAS no. 50-99-7), Folin-Ciocalteu (FC) reagent and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were provided by Sigma-Aldrich (USA). Choline chloride (ChCl) (<98%, CAS no. 67-48-1) was obtained from Thermo Scientific (Germany). Ethanol (EtOH) (CAS no. 64-17-5) was purchased from VWR Chemicals (France).

AgNO₃ (≥99%, CAS no. 7761-88-8) was purchased from Carlo Erba Reagenti (Italy) and used to prepare a 1 M AgNO₃ stock in deionized water. Sodium hydroxide (NaOH) (CAS no. 1310-73-2) was supplied by Merck (Germany) and used to prepare a 1 M NaOH stock in deionized water. Hydrochloric acid (HCl) 0.1 M and sodium carbonate (Na₂CO₃) 10% (w/v) were previously prepared and used.

3.2. Raw Material

A batch of 3 kg of oranges from Egypt (*Citrus sinensis* var. Valencia) was purchased in July 2023, at a local supermarket in Rome, Italy. OP were removed from the pulp, cut into small pieces and washed with distilled water. Finally, they were milled with a food grinder (G3 Ferrari) and stored at 4 °C until use. A sample was oven-dried (Heraeus D-6450 Hanau) at 100 °C until constant weight was acquired.

3.3. Preparation of NADES

NADES were prepared according to the method of Fanali *et al.* (2021) and Viñas-Ospino *et al.* (2021). Reagents were mixed in specific molar ratios and the mixture was stirred at 60-80 °C in a water bath (Heidolph MR 3001), until an homogeneous transparent liquid formed. An addition of 25% (v/v) water in the NADES solutions was performed. Four different NADES systems with two components (HBA and HBD) were obtained. Table 6 shows their composition, molar ratio and % of water in NADES.

Table 6 - Composition of NADES. HBA: hydrogen bond acceptor; HBD: hydrogen bond donor.

Abbreviation	Component 1 (HBA)	Component 2 (HBD)	Molar Ratio	Water (%)
LP:MA	L-Proline	DL-Malic acid	1:1	25
MA:Gluc	DL-Malic acid	Glucose	1:1	25
ChChl:Fruc	Choline chloride	Fructose	2:1	25
ChChl:Gluc	Choline chloride	Glucose	2:1	25

Density of the different NADES was determined according to the method of Grozdanova *et al.* (2020). 10 mL of NADES were put in a volumetric flask at 20 °C and the weight of the liquid was measured in an analytical balance (Sartorius CP324S-0CE) with an accuracy of ±0,0001 g. Density was calculated using Equation 2.

$$\rho = \frac{m_{NADES}}{V_{NADES}} \quad (2)$$

where ρ is density at 20 °C in g/mL, m_{NADES} is the weight at 20 °C in g, and V_{NADES} is the volume at 20 °C in mL.

pH was measured in a pH meter (HANNA HI 3222).

3.4. Extraction of Bioactive Compounds

Extraction of compounds with NADES was done according to the optimized extraction of Viñas-Ospino *et al.* (2021) with some modifications. OP samples and NADES were mixed in a solid-liquid ratio of 2:20 g/mL for 30 min, at 45 ± 5 °C. Extraction was carried out by magnetic stirring and heating in a circulating bath (Neslab® RTE 7). Samples were then centrifuged at 10000 rpm for 10 minutes (MPW-380). The supernatant layer was stored in dark tubes at 4 °C until analysis.

Extraction of compounds with water was done according to Rao *et al.* (2015). OP samples and water were mixed and boiled in a solid-liquid ratio of 20:100 g/mL for 2 h. Extraction was carried out by magnetic stirring and heating (Heidolph MR 3001). Extracts were then filtered through Whatman No. 1 filter paper and stored in dark tubes at 4 °C until analysis.

3.5. Green Synthesis of OP-Ag-NPs

Ag-NPs synthesis was carried out in glass vials equipped with a magnetic bar, at room temperature (25 °C) for 24 hours. Different ratios of $[Ag^+]/mM_{GAE}$ were studied by using OP water-assisted extract as a reducing agent and $AgNO_3$ salt solution (1 M) as an Ag^+ source. Deionized water was added until the total volume was 10 mL. NaOH (1 M) was added in appropriate amount to change the pH from acid (4) to basic (11).

Table 7 shows the volume, in mL, and concentration, in mM, of the reactants.

Table 7 - Volume and concentration of reactants used for the synthesis of Ag-NPs.

$[Ag^+]/[mM_{GAE}]$	$AgNO_3$ (mL)	$AgNO_3$ (mM)	Extract (mL)	Extract (mM) GAE	H_2O (mL)
0.75	0.499	5	3.81	6.67	5.69
1.5	0.499	5	1.91	3.33	7.60
3	0.499	5	0.95	1.67	8.55
6	0.499	5	0.48	0.83	9.02
12	0.499	5	0.24	0.42	9.26

3.6. Analytical Methods

TPC was determined by oxidation of phenolic compounds using Folin-Ciocalteu reagent, as reported by Singleton *et al.* (1999). In a 10 mL glass vial, 200 μ L of each extract (ratio 1:5) was mixed with FC reagent (145 μ L) and HCl 0.1 M (5 mL); Na_2CO_3 10% (w/v) was added until the final volume. The mixture was incubated in the dark at room temperature for 30 minutes and absorbance was measured in a photometer (HANNA HI 83742) at 760 nm, using deionized water as a blank. Results were expressed as mg of gallic acid equivalents per g of OP (mg_{GAE}/g_{OP}) using a calibration curve obtained with gallic acid standards.

Antioxidant activity was measured using the DPPH radical assay, as described by Kedare & Singh (2011). 3.9 mL of DPPH solution was mixed with 100 μ L of sample and placed in the dark. After 30 minutes, absorbance at 517 nm was measured. Results were expressed as mg of trolox equivalents per g of OP (mg_{TE}/g_{OP}) (TEAC).

Periodic (15, 30, 60, 120, 1440 min) monitoring of Ag-NPs synthesis was assessed spectrophotometrically (Shimadzu UV-2700) in the wavelength range of 250-600 nm. A small aliquot of 30 μ L of sample was diluted in 3 mL of deionized water prior to the spectral characterization. Deionized water was used as a blank throughout the experiment.

Hydrodynamic diameter measurements were made by DLS on a LitesizerTM 500 (Anton Paar). Measurements were performed at room temperature (25 °C) and the angle was backscatter.

Figure 3 illustrates the main steps of the experimental scheme.

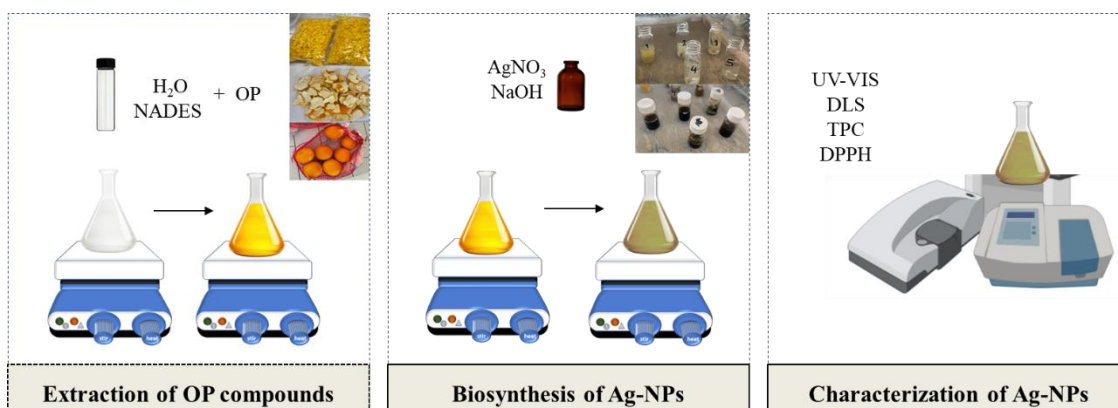


Figure 3 - Experimental scheme of the main steps.

Chapter 4. Results and Discussion

4.1. Production of OP Extracts

Green synthesis of Ag-NPs is based on the use of reducing agents present in the composition of biological sources. OP was chosen since it is an abundant and compound-rich industrial and household waste.

Moisture content of OP was 83.6% and Brezo-Borjan *et al.* (2023) refer that it contains several bioactive compounds with reducing activity such as polyphenols. It is well known that the overall extraction yield of these compounds is strongly affected by sample-solvent ratio, reaction temperature and selection of a suitable solvent (Gómez-Urios *et al.*, 2022).

Sample-solvent ratios were chosen according to the optimal extractions done by Viñas-Ospino *et al.* (2021) and Rao *et al.* (2015). Literature suggests an optimal time between 30 to 180 minutes for polyphenolic compounds extraction; however, prolonged operation can lead to oxidation (Mogole *et al.*, 2021). Hence, reaction time was set to 30 and 120 minutes, according to each solvent. Interestingly, H₂O is the most used solvent for extraction of biomolecules for the purpose of NPs synthesis; for this reason, different NADES were also chosen to establish and compare greener solvents.

Figure 4 displays the results of TPC and TEAC of OP extracts.

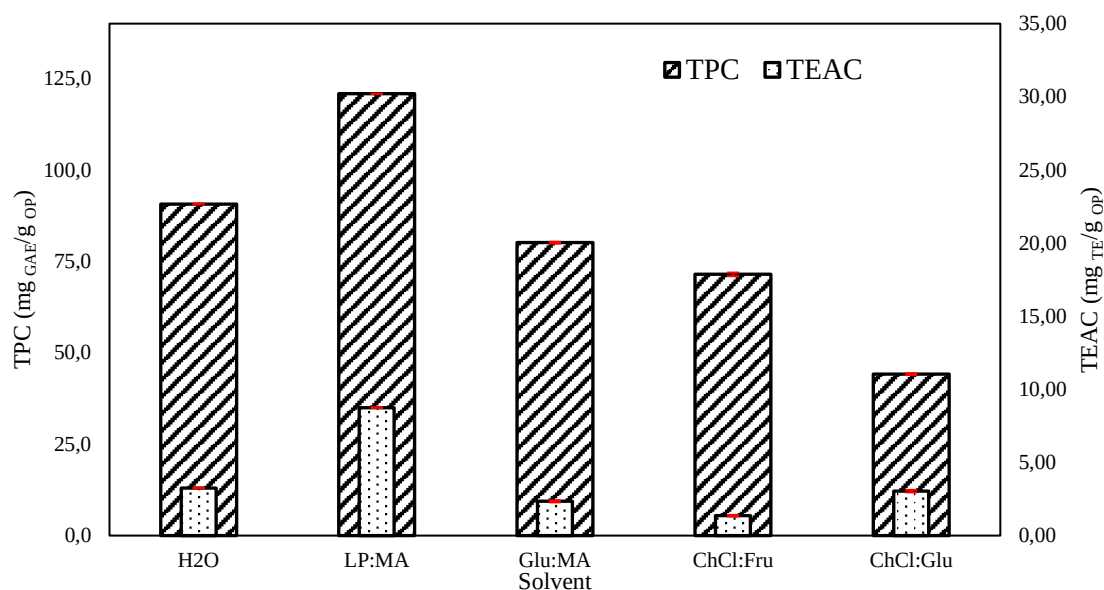


Figure 4 - OP extracts: total phenolic content (TPC (mg GAE/g OP) and trolox equivalent antioxidant activity (mg TE/g OP). Data are mean $\pm$ SD (n = 3).

Results shown in Figure 4 indicate that LP:MA achieved the highest TPC (120.8 mg GAE/g OP) and TEAC (8.75 mg TE/g OP) content. Overall, NADES-assisted extracts ranged from 44.1-120.8 mg GAE/g OP and 1.36-8.75 mg TE/g OP. H₂O as solvent also presented a high value of TPC (90.7 mg GAE/g OP) and TEAC (3.2 mg TE/g OP). These results confirm LP:MA and H₂O were the most efficient solvents to absorb phenolic compounds on the matrix by physical adsorption and chemical interactions. To note that H₂O can only extract polar compounds and it is possible LP:MA could extract both polar and non-polar compounds.

Previous studies reported a range of TPC (10.9-5180 mg GAE/100 g OP dry weight) (Tejero *et al.*, 2021; Gómez-Urios *et al.*, 2022; Viñas-Ospino *et al.*, 2023). These results may differ due to

several factors such as climate, season and geographical conditions of OP and solvent properties (Mogole *et al.*, 2021).

4.1.1. Screening of Solvents

In this work, H₂O and NADES derived from sugars (glucose, fructose), choline chloride and acids (malic and proline), were utilized as green solvents. Visual appearance and physicochemical properties (density and pH) of the NADES under study were evaluated; the latter are expected to affect the extraction of the target compounds (Gómez-Urios *et al.*, 2022). Table 8 shows the density, pH and visual appearance of NADES.

Table 8 - Physicochemical properties and visual appearance of NADES.

	MA:Glu	LP:MA	ChCl:Glu	ChChl:Fru
Visual appearance				
	Transparent light-yellow liquid	Transparent yellow liquid	Transparent liquid	Transparent liquid
Density (g/cm³)	1.37	1.34	1.18	1.19
pH	0.78	2.58	4.84	6.88

NADES could be great stabilizing agents in Ag-NPs formation, as confirmed by Liu *et al.* (2022). Besides, it would be interesting to produce NADES-assisted OP extracts since it has been rarely investigated (Panić *et al.* (2021) and further apply them as reducing agents for Ag-NPs formation, which was also not found in literature. However, as aforementioned, viscosity of NADES hinders their efficiency as extraction and medium solvents due to low mass and time-consuming transport efficiency.

Addition of water and an increase in temperature may effectively reduce viscosity; an excessive water content increases the polarity of extraction media and a very high temperature can damage thermolabile compounds (Socas-Rodríguez *et al.*, 2021). Thus, in this work, 25% of water was added to each NADES and 45 °C was selected as the optimal temperature for extraction, resulting in diverse OP extracts.

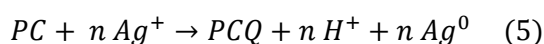
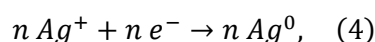
A screening of solvents was made to decide which OP extract could be used for Ag-NPs synthesis. Accordingly, LP:MA and H₂O resulted in the best solvents to extract phenolic compounds (see Figure 4). ChCl:Glu and ChChl:Fru were discarded since chloride ions precipitate Ag⁺ as AgCl (Liu *et al.*, 2022). Ultimately, LP:MA was rejected due to small amount of solvent and very acidic medium (2.58); the synthesis of LP:MA took a long time to successfully conclude and more quantity of NaOH would be needed to reach an alkaline medium in Ag-NPs synthesis. Hence, H₂O-assisted OP extract was ultimately chosen to be utilized as reducing agents in the synthesis of Ag-NPs and this established a further add-on to green chemistry policy.

4.2. Synthesis and Characterization of OP-Ag-NPs

Ag-NPs were synthesized using different volumes of aqueous OP extract mixed with a constant AgNO₃ concentration in alkaline medium at room temperature, resulting in various ratios: R0.75, R1.5, R3, R6 and R12.

As it was mentioned before, green synthesis mechanism of Ag-NPs is not yet fully understood. It is likely that reduction of Ag⁺ to Ag⁰ occurs by interplay of phenolic compounds of OP in alkaline conditions, due to dissociation of H-bonding (Konwarh *et al.*, 2011), and temperature and pH are major physical factors that control the nucleation process of Ag-NPs formation.

FTIR analysis was not conducted in this work, but Niluxsshun *et al.* (2021) identified and linked flavonoids, alkaloids, coumarins and phenolics to the mechanism due to absorption bands around 2950-3670 and 1636 cm⁻¹. These are mainly associated to -OH and -C=O stretching vibrations, suggesting the involvement of phenolic groups. Thus, it is assumed the following electron transfer process by Zuorro *et al.* (2022), regarding oxidation of the phenolic compounds (PC) to its quinone form (PCQ) (Equation 3) and further reduction of Ag⁺ to Ag⁰ (Equation 4):



For this reason, pH was set to 11 since alkalinity favors Ag⁺ bioreduction and subsequent formation of Ag-NPs due to the release of H⁺ ions during the oxidation of PC (Equation 3) (Zuorro *et al.*, 2022). Biomolecules are likely to be inactivated under extremely acidic conditions, occurring slow rate of formation of Ag-NPs (Konwarh *et al.*, 2011; Omran *et al.*, 2017). 25 °C was chosen as an optimal temperature since fast formation and aggregation of NPs could occur in temperatures higher than 30 °C (Shaikh *et al.*, 2021); besides, heating energy costs were avoided.

4.2.1. Spectral Characterization of OP-Ag-NPs

Ag⁺ reduction and formation of OP-Ag-NPs was preliminarily confirmed by the chromatic change of the reaction mixture from colorless or pale yellow to dark brown as reported by other authors (Tarannum *et al.*, 2019; Mogole *et al.*, 2021). Manifestation of the brown hue is attributed to SPR oscillation on the Ag-NPs surface (Omran *et al.*, 2017) and was gradually visible in the first 15 minutes.

UV-VIS spectroscopy was employed in the region 280-550 nm to evaluate the time course of the reaction kinetics by taking measurements at intervals of 15 and 30 minutes, 1, 2 and 24 h. Figure 5 displays the spectral portrait of the synthesized OP-Ag-NPs and the color change.

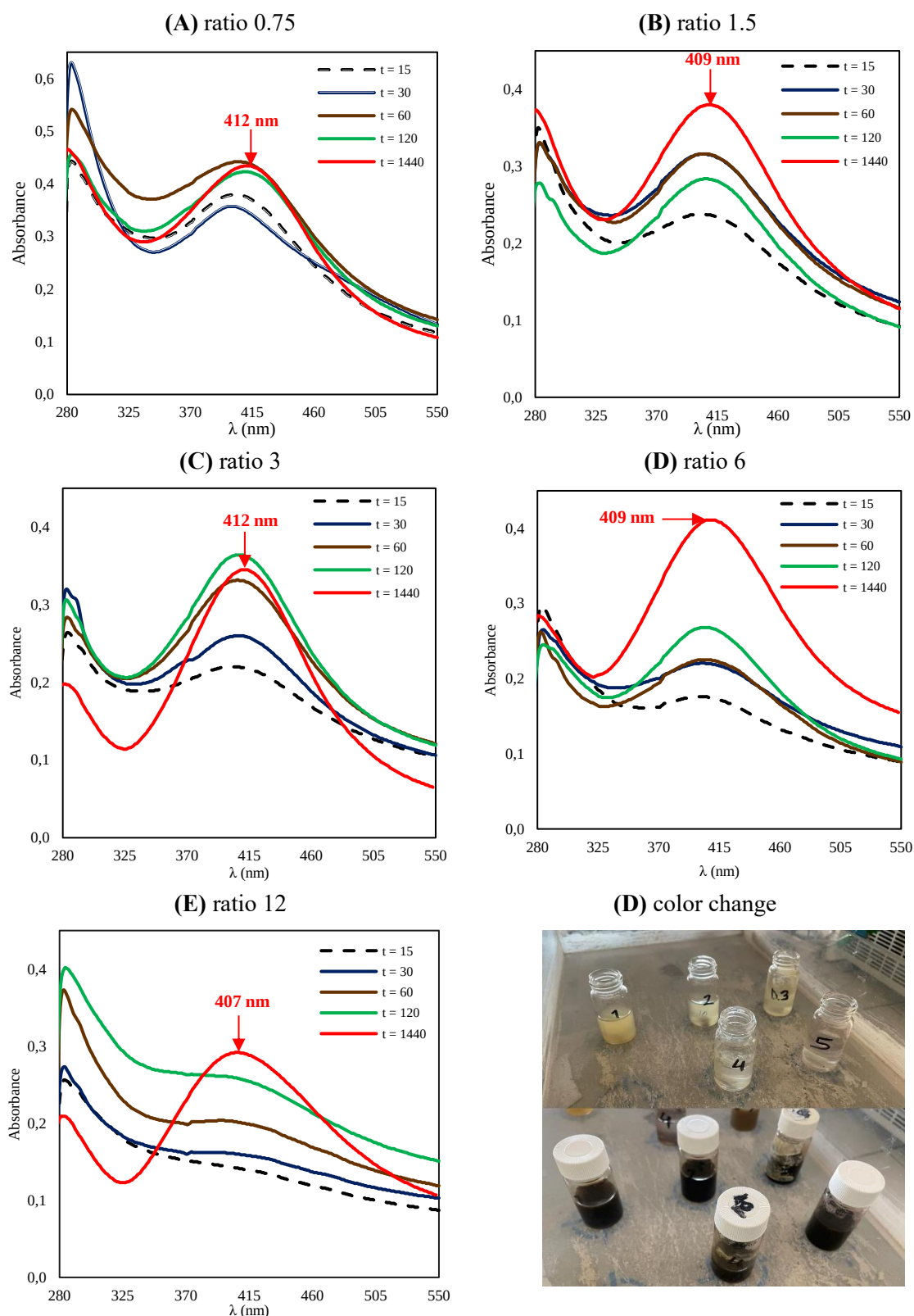


Figure 5 - Spectral portrait of OP-Ag-NPs using: (A) ratio 0,75; (B) ratio 1,5; (C) ratio 3; (D) ratio 6 and (E) ratio 12. (D) Color change of the extract and the corresponding OP-Ag-NPs.

SPR peaks of the samples ranged between 407-412 nm after 24 hours; this is consistent with the plasmon resonance absorption band of Ag-NPs at $\lambda_{400-460}$, which occurs due to the collective excitation of free electrons in the conductive band around Ag-NPs and confirms the formation of Ag-NPs (Tarannum *et al.*, 2019). Other authors who conducted a similar experiment with *C.*

sinensis reported a SPR range between 420-446 nm (see Table 6). In fact, position, intensity and sharpness of SPR is determined by various parameters, including particle size distribution, dielectric constant of the medium and surface absorbed species (Konwarh *et al.*, 2011). According to Zuorro *et al.* (2022), the sharper the peak, the narrower the size distribution.

As the reaction occurred, (A), (C) and (E) showed shifts in the absorption peaks which may be an evidence of aggregation of particles (Niluxsshun *et al.*, 2021). Besides, it is assumed that (A) and (C) may be spherical, according to Nikolova *et al.* (2023) who reported that this shape is observed in the region 410-450 nm.

In Figure 5, it is also noticeable a peak at about 280 nm. This decreasing peak is associated with the UV absorption of polyphenols and it indicates the interaction of these compounds with Ag^+ ions (Ihsan *et al.*, 2015). It is still very present in all spectral portraits at $t = 24$ h, which reveals that OP extract is not capable of completely reducing Ag^+ to Ag^0 in this period. Likewise, this was reported by Ihsan *et al.* (2015) at room temperature; however, when the authors heated the reaction mixture, the 280 nm peak almost disappeared, which suggests that most of the phenolic compounds reduced Ag^+ and temperature plays an important role in Ag-NPs synthesis.

4.2.2. DLS of OP-Ag-NPs

DLS technique permits the measurement of the average particle size distribution of the synthesized OP-Ag-NPs and validates the polydispersity index (PDI) (Figure 6).

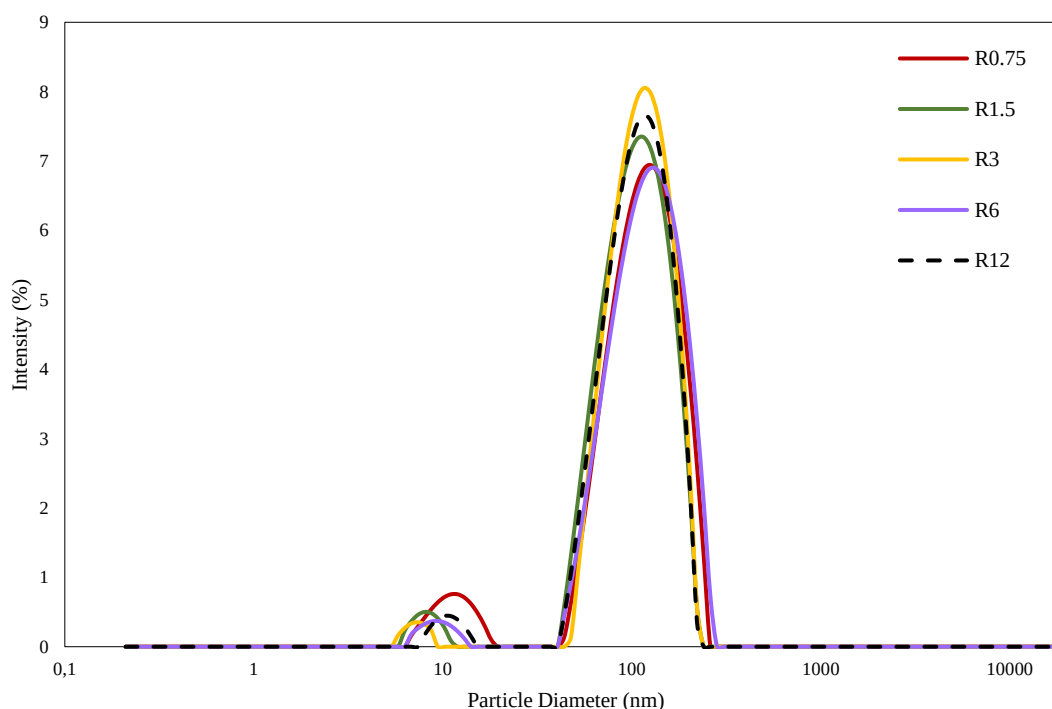


Figure 6 - OP-Ag-NPs DLS intensity size distribution.

DLS data confirmed OP-Ag-NPs biosynthesis with an average hydrodynamic diameter of 99-112 nm and PDI of 0.210-0.263. It is assumed that different ratios did not produce significant changes in particle size. As stated before, it is worth to mention that DLS average particle size is slightly bigger than values derived from TEM; which was not applied in this work.

It is possible to conclude that the resulting Ag-NPs are monodisperse, since PDI is less than 0.7. Usually, monodispersed Ag-NPs show higher performances and applications compared to polydisperse Ag-NPs (Liaqat *et al.*, 2022). Stable NPs present values more positive than +30.0

mV and more negative than -30.0 mV (Al-Ghamdi *et al.*, 2020); therefore, OP-Ag-NPs are stable since a zeta potential of -21.0 mV was recorded.

4.2.3. Antioxidant Activity of OP-Ag-NPs

Ag-NPs can exhibit potent antioxidant activity by reducing oxidative stress in cells, which is beneficial for the treatment of diseases. This was confirmed by Sarwer *et al.* (2022) who developed Ag-NPs using *Myrsine africana* leaf extract.

Figure 7 displays TPC and TEAC content of OP-Ag-NPs in comparison to TPC and TEAC of H₂O-assisted OP extract.

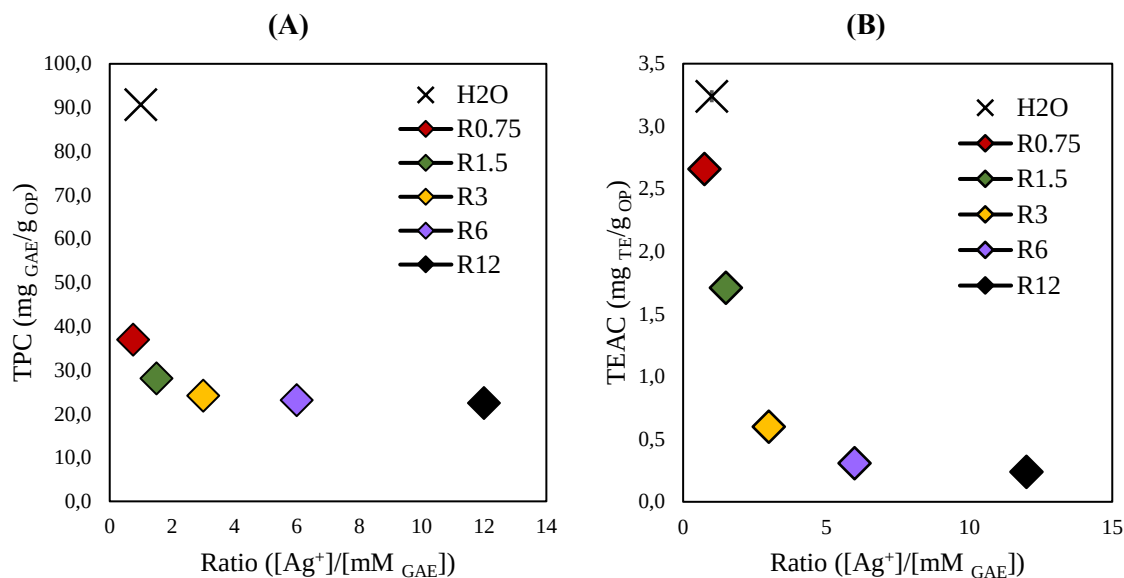


Figure 7 - Antioxidant activity of OP-Ag-NPs in comparison to H₂O-assisted OP extract: (A) TPC (mg GAE/g OP) and (B) TEAC (mg TE/g OP). Data are mean $\pm$ SD (n = 3).

As seen in Figure 7, values of TPC and TEAC from OP-Ag-NPs are significantly lower than the H₂O-assisted OP extract (90.7 mg GAE/g OP and 3.24 mg TE/g OP), ranging from 24.2-37.0 mg GAE/g OP and 0.24-2.66 mg TE/g OP. It is assumed that loss of antioxidant activity of OP-Ag-NPs in comparison to OP extract is due to the interaction of the reducing agents in the bioreduction and capping of Ag⁺ to Ag⁰; overall, OP-Ag-NPs were expected to exhibit antioxidant activity.

Furthermore, there is a consistent trend between TPC and TEAC values and ratio; lowest TPC and TEAC content is related to bigger ratios (with less OP extract volume) whereas higher TPC and TEAC are present in smaller ratios (with more OP extract volume).

4.3. Effect of Ratio Ag⁺:TPC

AgNO₃ concentration was kept constant (5 mM in each flask) and concentration of polyphenols of OP extract was changed (6.67, 3.33, 1.67, 0.83 and 0.42 mM GAE); this resulted in different ratios ($[Ag^+]/[mM_{GAE}]$): R0.75, R1.5, R3, R6 and R12.

Figure 8 represents the spectral portrait of OP-Ag-NPs taken at t = 24 h (A) and the highest SPR peaks of OP-Ag-NPs during the 24 hours (B).

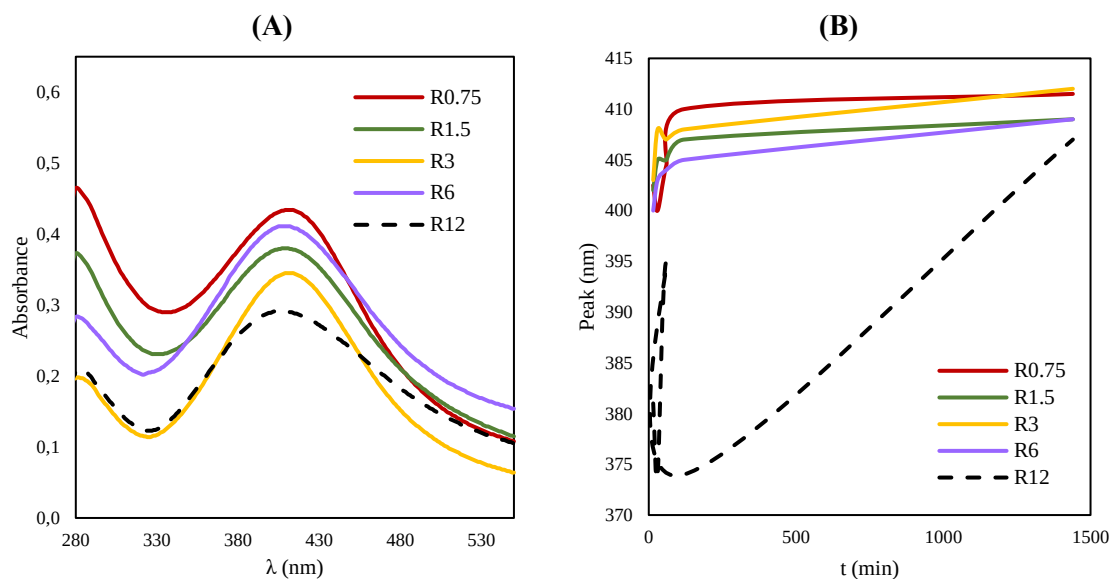


Figure 8 - Effect of ratio: (A) spectral portrait of different ratios of OP-Ag-NPs at $t = 24$ h; (B) highest SPR peaks of different ratios for time intervals of 15 and 30 minutes, 1, 2 and 24 hours.

An increasing trend of SPR peak is observed with decreased ratio (or more OP extract volume) at $t = 24$ h. Thus, as the OP extract volume is increased, so is the concentration of polyphenols, and a higher content of biomolecules become involved in the reduction of Ag^+ to Ag^0 .

However, this is not detected with R6; and it can be ascribed to a change in volume ratio and dilution of the resulting colloidal solution, making R6 more optimal than R1.5, despite the latter having more volume of OP extract and, consequently, more polyphenol content. Similarly, Aminu & Oladepo (2021) reported a variation in the SPR absorption bands on increasing OP extract volume. The authors stated that 3 mL of OP extract produced a flat SPR absorption and did not reduce Ag^+ ions, while 5 mL resulted in the optimal volume and 7 and 10 mL of extract volume resulted in a decrease in absorbance. Hence, optimal ratios may not be associated with an increase in polyphenol content.

In addition, it is reported the synthesis of OP-Ag-NPs in the first 15 minutes of reaction for all samples, except for R12. A SPR peak above or equal to 400 nm, which is in accordance with the plasmon resonance absorption band of Ag-NPs, was shown rapidly (Tarannum *et al.*, 2019). This concludes the reaction was fast and OP extracts had more available phenolic compounds to reduce Ag^+ to Ag^0 . Meanwhile, OP-Ag-NPs of R12 exhibited a SPR peak of 381 nm at $t = 15$ min; at $t = 24$ h, it presented a peak of 407 nm, indicating slow transformation of Ag^+ to Ag^0 . As such, this was the slowest reaction and it may be attributed to the low polyphenol content in OP extract, making biomolecules less available to act as reducing agents.

5. Conclusion and Future Perspectives

Green synthesis of Ag-NPs is a relatively new field of research, so the potential of many biogenic sources have not been widely explored; an urgent need for expansion of waste materials is necessary. In addition, it is challenging to ascribe the optimal conditions for Ag-NPs synthesis since every biogenic source interacts differently with the medium. Thus, this master thesis aimed to pursue a green route for the synthesis of Ag-NPs from OP extract. In summary, the reduction of AgNO₃ and capping of Ag-NPs was thought to occur through the action of phenolic compounds present in OP extracts, forming Ag-NPs. Ag⁺:TPC ratios of 0.75, 1.5, 3, 6 and 12 were evaluated.

Green solvents are one of the basis of an environmentally-friendly protocol; so, a screening of four NADES and water OP extracts was made, taking into account TPC and TEAC content. Due to high acidic and viscosity of NADES, water was ultimately chosen to extract OP extract, also showing high antioxidant activity (90.7 mg GAE/g OP and 3.2 mg TE/g OP).

A simple one-pot synthesis of Ag-NPs was performed at 25 °C in alkaline medium. UV-VIS data monitorization of 24 hours revealed formation of Ag-NPs due to SPR peaks in the range 407-412 nm, as well as chromatic change of colorless to dark brown. In addition, OP-Ag-NPs were monodispersed and very stable, presenting a mean PDI of 0.210-0.263 and a zeta potential of -21.0 mV. A peak at 280 nm was also outlined in all samples, indicating that OP extracts were not capable of completely reducing Ag⁺ to Ag⁰. Overall, ratios did not significantly alter particle size but antioxidant activity of Ag-NPs (24.2-37.0 mg GAE/g OP and 0.24-2.66 mg TE/g OP) was reported higher in bigger ratios and lower than the H₂O-assisted OP extract.

Future studies involving Ag⁺:TPC ratio could focus on altering Ag⁺ volume or changing Ag precursor. Furthermore, besides ratio, other main external factors (i.e., reaction temperature, pH, time and exposure to light) influence the synthesis. Storage of synthesized Ag-NPs should also be studied. Hence, a diverse approach could be to focus on a recurrent waste material and re-evaluate these parameters by experimental design; besides, if a proper identification of present biomolecules is performed, a one-step protocol could be implemented and possibly taken to large scale production.

As stated before, a large number of natural materials are yet to be explored; it would be interesting to screen frequent waste from a specific organization or area to evaluate their potential as reducing agents, thus helping communities in upcycling.

Briefly, this thesis contributed to the three pillars of sustainability. Reduction of energy consumption and the use of water as solvent in the synthesis complies with green protocols and averts physical or chemical routes; in addition, upcycling of OP waste comprises social, economic and environmental issues, since it is a waste easily available and provided by households, avoiding incineration or dump in landfills. Overall, the outcome of this thesis adds to the repertoire of simple and green Ag-NPs synthesis and could be beneficial for nanotechnology-based applications.

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