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Fitossíntese de nanopartículas de prata: uma revisão e um estudo do potencial redutor do extrato da casca de romã e da atividade antifúngica dessas nanopartículas

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Fitossíntese de nanoparticulas de prata: uma revisão e um estudo do potencial redutor do extrato da casca de romã e da atividade anifúngica dessas nanopartículas

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Aos meus pais Amauri Sauvesuk e Ingridia Barbara Siqueira Sauvesuk

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EPÍGRAFE

Sonho que se sonha só

É só um sonho que se sonha só

Mas sonho que se sonha junto é realidade…

Raul Seixas

Sauvesuk L. **Fitossíntese de nanoparticulas de prata: uma revisão e um estudo do potencial redutor do extrato da casca de romã e da atividade anifúngica dessas nanopartículas** [dissertação]. Araçatuba. Universidade Estadual Paulista, 2017.

RESUMO GERAL

O presente estudo revisou na literatura trabalhos entre 2006 e 2017 relacionados à fitossíntese de nanopartículas de prata (AgNP) e destacar a variedade de plantas passíveis de serem utilizadas e o quanto estas influenciam as características e propriedades dessas nanopartículas. Avaliou-se também como a temperatura (25, 50, 70 e 95°C) e a concentração de extrato da casca de romã (*Punica granatum*) (7, 14 e 28 mg mL^{-1}) interferem na efetividade da reação fitoquímica por meio da quantificação dos íons Ag^{+} livres na solução de AgNP. A atividade antifúngica das AgNP produzidas foi avaliada por meio do método da microdiluição (CLSI M27-A2) contra cepas padrão (ATCC) de *Candida albicans* e *Candida glabrata*. As AgNP foram caracterizadas por microscopia eletrônica de varredura (MEV), difração de raios-X e espectroscopia UV/visível. O extrato da casca desidratada da romã foi obtido por maceração seguido de percolação em etanol (70%) e desidratado em rota-evaporador. Quantificou-se os fenóis totais expressos em ácido gálico por método colorimétrico e o ácido elágico por HPLC, sendo respectivamente 158,61 e 4,21 mg mL^{-1}. Foram revisados mais de duzentos artigos relacionados à fitossintese de AgNP, onde cerca de cento e setenta plantas foram utilizadas para esta síntese e sendo a maioria delas geograficamente concentradas nos continentes asiático e europeu. As AgNP fitossintezadas no presente estudo apresentaram concentração de íons livres de Ag^{+} proporcional à quantidade de extrato utilizada na reação e maior nas temperaturas mais elevadas, e foram efetivas contra ambas cepas padrão de *Candida* avaliadas.

Palavras-chave: Nanopartículas, prata, G*ranatum*, *Candida albicans* e *Candida glabrata.*

Sauvesuk L. **Phythosynthesis of silver nanoparticles: review of literature and a study of the reductor potential of pomegranate peel extract and its antifungal potential**. [dissertation]. Araçatuba. Universidade Estadual Paulista, 2017.

GENERAL ABSTRACT

The present work reviewed in the literature studies of phythosynthesis of silver nanoparticles (AgNP) between 2006 and 2017, highlighting the variety of plants used to and how they can influence the characacteristics and properties of AgNP. This work also evaluated the influence of temperature (25, 50, 70 and 95°C) and concentration of peel extract of pomegranate (*Punica granatum*) (7, 14 and 28 mg mL^{-1}) on the effectiveness of phytosynthesis reaction by quantifying the free Ag^{+} ions in the AgNP soluction. The antifungal activity of AgNP against reference strains (ATCC) of *Candida albicans* and *Candida glabrata* were evaluated by the microdilution method (CLSI M27-A2). AgNP were characterized through scanning electron microscopy (SEM), X-Ray diffraction and UV-Vis spectroscopy. The pomegranate peel was dehydrated and the hydroalcoholic extract was obtained by maceration followed by percolation, and then obtained the crude extract using a rotary evaporator. Total phenols expressed in galic acid and elagic acid were quantified in the extract using a colorimetric method and by HPLC, and the values found were 158.61 and 4.21 mg mL^{-1}, respectively. It was found more than two hundred studies using about an hundred seventy plants to synthesize AgNP, being the majority of those plants from the Asiatic and European Continents. The AgNp phytosynthesized in the present study presented the concentration of free Ag^{+} ions proportional with the quantity of pomegranate peel extract and it was high when the temepratures used in the reaction were above 50ºC. Also, AgNP were effective against both *Candida* reference strains evaluated.

Keywords: nanoparticles, silver, *Granatum*, *Candida albicans* and *Candida glabrata.*

LISTA DE FIGURAS

sample: a) SEM image; b) chemical mapping of silicon element present in the substrate, where the electron beam was focused directly on the substrate and is showed in red color; c) chemical mapping of carbon element present in the alcoholic extract in green color; d) chemical mapping of silver element, in blue color.

Figure 4 Dinamic light scattering pattern of AgNP phytosynthesized at different conditions.

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LISTA DE ABREVIATURAS

AEC – Aqueous extract of *Croton bonplandianum*

AgNP - Nanopartículas de prata

ATCC – American Type Culture Collection

CIM /MIC – Concentração inibitória mínima/ Minimum Inhibitory Concentration

CLSI M27 – A2 - Clinical and Laboratory Standards Institute, Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard - Second Edition

DLS - Dinamic light scattering

EDX - Energy Dispersive X-Ray Detector

ELISA – Enzyme Linked Immuno Sorbent Assay

FEG-SEM - Microscope with field emission gun electron effect

HPLC – High Performance Liquid Chromatography

MEV/SEM - Microscopia eletrônica de varredura/scanning electron microscopy

MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

ROS – Reactive oxygen species

SDA - Sabouraud dextrose agar medium

UV/VIS -Absorption spectroscopy in the Ultraviolet/Visible

XRD - X-Ray diffraction

SUMÁRIO

INTRODUÇÃO GERAL

1. Introdução Geral

Os patógenos isolados de espécies clínicas são identificados visando confirmar o diagnóstico médico e guiar a terapia antimicrobiana. Os antibióticos são compostos naturais ou sintéticos capazes de inibir o crescimento ou causar a morte de fungos ou bactérias. Podem ser classificados como bactericidas, quando causam a morte da bactéria, ou bacteriostáticos, quando promovem a inibição do crescimento microbiano (Pupo 2010). Contudo, para que o antimicrobiano exerça sua atividade, primeiramente deverá atingir concentração ideal no local da infecção, ser capaz de atravessar, de forma passiva ou ativa, a parede celular, apresentar afinidade pelo sítio de ligação no interior da bactéria e permanecer tempo suficiente para exercer seu efeito inibitório (Anvisa 2007).

A definição de resistência é a capacidade de um microrganismo para resistir aos efeitos de uma droga a que é normalmente sensível, sendo que diversos fatores contribuem para o desenvolvimento da resistência microbiana aos antimicrobianos. Um dos fatores de risco mais importante é a exposição repetida a concentrações de antimicrobianos abaixo da adequada uma vez que concentrações subletais de um antimicrobiano exercem pressão seletiva sobre sua população sem erradicá-las (Anvisa 2007).

Os mecanismos convencionais de resistência a antibióticos e biocidas se enquadram em quatro categorias: inativação direta da molécula ativa, que altera a sensibilidade do organismo alterando o seu alvo de ação, reduzindo a concentração do fármaco e atingindo o seu alvo, inalterando a sua composição química e os sistemas de efluxo (Hogan & Kolter, Poole *et al.* 2002).

Em vista desta resistência crescente pesquisadores buscam a obtenção de novos produtos farmacologicamente ativos em espécies vegetais aproveitando a diversidade de ecossistemas do planeta, através dos avanços dos estudos químicos e farmacológicos (Nobakht *et al.* 2017).

A *Punica granatum* L. (romã) é uma planta cujos extratos têm demonstrado ação antibacteriana e antiaderente in vitro, sobre os microrganismos Gram-positivos e Gram-negativos; os *Streptococcus mutans*, *S. mitis*, *S. sanguis* têm apresentado sensibilidade ao extrato da romã no que se refere ao crescimento e à capacidade de aderência sobre a superfície dental (Pereira 2006).

O extrato da romã é composto principalmente de alcalóides e polifenóis. Os compostos fenólicos da romã tem demonstrado uma variedade de funções benéficas, incluindo atenuação de fatores aterogênicos, modulação das respostas antiinflamatórias e de enzimas do sistema de defesa antioxidante endógeno (superóxido dismutase, catalase e glutationa peroxidase). Também os flavonóides extraídos do suco fermentado e do óleo da romã tiveram atividade inibitória das enzimas oxidantes ciclooxigenase e lipooxigenase (Jardini & Filho 2007). O extrato da fruta é amplamente utilizado em vários sistemas tradicionais da medicina para tratamento de artrite e outras doenças (Ahmad, Sharma & Rai 2012).

O suco da romã apresenta em sua composição compostos fenólicos como: antocianinas (delfinidina, cianidina e pelargonidina), quercetina, ácidos fenólicos (caféico, catequínico, clorogênico, orto e paracumárico, elágico, gálico e quínico) e taninos (punicalagina) (Jardini & Filho 2007). O ácido elágico tem uma capacidade de perder elétrons, o que resulta na formação de radicais H^+, e também na redução dos íons de prata e de ouro e consequente formação de partículas em tamanho nanométrico e com significativa redução do tempo de reação, conforme demonstrado na figura 1

abaixo com um tanino, demonstrando a redução na punicalagina (Ahmad, Sharma & Rai 2012).

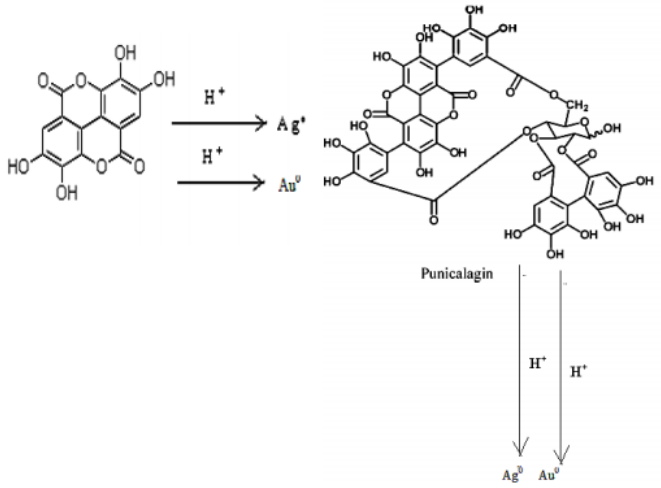

Figura 1. Mecanismo de biossíntese de nanopartícula de prata e de ouro utilizando o extrato da casca do fruto da *Punica granatum* (Ahmad, Sharma & Rai 2012).

Além dessa capacidade redutora, o ácido elágico protege eficazmente as células dos radicais livres prejudiciais. Sendo que alguns estudos demonstram atividade antiproliferativa e indução de apoptose em cultura de células carcinogênicas do epitélio cervical, prevenção de câncer do trato gastrointestinal atribuída ao acúmulo seletivo de ácido elágico em células epiteliais de rato e atividade antimicrobiana seletiva em microrganismos patogênicos para o homem (Gonçalves 2008).

Esse potencial de redução dos íons prata pela romã tem se mostrado bastante eficaz na produção de nanopartículas e assim permite a aplicação de um método alternativo para síntese de nanopartículas metálicas, podendo fornecer compostos menos tóxicos e economicamente mais viáveis (Takamiya 2013). A figura 2 abaixo ilustra como as partículas de prata são revestidas pelas moléculas do extrato fitoterápico.

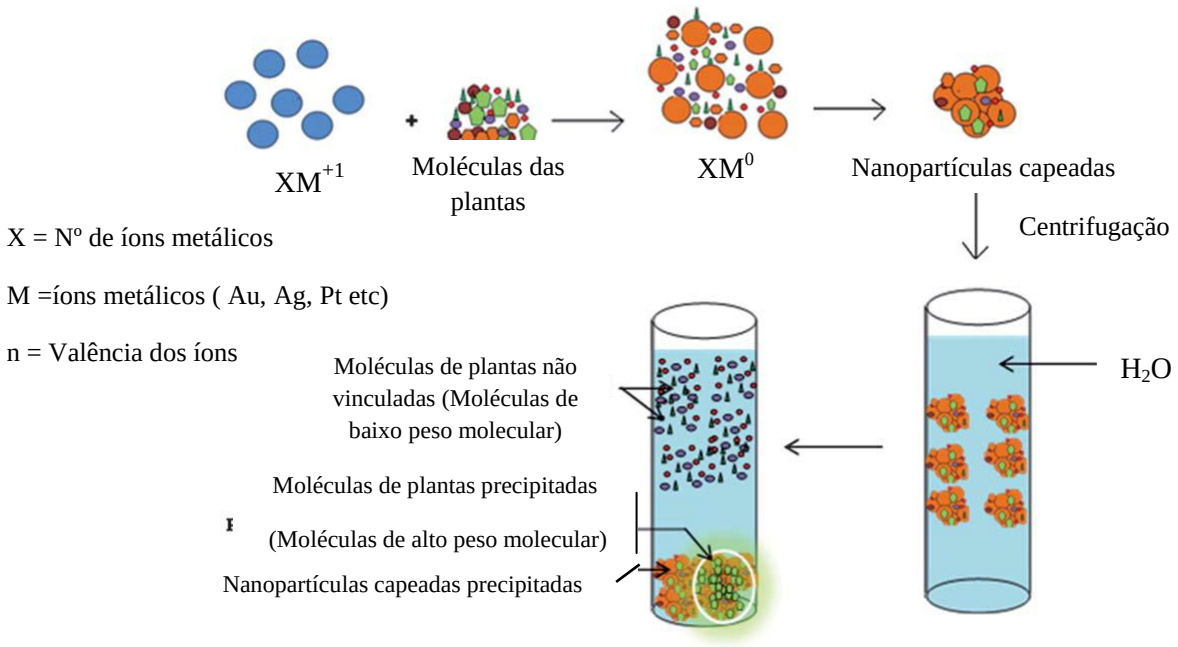

Figura 2. O papel das moléculas das plantas em conferir biocompatibilidade as nanopartículas (Brar & Das 2013).

As nanopartículas de prata (AgNP) para exercerem o efeito antimicrobiano aderem à superfície da célula, alteram as propriedades físicas e químicas da membrana e parede celular, e produzem importantes distúrbios nas funções tais como a permeabilidade, osmose, transporte de elétrons e respiração celular (Garcia 2014).

As AgNP podem causar estresse oxidativo nas células em diferentes níveis: moléculas, organelas, e em toda a célula, de acordo com esquema ilustrado na figura 3. AgNP induzem forte dano oxidativo na membrana celular e em organelas como lisossomos e mitocôndrias, e nos núcleos, resultando diretamente em apoptose ou necrose. O estresse oxidativo causado pelas AgNP pode desencadear a resposta inflamatória, incluindo a ativação da imunidade inata, e aumenta a permeabilidade do endotélio celular. AgNP na dose não citotóxica induz dano ao DNA, anormalidade cromossomal e possível mutagenicidade. Vários fatores como dose, tempo de exposição, tamanho, superfície química e tipo celular possuem importantes papéis na resposta celular mediada, ilustrado na figura 3 (Zhang *et al.* 2014).

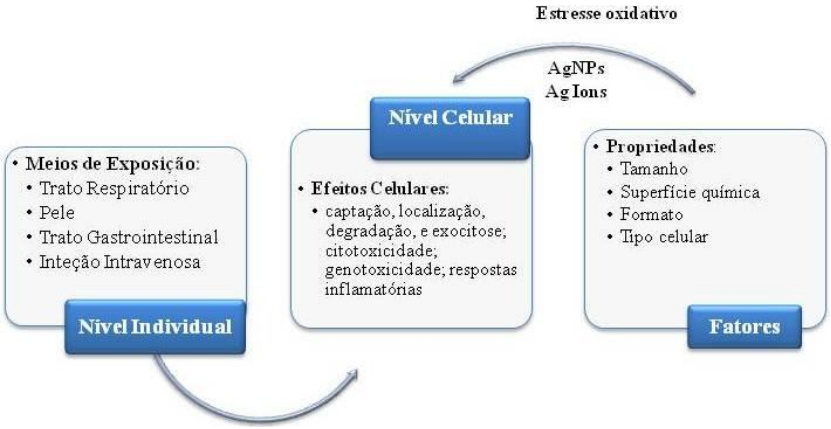

Figura 3: Efeito potencial das AgNP na célula e os principais fatores que medeiam estes efeitos (Zhang *et al.* 2014).

A toxicidade à célula e ao microorganismo aumenta com o decréscimo no tamanho do material. Pequenas partículas podem mais facilmente contatar a membrana celular e ser internalizada pela célula aumentando as chances de induzir efeito citotóxico. Além do tamanho há outros fatores que interferem como: a forma, as propriedades da superfície, ou mesmo parcialmente atribuído a presença de agentes estabilizantes (Takamiya 2013).

O objetivo geral deste trabalho foi analisar os avanços realizados no campo da síntese green, a variedade de plantas passíveis de serem utilizadas e o quanto estas influenciam as características e propriedades das nanopartículas e como o processo de síntese com pequenas modificações como temperatura, concentração de extrato e tempo podem modificar a estabilidade da nanopartícula produzida pela síntese "green" através do extrato da casca da *Punica granatum.* Além disso, quantificar os íons livres e avaliar a atividade antifúngica da nanopartícula de prata perante cepas padrão (ATCC) de *Candida albicans* e *Candida glabrata.*

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CAPÍTULO 1

Phytosynthesis of silver nanoparticles: is it really a promising alternative method?

*Artigo enviado para o periódico Environmental Science & Technology

Phytosynthesis of silver nanoparticles: is it really a promising alternative method?

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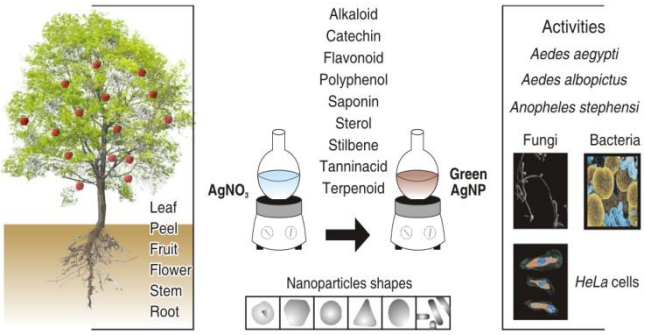
Abstract

Bionanotechnology has been growing exponentially, and the use of plants to reduce metals has been explored in the literature. The objective of this study was to review published literature about silver nanoparticle (AgNP) biogenic synthesis using plants. It intended to show how intense the studies involving phytosynthesis have been in the nanotechnology field from 2006 to 2017. The synthesis methods and the bioactive reductor in the plants, as well as the antimicrobial potential and cytotoxicity of green AgNP, were discussed briefly. Phytofabrication of AgNP demonstrated advantages of being energy efficient, inexpensive, and cost effective. It also provides a healthier work and community environment, preserving human health by producing less waste and safer products. Nevertheless, after meticulous reading of selected literature, it was noted that there is a relevant issue in the selection of the plant, and in the standardization of not only the methods used to phytosynthesize AgNP, but also of those to obtain the plant extract. The present review clearly demonstrated the vertiginous increase in studies of plant-mediated biogenic synthesis of AgNP, which demonstrates the significant awareness towards developing environmental and human health-friendly techniques for the nanotechnology industry.

Keywords: Plant, silver, nanoparticles, antibacterial agents



Introduction

The application of nanotechnology has been making increasingly rapid advances and great impact in the field of health.[1,2] Nano-sized tools are used for the diagnosis, treatment, and prevention of disease, and to increase comprehension of the intricate subjacent pathophysiology of disease.[3] Bionanotechnology has been growing exponentially, and a plethora of plants from different genres and species used to reduce metals is found in the literature. The phytosynthesis method has the advantage of being rapid, simple, and cost-effective, as well as being ecofriendly, less hazardous, and fabricating nanoparticles which present better biocompatibility.[4-7] The selection of the plant, the extract preparation, and the reaction conditions are crucial since they can interfere directly in the characteristics of metallic nanoparticles, including their size and stability. Phytochemicals vary vastly in each plant and in different parts of a single plant, and they play a preponderant role in the oxidation-reduction systems which are responsible for reducing the metallic ions in the synthesis reaction.[8-10]

The present study aimed to review published literature which studied biogenic synthesis of AgNP using different plants. It intends to show the intensity of studies involving green chemistry in the nanotechnology field from 2009 to 2017, and the extensive variety of plants used in phytosynthesis reactions to obtain AgNP. Also discussed briefly the synthesis methods and the bioactive reductor in the plants, as well as the antimicrobial potential and cytotoxicity of green AgNP.

Plant groups and mechanisms of green synthesis

The biosynthesis of nanoparticles takes advantage of a vast extent of plants, which include vascular (angiosperm and gymnosperm.[9,12]) and non-vascular (algae, bryophytes and pteridophytes[11]) plants.

Research has shown the influence of plant extracts on the characteristics of

nanoparticles. Each plant and each part of the plant, such as the peel, fruit, stem,[12] root,[12] and rhizome,[11] have different combinations of organic reducing agents in different concentrations. Moreover, climatic conditions influence the functional compounds of the plants.[13] Asard *et al*. verified an increase of polyphenol levels with drought and CO_2 concentration.[13]

There are different green methods of silver synthesis,[12] and the reduction of silver ions to AgNP is observed visually by the coloring modification of the solution.[14,15] The probable mechanism of nanoparticle synthesis is shown in Figure 1.

A capped biomolecule surface containing metallic nanoparticles is formed to prevent the agglomeration of particles, there by stabilizing the AgNP.[16] Other authors explained that most plants liberate hydrogen and this active hydrogen may be accountable for the reduction of silver ions and the formation of AgNP.[17,18] The reaction process was described by Vidhu[18] and is illustrated in Figure 2. Also, the carbonyl group of amino acids and proteins has the consistent ability to bind with AgNP, which is demonstrated by spectral analysis and the presence of the –OH group in most plant extracts. Thus, it could be related to the reduction of Ag^+ to Ag^0 through the oxidation of alcohol to the aldehyde group,[14,16] as shown here:[16]

$$2Ag^+ NO^-_3 + ROH \rightarrow 2Ag^0 + RO + 2HNO_3$$

Qu *et al*. observed FTIR spectra characteristic peaks at 1.36 and 3.23 cm/L, which were attributed to the stretching and bending vibrations of hydroxyl groups. This suggests that the consumption of hydroxyl groups was accountable for the reduction of Ag^+.[17]

However, it is necessary to elucidate the phytochemistry involved in green synthesis since it is not completely clarified. Phenols and polyphenols are some of the largest and most frequent classes of secondary metabolites in plants. Simple phenols

comprise structures with one aromatic ring, substituted with one or more hydroxyl parts, and polyphenols have at least two phenolic rings in their structures, as well as complex polyphenolic compounds of a polymeric nature.[19] Therefore, after the advent of chromatographic techniques and the development of phytochemistry, the term polyphenol was expanded to include a wider range of molecules with at least two phenolic rings in their skeleton. They include xanthones, stilbenes and stilbenoids, depsides, phenylpropanoid derivatives, lignans and lignin, and most importantly, flavonoids and tannins.[19]

The hydroxyl groups, carbonyl group, amino acids, and proteins are constituents of metabolites in the plant, and they may vary depending on each plant and part of the plant. Studies have suggested that the phytochemicals related to the reduction reaction are phenols, which would include tannins[17,20-22] and flavonoids.[14,22] Table 1 shows the bioactive compounds present in several plants involved in the production of AgNP in the green process.

Table 1

Plants have a large variety of metabolites, including primary metabolites, associated with growth and metabolism (proteins, carbohydrates and lipids), and secondary metabolites, reputable as final products of primary metabolism, which are not included in metabolic activity and operate as defense chemicals (alkaloids, essential oils, lignins, phenolics, sterols, steroids, and tannins, etc.).[23] Plant secondary metabolites can be divided into three major groups: terpenoids; flavonoids and allied phenolic and polyphenolic compounds; and alkaloids containing nitrogen and/or sulpher.[23]

The first group of terpenoids includes monoterpenes, diterpenes, triterpenes, tetraterpenes, sesquiterpenes,[23]and saponins.[24] The second group comprises

polyphenols, which are a general class of secondary metabolites[25] that encompass groups such as catechins,[26] flavonoids,[25] stilbenes,[25]and tannins.[25] The last group includes the following alkaloids: amaryllidaceae, betalain, diterpenoid, imidazole, indoles, isoquinoline, methylxanthines, monoterpenoid, peptide, phenethylamines, piperidines, pyridine, pyrrolidines, pyrrolizidine, quinoline, quinolizidine, steroidal, and tropaneds.[23] The large amount of compounds creates enormous difficulty in sorting them correctly, and a wide variety of options have been found in the reviewed articles, from general to specific classifications. Despite this, Ahmad et al. assigned the flavonoid luteolin as the bioactive reductor of *Ocimum sanctum.*[12] Figure 3 illustrates how the phytochemicals act to reduce silver.

Also, a mechanism for the reduction process which involves silver and the following specific chemical groups is proposed (Table 3): alcohols (*Ficus carica),* aldehydes (*Butea monosperma; Curcuma longa*), alkanes, alkenes, alkynes (*Ficus carica*), amine group *(Phoenix dactylifera; Ziziphora tenuior),* aromatic amide *(Acorous calamus),* carbonyl groups *(Acorous calamus, Vitex negundo, Ziziphora tenuior, Ziziphus jujuba),* carboxyl group *(Erythrina indica, Phoenix dactylifera),* chlorine (*Morinda citrifolia),* hydroxyl groups (*Erythrina indica, Leonuri herba, Phoenix dactylifera, Vitex negundo, Ziziphora tenuior),* fructose (*Emblica officinalis),* glucose (*Emblica officinalis),* glycosides (*Chrysanthemum indicum*), carbohydrates (*Abutilon indicum, Gloriosa superba, Solanum xanthocarpum),* proteins (*Anacardium occidentale, Andrographis paniculata, Butea monosperma, Cymodocea serrulata, Eucalyptus globulus, Gloriosa superba, Leucas aspera, Mulberry, Sesbania grandiflora, Solanum xanthocarpum, Ziziphus jujuba),* enzymes/proteins (*Acalypha indica),* vitamins (*Acalypha indica),* ketones (*Butea monosperma),* peptides (*Jatropha*

curcas), amino acids (*Gloriosa superba*) and polyols (*Anacardium occidentale, Mulberry*, *Piper longum*).

Moreover, the reduction of silver is also bound to the organic acids (*Acalypha indica),* and even to specific acids like ascorbic acid (*Hibiscus cannabinus; Iresine herbstii),* caffeic acid *(Macrotyloma uniflorum),* citric acid (*Citrus limon; Citrus sinensis; Orange),* and rosmarinic acid (*Ocimum sanctum).*

The phytochemical composition of plants is quite complex and diversified in terms of quantity and components. Thus, it is indispensable to deepen the research in phytonanotechnology in order to clarify which biomolecules or compounds would be involved in the reduction reaction to produce metal nanoparticles, as well as to classify the suspected phytochemical of silver reduction more specifically.

Factors that influence AgNP characteristics

Research has demonstrated that the plant type, the part of plant (flower, leaf, peel, root, etc),[27,37] the extraction time[17] and even its exposure to sun-light,[11, 28-31] can interfere in AgNP synthesis. The synthesis process is also affected by the solvent,[32] pH,[33] temperature,[33] extract concentration,[34] and sonication.[35]

The plant part significantly influences the size of the nanoparticles. For instance, leaf and fruit extracts of *Couroupita guianensis* synthesize AgNP with cubic crystalline forms and sizes ranging between 10–45 nm using leaf extracts and 5–15 nm using fruit extracts.[32] Moreover, *Leucas aspera* aqueous extracts produced clustered AgNP with irregular shapes and sizes varying from 25–80 nm[27] using leaf extracts, and spherical shape particles from 29-45 nm using bark extracts as reducing agents of silver ions.[36]

Solvent used in the extraction process may also influence the antimicrobial activity of AgNP, as Vimala *et al.* demonstrated using *Couroupita guianensis* against different larvae stages of *Aedes aegypti* (Table 2).[32]

According to Iravani *et al*., the reduction of silver ions to colloidal AgNP was elevated with the increase of the pH solution during the reaction process using *Pinus eldarica* bark extract. Larger nanoparticle size was noticed at lower or acidic pH, while smaller and highly dispersed particles tended to form at higher or alkaline pH. These authors also noticed that the rate of reduction of silver ions was proportional to the increase of the temperature reaction. When the reaction temperature was increased from 25 to 150°C, the AgNP size became smaller.[33]

The time and temperature of the extraction process of the plant also influence the rate of silver nanoparticle formation. Qu *et al.* cited that an extraction time longer than 15 minutes of boiling might destroy the reductor compounds and lead to a decrease in the effectiveness of AgNP synthesis. They also observed that the synthesis rate was reduced at temperatures higher than 25°C during the extraction process.[17]

Plants have a big role in conferring biocompatibility to metallic nanoparticles and antimicrobial properties

AgNP are effective against a broad variety of microorganisms, and their antimicrobial activity may be influenced by the characteristics of the plant selected for the silver reduction process when phytosynthesized. Table 3 illustrates the vast variety of plants used in that process and the microorganisms tested with them.

AgNPs also have the most diverse applications in the environmental,[38,39-41] biomedical,[4,42,43] and eletronic[44] fields. There are several methods proposed to fabricate AgNP, and green synthesis has been shown as cost effective,[45] simple,[45] non-toxic,[45] and ecofriendly.[45,46] Also, apart from reducing the silver ions, phytochemicals have synergistic properties.[47] Phytosynthesized AgNP would also aggregate the properties of the plant, and thus may present higher antimicrobial,[22,48-62] anti-inflammatory,[38] antioxidant,[63,64] anticancer,[17,65-69] and catalytic activity,[41,49,70] as well as

biocompatibility, than commercial nanoparticles synthesized by conventional chemical means. Development of new green methods for nanoparticle synthesis has been in growing demand due to its eco-friendly nature for bulk scale synthesis and cost effectiveness without using hazardous chemicals.[71]

Localization of the plants

The plants studied are located predominantly on the Asian continent, with most of the plants collected in Central Saudi Arabia, The Republic of China, Burma, Ceylon (Sri Lanka), Iran, Iraq, Malaysia, Northern Japan, Pakistan, and South Korea. However, they can also be found on the European continent, primarily from Finland, Greece, Portugal, and Spain, followed by the African continent, where the plants were collected from South Africa, Egypt, and Nigeria. North America (USA and Mexico), Oceania (Australia), and South America (Brazil) followed on the list of the main publication origins.

The possible explanation for the predominance of the Asian and European continents in the phytofabrication of metal nanoparticles would be related to the three main systems prevailing in medicine: Ayurveda, Greek, and Chinese medicine.[72] These systems incorporate the philosophy of plants in the healing process. They also share perceptions, ideologies, and aspirations which create 'interconnectivity' among the Asian, European, African, and North and South American continents. Greco-Arabic medicine has influenced the Islamic world to establish secular medical institutions catering to the sick, regardless of their religion or status[72]. Furthermore, India has significant political, strategic, and economic stakes in engaging with East Africa, as well as state reforms which have dramatically changed the policy environment for India's pharmaceutical industry.[73]

Cellular mechanism of AgNP action

The mechanisms of nanoparticle action have been reported as: 1) the ability to interfere with reactive oxygen species (ROS) generation[74,75] mediated by the stimulation of a signaling cascade;[76] 2) DNA damage[74] with structural changes, which could alter the metabolic function,[77] protein carbonylation,[74] and protein damage leading to ubiquitination and degradation by way of proteasomes;[74] 3) reduction in the mitochondrial activity as an outcome of down-regulation of proteins involved with the electron transport chain,[74,76] combined with the protease enzyme, respiratory enzyme, and the DNA of bacteria, leading to the process of indigestion, suffocation, inhibition of cell replication, and thus, apoptosis.[60,74,76] Also, nanoparticles easily penetrate the cells because of their smaller size,[74,76] and then regulate cellular uptake. In contrast, cell proliferation could be independent of AgNP particle size, indicating a different regulating process and reinforcing the ROS-independent pathway.[76]

On the other hand, Verano-Braga et al.[74] sought to elucidate the role of particle size on the cell, and demonstrated that AgNP at 100 nm were located on the plasma membrane of the LoVo cells after 24 hours of exposition, and promoted indirect effects via phosphatase 2A pathways, serine/threonine protein kinase (PAK), and mitogen-activated protein kinase (MAPK) (Figure 4). However, 20 nm nanoparticles were assimilated by the cell, forming large clusters of aggregated nanoparticles in the cell, promoting morphological changes in the LoVo cell with direct effect on cellular stress as generation of ROS and protein carbonylation. Moreover, by CLSM measurements, Miethling-Graff *et al.*[76] verified that silver particles exceeding 20 nm rarely internalized in the cell, decreasing toxicity and damage to the cell.

Also, there are expressive determinatives that can interfere with ROS generation, and implicate in the cytotoxicity/genotoxicity of AgNP, as shown in the following

scheme (Figure 5). The production of ROS would be directly related to the surface shape and characteristics of the nanoparticles, their dissolution and aggregation, the release of metallic ions from nanometals and nanometal oxides, the mode of interaction of nanoparticles with cells, and the pH of the medium, as well as the UV light activation. (Figure 5)[75]

Biocompatibility and cytotoxicity studies

Green synthesis has been an increasing focus of attention for the promise of yielding large amounts of nanoparticles with significant advantages over some chemical and physical methods,[17] as demonstrated by Qu *et al*,.[17] which verified lower cytotoxicity of AgNP synthesized by *Agrimoniae herba* than those particles reduced by *sodium citrate*.[17] Particular attention should be given to the extract concentration used to synthesize AgNP, since it could negatively influence the cytotoxicity of the particles.[78] According to Swamy *et al.*,[78] L6 rat skeletal muscle cell viability decreased when the concentration of *Momordica cymbalaria* fruit extract was increased.

A variety of cell lines have been used to investigate the cytotoxicity of AgNP, including mouse fibroblast cell lines L929[51] and 3T3,[71] HeLa cell line,[79] MCF-7 breast cancer cells, acute monocytic leukemia NCCS cell line THP-1, lung adenocarcinoma A549 cell line,[17,68] human lung cancer H1299 cells,[80] human adenocarcinoma cell lines (HCT15 and HT29),[81] human embryonic kidney HEK 293 cell line,[82] and pancreas adenocarcinoma PANC-1cell line.[6] The InQ Plus Benchtop Cell Research System[82] and the mortality of *Daphnia magna* and *Ceriodaphnia dúbi*[83] have also been used to evaluate the cytotoxicity of AgNP . In vitro cytotoxicity is determined using the MTT assay,[17] in which the viability of the cells is signalized through the conversion of tetrazolium salt MTT to a colored formazan by the mitochondrial dehydrogenases activity.[22] This conversion occurs only in viable cells,[60] and the color change is

measured photometrically using a spectrophotometer[22] or ELISA reader.[60] The absorbance of untreated cells is used as the control reference,[22] and the viability of the cells (%) is calculated by the mean OD/control OD x100. Cell Counting Kit-8 (CCK-8) was utilized by Maddinedi et al.[71] in the cytotoxicity assay where the mouse fibroblast (3T3) cells viability was checked after being treated with native diastase powder-stabilized AgNP.

The cotton-fabric ointments containing phytosynthesized AgNP using *Cassia roxburghii* were tested in vivo in male Wistar albino rats with burn wounds by Pannerselvam et al.[84] These particles promoted better wound healing when compared to a commercial burn healing ointment.[84] In another in vivo study, treatment with AgNP stabilized by *Piper nigrum* led to a reduction in the elevation of liver enzymes, bringing them to normal levels. Moreover, when compared to a commercial AgNP, they presented better results that did not lead to liver fibrotic changes and preserved the normal hepatic architecture.[85]

Attention should also be given to the toxicity of AgNP to the environment, since its effects on the environment have been still poorly understood.[89] The attendance of natural organic matter stabilized the particles and reduced toxicity in freshwater[217]. These results suggest that dissolved silver was answerable for the toxicity and highlight the need to account for matrix components such as chloride and organic matter in natural waters that induce AgNP fate and mitigate toxicity.[217]

Drimia indicta (Roxb.) Jessop (family Asperagaceae) is a well-known environmental mutagen plant commonly used for genetic monitoring studies.[218] For instance, AgNP produced using leaf extract of *Albizia saman* induced cytotoxicity and DNA damage in root tip meristematic cells of *D. indica* leading to cell death. Indeed,

those nanoparticles presented inhibitory and mitodispersive effect on cell division and behavior of chromosomes of *D. indica.*[219]

Concluding remarks

Plant extract-mediated phytosynthesis of AgNP has advantages, such as being energy efficient, inexpensive, and cost effective, as well as providing healthier work and community environments. It also preserves human health and the environment by producing less waste and safer products. It is noteworthy that, apart from the variety of the plants used in green synthesis and the quantity of phytochemicals present in different parts of a single plant, the same species of a plant may have its growth and composition affected by several factors, including climate, soil, location, humidity, and planting conditions.

Notably, there is a relevant issue in the selection of the plant and in the standardization of the methods not only used to phytosynthesize AgNP, but also to obtain the extract of the plant, where previously the plant materials were authenticated by sensory, macroscopic, microscopic, physicochemical, and chromatographic fingerprint characteristics to establish the quality and the degree of purity of plant exsiccate.[90] Finally, the present review clearly evidenced the vertiginous increase in the studies of plant-mediated biogenic synthesis of AgNP, which demonstrates the significant awareness towards developing environmental and human health-friendly techniques for the nanotechnology industry.

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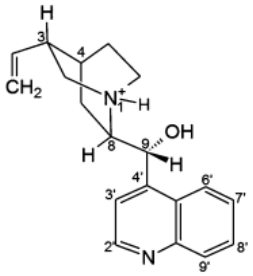
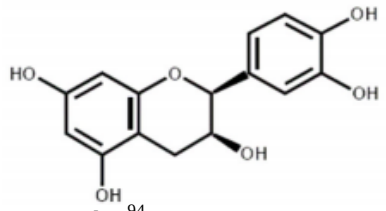
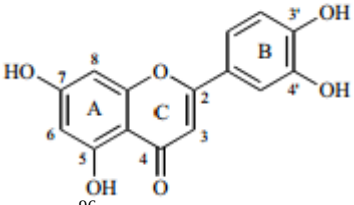
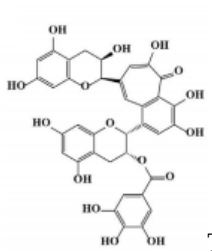
Legends of Tables:

Table 1. Relationship between bioactive components and plants studied.

Table 2. Minimal larvicidal concentration at 50% (LC 50) and 90% (LC 90) of silver nanoparticles obtained by leaf and fruit extracts using methanolic and ethyl acetate solvents.[32]

Table 3. Plant, part of plant used in nanophytosynthesis, characteristics of silver nanoparticles, and their effectiveness.

Table 1. Relationship between bioactive components and plants studied.

Bioactive compounds	**Plants**
Alkaloid  (-)-Cinchonidine (Cd)(1S, 3R, 4S,8S,9R).[29]	*Coscinium fenestratum;*[43] *Cymodocea serrulatal;*[67] *Erythrina indica;*[22] *Gloriosa superba;*[40] *Gymnema sylvestre;*[48] *Hibiscus sabdariffa;*[92] *Leucas martinicensis;*[49] *Piper longum*[44,93]
Catechins  Epicatechin[94]	*Rumex hymenosepalus*[95]
Flavonoids  Luteolin[96]	*Abutilon indicum;*[14] *Acalypha indica;*[97,98] *Agrimoniae herba;*[17] *Chrysanthemum indicum;*[99,100] *Chrysanthemum morifolium;*[80,101] *Cymodocea serrulata;*[67] *Psidium guajava (Guava);*[42,102] *Hibiscus sabdariffa;*[92] *Lantana camara;*[70,103] *Morinda citrifolia;*[79,104,105] *Ocimum sanctum;*[12,106,107] *Potentilla fulgens;*[87] *Pulicaria glutinosa;*[108] *Rosa damascena;*[109] *Saraca indica;*[110] *Sesuvium portulacastrum;*[111] *Solanum xanthocarpum*[112]
Polyphenols  Theaflavin-3'-gallate[113]	*Dryopteris crassirhizoma;*[114] *Emblica officinalis;*[56] *Euphorbia helioscopia;*[115] *Leonuri herba;*[58] *Leucas áspera;*[27 36] *Malus domestica;*[16] *Pulicaria glutinosa;*[108] *Rosa damascena;*[109] *Rumex hymenosepalus;*[95] *Terminalia chebula*[116,117]

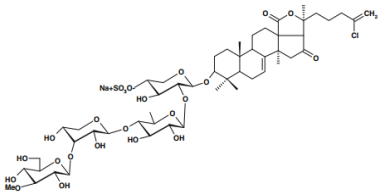
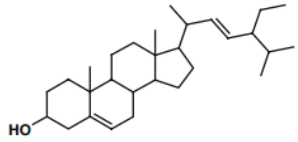
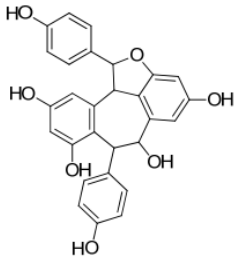
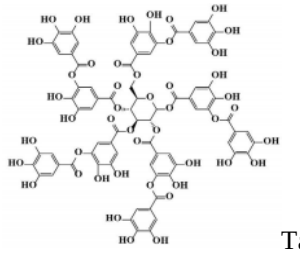
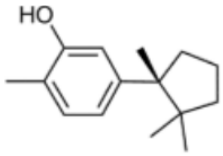
Saponins  118	*Abutilon indicum*[14] and *Memecylon edule*[119]
Sterol  Stigmasterol[120]	*Abutilon indicum*[14]; *Cymodocea serrulata*[67]; *Gymnema sylvestre*[48] and *Memecylon umbellatum*[59]
Stilbenes  Ampelopsin A[121]	*Rumex hymenosepalus*[95]
Tannin  Tannic acid[113]	*Agrimoniae herba;*[17] *Cymodocea serrulata;*[67] *Eucalyptus globulus;*[20, 122] *Gymnema sylvestre;*[48] *Piper longum;*[44,93] *Punica granatum;*[8,46,123] and *Terminalia chebula*[116,117]
TerpenoiBds  α -Cuparophenol[124]	*Chrysanthemum indicum;*[99,100] *Eucalyptus globulus;*[20] *Gymnema sylvestre;*[48] *Lantana camara;*[70,103] *Morinda citrifolia;*[79,104,105] *Ocimum sanctum;*[12,106,107,125] *Saraca indica;*[110] *Sesuvium portulacastrum;*[111] *Solanum xanthocarpum*[112]

Table 2. Minimal larvicidal concentration at 50% (LC 50) and 90% (LC 90) of silver nanoparticles obtained by leaf and fruit extracts using methanolic and ethyl acetate solvents.[32]

	Methonolic	Ethyl acetate
LC 50	67.78 - 85.75 ppm	44.55 - 49.96 ppm
LC 90	598.63 - 714.45 ppm	318.39 - 568.84 ppm

Table 3. Plant, part of plant used in nanophytosynthesis, characteristics of silver nanoparticles, and their effectiveness.

Plant	Plant part	Shape/Size (nm)	Activity
Abutilon indicum	Leaf	Spherical/7-17	*Bacillus substillus, Escherichia coli, Staphylococcus aureus*[14]
Acacia auriculiformis	Pod	Spherical/20-50	*Escherichia coli, Bacillus cereus, Staphylococcus* spp. *and Klebsiella* spp.[126]
Acacia leucophloea	Bark	Spherical/17-29	*Bacillus cereus, Listeria monocytogenes, Shigella flexneri, Staphylococcus aureus*[127]
Acalypha indica	Leaf	Spherical/20-30	*Escherichia coli*[97]
	Leaf	Cubic face/10-50 [98]	-
Achillea bierbesteinii	Flower	Hexanogal, pentagonal and spherical/10-14 [128]	-
	Flower	Spherical and pentagonal/10-40	Anti-apoptosis effect against MCF-7 cell line (human breast cancer) [129]
Acorous calamus	Rhizome	Spherical/31 [130]	
Acoorus calamus	Rhizome	Spherical/11	*Bacillus cereus, Bacillus substillus* [65]
Actaea racemosa	Leaf	-	*Bacillus substillus, Escherichia coli, Kocura rhizophila, Pseudominas aeruginosa* [82]
Agrimoniae herba	Whole plant	Spherical/11 [17]	-
Albizia adianthifolia	Leaf [131]	-	-
Albizia saman	Leaf	Spherical, triangular and irregular/55-83	Silver nanoparticles induces cytotoxicity and DNA damage in root tip meristematic cells of *Drimia indica*[219]
Allium kurrat	Leaf [132]	-	-
Aloe sp	Leaf	Spherical/ 3-9	*Bacillus substillus, Escherichia coli, Kocura rhizophila, Pseudominas aeruginosa* [82]
Alternanthera dentata	Leaf	Spherical/ 50-100	*Escherichia coli* [133]
Anacardium occidentale	Leaf	Spherical/15 [134]	-
Ananas comosus	Leaf	Spherical/12	*Escherichia coli, Staphylococcus aureus, Staphylococcus pneumoniae* [135]
Andrographis paniculata	Leaf	Spherical/52-56	*Aspergillus niger, Enterococcus faecais, Penicillium* spp.[136,35]
	Leaf	Rectangular/15-23	
Anethum graveolens	Leaf [132]	-	-
Annona muricata	Leaf	Spherical/22 [137]	-
Arbutus unedo	Leaf	Spherical/30 [138]	-
Artemisia absinthium	Leaf	Spherical/5-20 [139]	-
	Leaf	-/5-20 [21]	-
Ashoka tree	Leaf	Spherical/5-20	*Plasmodium falciparum* [140]
Azadirachta indica	Leaf	Spherical/ 10-37 [141]	-
Bacopa monniera [142]	-	-	-
Barleria prionitis	Leaf	Polydisperse/10-120 [6]	-
Beetrots extract	Beetrot pieces	Spherical/15	*Staphylococcus aureus, Streptococcus aureus, Escherichia coli, Pseudomonus aeroginosa* [143]
Beta vulgaris	Leaf [132]	-	-
Brassica juncea	Leaf	Anomalous shape [144]	-
Bryophyllum sp	-	-/2-5 [145]	-
Butea monosperma	Leaf	Spherical/20-80 [146]	-
Caesalpinea sappan	-	Spherical/30-47 [147]	-
Capsicum frutescens	Leaf [132]	-	-
Cassia roxburghii	Leaf	Spherical/17-29	Antifungal activity against *Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, Penicillium sp., Candida albicans and plant pathogens such as Rhizoctonia solani, Fusarium oxysporum and Curvularia sp*[37]

Catharanthus roseus	Leaf	Spherical/25-32	*Escherichia coli, Lactobacillus, Pseudomonas fluorescens, Staphylococcus aureus* [148]
Catharanthus roseus	Root	Spherical/35-55	Larvae of *Aedes aegypti, Culex quinquefasciatus* [149]
Chenopodium album	-	Spherical/12 [150]	
	Leaf	Spherical/10-30 [151]	
Chrysanthemum indicum	Leaf	Spherical/37-72	*Escherichia coli, Klebsiella pneumoniae* [99]
	Flower	Spherical/25-59	Larvicidal and pupicidal activity against malaria vector *Anopheles stephensi* [100]
Chrysanthemum morifolium	Flower	Spherical/20-50	*Escherichia coli, Pseudominas aeruginosa, Staphylococcus aureus*[80]
Ciprus sp	-	-/2-5 [145]	-
Citrullus colocynthis	Leaf	Spherical/31 [69]	-
Citrus limon (lemon)	Juice	Spherical and spheroidal/50 [152]	-
Citrus sinensis	Orange juice	Oval/-	*Bacillus substillus, Escherichia coli, Shigella, Staphylococcus aureus* [47]
Coccinia grandis	Leaf	Spherical/20-30 [38]	-
Cocos nucifera	Flower	Spherical/22	*Bacillus substillus, Klebsiella pneumoniae, Pseudominas aeruginosa, Salmonella paratyphi*[53]
Coscinium fenestratum	Leaf	Rod-shaped/28-68 [43]	-
Croton sparsiflorus	Leaf	Cubic/16	*Bacillus substillus, Escherichia coli, Staphylococcus aureus*[153]
Cucurbita máxima	Petal	Spherical/19 [65]	-
Curcuma longa	Tuber	Spherical/6-9 [154]	-
Cymodocea serrulata	Leaf	Spherical/5-25 [67]	-
Daphnia magna	Neonates	Spherical and triangular/<100 [89]	-
Delphinium denudatum	Root	Spherical/85	*Bacillus cereus, Escherichia coli, Pseudominas aeruginosa, Staphylococcus aureus* [155]
Desmodium gangeticum	Leaf	Spherical/18-39	*Escherichia coli* [57]
Dillenia indica	Fruit	-/40-100 [156]	-
Diopyros kaki	Leaf	Spherical/32 [157]	-
Dioscorea bulbifera	Tuber	Rod-shape and triangular/8-20 [50]	-
Dragon's blood		Shaped/4-50 [158]	-
Dryopteris crassirhizoma	Rhysome	Spherical/5-60	*Bacillus cereus, Pseudominas aeruginosa* [114]
Drosera binata	-	-/5	*Staphylococcus aureus* [52]
Eclipta Alba	Leaf	Spherical/2-6 [145]	-
Emblica officinalis	Fruit	Spherical/15	*Bacillus substillus, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus*[56]
Erythrina indica	Root	Spherical/20-118	*Bacillus substillus, Escherichia coli, Staphylococcus aureus* [22]
Eucalyptus angophoroides	Leaf	Spherical/3-9	*Escherichia coli, Kocura rhizophila, Pseudominas aeruginosa, Salmonella* [82]
Eucalyptus globulus	Leaf	Spherical/1-4; 5-25	*Bacillus substillus, Escherichia coli, Pseudominas aeruginosa, Staphylococcus aureus* (MRSA and MSSA)[20]
Euphorbia condylocarpa	Root	Spherical/- [159]	
Euphorbia helioscopia	Leaf	Spherical/2-14 [115]	
Euphorbia prostrata	Leaf	Rod-shaped/25-80 [83]	
Euphorbia prostrata	Leaf	Spherical/10-15	*Leishmania parasites* [83]
Fagopyri Dibotryis	Rhizoma [160]	-	-
Festuca rubra	Leaf	Anomalous shape [144]	-

Ficus religiosa	Leaf	Spherical/5-35 [161]	Anti-angiogenic and antioxidant activity
Ficus Carica	Leaf	Spherical/13	[31]
Garcinia xanthochymus	Leaf	Spherical/20-40	*Escherichia coli, Klebsiella pneumoniae, Pseudominas aeruginosa, Salmonella typhi, Staphylococcus aureus*[162]
Gentiana asclepiadea	Haulm and flower	-/20 [163]	-
Ginko biloba	Leaf [157]	-	-
Gloriosa superba	Leaf	Cubic/10-25 [40]	-
Gmelina asiatiaca	Leaf	Spherical, triangular and decahedral/ 20-64	Larvae of *Anopheles stephensi, Aedes aegypti, Culex quinquefasciatus* [164]
Guava	Leaf	Spherical/-	*Escherichia coli* [42]
Gymnema sylvestre	Leaf	Spherical/- [48]	-
Hevea brasiliensis	Latex	Spherical/2-100 [165]	-
Hibiscus arboreus	Leaf	95-105 [103]	-
Hibiscus cannabinus	Leaf	Spherical/9	*Escherichia coli, Proteus mirabilis, Shigella flexneri*[166]
Hibiscus rosa sinensis	Petals	Cubic/76	*Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Vibrio cholerae* [55]
Hibiscus sabdariffa	Leaf and stem	Spherical/5-30	*Escherichia coli* [92]
Hydrilla sp.	-	-/2-5 [145]	-
Hypnea musciformis	Leaf	Spherical/40-65 [167]	-
Illicium verum	Seed	Spherical/5-300 [168]	-
Impatiens balsamina	Leaf	-	*Bacillus substillus, Escherichia coli, Kocura rhizophila, Pseudominas aeruginosa* [82]
Iresine herbstii	Leaf	Spherical/44-64	*Enterococcus faecais, Escherichia coli, Klebsiella pneumoniae, Pseudominas aeruginosa, Staphylococcus aureus*[169]
Jatropha curcas	Latex	Cubic/10-20 [170]	-
Lantana camara	Leaf	Spherical, triangular, hexagonal and polygonal/ 57-65	*Escherichia coli* [103]
Lantana camara	Leaf	Spherical/11-24	*Bacillus spp., Escherichia coli, Pseudomonas* spp., *Staphylococcus* spp.[70]
Lemon	Peels	Spherical/17-61	*Trichophyton mentagrophytes, Trichophyton rubrum, Candida albicans* [61]
Leonuri herba	Leaf	Spherical/9-13	*Enterobacter cloacae, Escherichia coli, Pseudominas aeruginosa* [58]
Leucas áspera	Bark	Spherical/89	*Aeromonas hydrophila* [36]
	Leaf	Irregular shapes/25-80	*Aedes aegypti* [27]
Leucas martinicensis	Leaf	Spherical/20-30	*Bacillus substillus, Escherichia coli, Salmonella typhi, Staphylococcus aureus* [49]
Lippia citriodora	Leaf	Spherical/15-30 [34]	-
Lonicera japônica	Flower	-/3	*Escherichia coli* [171]
Macrotyloma uniflorum	Seed	Spherical/12 [18]	-
Magnolia grandiflora	Leaf	-	*Bacillus substillus, Escherichia coli, Kocura rhizophila, Pseudomonas aeruginosa* [82]
Magnolia kobus	Leaf	-/100-800 [157]	-
Malus domestica	Fruit	Spherical/10-40	*Bacillus cereus, Brevibacterium linens, Citrobacter koseri, Escherichia coli, Pseudominas aeruginosa, Staphylococcus aureus* [16]
Malva parviflora	Leaf	Spherical/19-25 [132]	-

Medicago sativa	Seed	Hexagonal and nanotriangles/ 86-108	*Antibacterial activity* [172]
Medicago sativa	Leaf [144]	-	-
Melia dúbia	Leaf	Spherical/7 [66]	-
Memecylon edule	Leaf	Square-shaped/50-90 [119]	-
Memecylon umbellatum	Leaf	Spherical/15-20	*Bacillus substillus, Klebsiella aerogenes, Salmonella typhimurium, Staphylococcus aureus, Streptococcus pneumonia* [59]
Mentha piperita	Leaf	Spherical/90	*Escherichia coli, Staphylococcus aureus* [54]
Mimosa pudica	Leaf	Spherical/25-50	*Aspergillus Níger, Escherichia coli, Pseudominas aeruginosa, Rhicephalus (Boophilus)microplus Canestrini* [173]
Mimusops elengi	Fruit	Spherical/ 12-30	*Staphylococcus aureus, Escherichia coli* [174]
	Leaf	Spherical/55-83	*Klebsiella pneumoniae, Staphylococcus aureus, Micrococcus luteus* [175]
	Leaf	Spherical and hexagonal/25-40	Larvicidal and pupicidal toxicity against malaria vector *Anopheles stephensi* and arbovirus vector *Aedes albopictus* [176]
Morinda citrifolia	Leaf	Spherical/10-60	*Bacillus cereus, Enterobacter aerogenes, Enterococci* spp., *Escherichia coli, Klebsiella pneumoniae, Pseudominas aeruginosa* [104, 105, 79, 41]
	Leaf	Spherical/12	
	Root	Spherical/30-55	
	Leaf	Spherical/12	
Moringa oleifera	Leaf	Spherical/11 [65]	
	Seed	Spherical/100	*Dengue serotype DEN-2, Aedes aegypti* [177]
Musa paradisíaca	Leaf	Rod/60-150	*Haemaphysalis bispinosa, Hippobosca maculata,* larvae of *Anopheles stephensi* and *Culex tritaeniorhynchus* [178]
Neem	Leaf	Spherical/2-8	*Plasmodium falciparum* [140]
Nelumbo nucifera	Leaf	Spherical, triangular and decahedral/25-80	Larvae of *Anopheles subpictus Grassi* and *Culex quinquefasciatus* [179]
	Seed	Spherical/5-16	Gram negative bactéria [180]
	Root	Spherical/16	Antioxidant and cytotoxicity activities against HeLa cell lines [64]
Nigella sativa	Leaf	Spherical/15 [45]	-
Nyctanthes arbortristis	Flower	Spherical and oval/5-20	*Escherichia coli* [51]
Ocimum sanctum	Stem and root	Spherical/10 [12]	-
	Leaf	Spherical/10-20 [181]	-
	Leaf	Triangular/42 [107]	-
	Leaf	Spherical/18-35 [125]	-
Orange	Peel	Spherical/60nm	*Aeromonas hydrophila* [182]
Origanum vulgare	Leaf	Spherical/126-146	*Aeromonas hydrophila, Escherichia coli, Salmonella paratyphi, Salmonella* spp., *Shigella dysenteriae, Shigella sonnei* [68]
Parmotrema praesorediosum	Lichen	Cubic/5-40	*Proteus vulgaris, Pseudominas aeruginosa, Salmonella typhi, Serratia marcescens* [183]
Parthenium hystrophorus	Leaf	Spherical/20-70	*Bacillus cereus, Escherichia coli, Pseudominas aeruginosa, Staphylococcus aureus* [184]
Pelargonium graveolens	Leaf	-/15	*Bacillus substillus, Escherichia coli, Kocura rhizophila, Pseudominas aeruginosa* [82]
Phytolacca decandra	Root	Spherical/91	*Escherichia coli* [77]
Phyllanthus amarus	Wole plant	Spherical/15-30	*Pseudominas aeruginosa* [185]
Phoenix dactylifera	Palm pit	Spherical/1-40	*Acinetobacter baumannii, Klebsiella pneumoniae, Rhizoctonia solani, Rhizoctonia solani* [186]

Pimenta dioica	Leaf	Spherical/200-500 [187]	-
Pinus densiflora	Leaf	-	*Bacillus cereus, Brevibacterium linens, Propionibacterium acnes, Staphylococcus epidermidis*[157, 188]
	Young cone	Oval and triangular/30-80	
Platanus orientales	Leaf [157]	-	-
Pinus eldarica	Bark	Spherical/ 10-40 [33]	-
Piper longum	Leaf	Spherical/17-41	*Bacillus cereus, Bacillus substillus, Pseudominas aeruginosa, Staphylococcus aureus* [44]
	Fruit	Spherical/46 [93]	
Piper nigrum	Leaf	Spherical/7-50	*Citrobacter freundii, Erwinia cacticida* [189]
	Stem	Spherical/9-30	
Pistacia atlântica	Seed	Spherical/10-50	*Staphylococcus aureus* [190]
Plumbago zeylanica	Root	Spherical/60	*Acinetobacter baumannii, Escherichia coli, Staphylococcus aureus* [191]
Plumeria Alba	Flower	Spherical/36	*Escherichia coli* [192]
Polygala tenuifolia	Root	Spherical and irregular shapes/15 [193]	-
Polygonum cuspidatum	-	Spherical/37	*Pseudominas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis* [194]
Populus Alba	Leaf	Spherical/71-92	*Escherichia coli* [103]
Potentilla fulgens	Roots	Spherical/10-15	*Bacillus substillus, Escherichia coli* [87]
Prosopis farcta	Leaf	Spherical/8-11	*Bacillus substillus, Escherichia coli, Pseudominas aeruginosa, Staphylococcus aureus* [195]
Prosopolis juliflora	Leaf	Triangle, tetragonal, pentagonal and hexagonal/11-19 [196]	-
Psidium guajava	Leaf	Spherical/30-60 [102]	-
Pterocladiella calippacea	Seaweed	-/11-14	*Bacillus substillus* [197]
Pulicaria glutinosa	Whole plant	Spherical/40-60	*Escherichia coli, Micrococcus luteus, Pseudominas aeruginosa* [108]
Punica granatum	Peel	Spherical/30 [46]	-
Psidium guajava	Leaf	Spherical/30-60 [102]	-
Quercus brantii	Leaf	Spherical/0-6 [198]	-
Rhinacanthus nasutus	Leaf	Spherical/11-22	*Alternaria alternata, Aspergillus flavus, Aspergillus Níger, Klebsiella pneumoniae, Staphylococcus aureus*[199]
Rosa damascena	Flower	SSpherical/10-30 [109]	-
Rosa indica	Petals	Spherical/23-60 [200]	-
Rumex hymenosepalus	Root	Cubic and hexagonal/2-40 [95]	-
Saliva officinalis	Aerial plant	Spherical and pentagonal/1-40 [201]	-
Sansevieria trifasciata	Leaf	-	*Bacillus substillus, Escherichia coli, Kocura rhizophila, Pseudominas aeruginosa* [82]
Saraca indica	Flower	Spherical/16-20 [110]	-
Sesbania grandiflora	Leaf	Spherical/10-25	*Salmonella entérica, Staphylococcus aureus* [202]
Sesuvium portulacastrum L	Callus and leaf	Spherical/5-20	*Alternaria alternata, Brevibacterium linens, Fusarium equisetii, Klebsiella pneumoniae, Listeria monocytogenes, Micrococcus luteus, Penicillium* spp., *Pseudominas aeruginosa, Staphylococcus aureus* [111]
Sida acuta	Leaf	Spherical, triangular and decahedral/18-35 [203]	-

Sterculia foetida	Seed	Spherical	Larvicidal activity against *Aedes aegypti (L.), Anopheles stephensi, Liston* and *Culex quinquefasciatus* [204]
Syzygium aromaticum	Clove bud	Spherical/20-30 [102]	-
Syzygium cumini	Bark	Polydisperse /9-35 [6]	-
	Fruit	Spherical/5-20 [205]	-
Solanum xanthocarpum	Berry extract	Crystallite/10-15	*Helicobacter pylori* [112]
Tanacetum vulgare	Flower	Spherical/16 [206]	-
Terminalia chebula	Fruit	Cubic/25	*Escherichia coli, Staphylococcus aureus* [207, 208]
	Fruit	Pentagonal, spherical and triangular/100	
Trianthema decandra	Roots	Spherical/36-74	*Escherichia coli, Proteus vulgaris, Staphylococcus aureus, Streptococcus faecalis, Proteus vulgaris, Yersinia enterocolitica* [209]
Tribulus terrestris	Leaf	Spherical/15-40	*Bacillus substillus, Escherichia coli, Pseudominas aeruginosa, Staphylococcus aureus, Streptococcus pyogens* [39,210]
	Fruit bodies	Spherical/16-28	
Tridax procumbens	Leaf and stem	-	*Aspergillus flavus. Aspergillus Níger, Escherichia coli, Vibrio cholerae* [211]
Triticum aestivum	Seed	-/20-96 [212]	-
Turbinaria conoides	Brown seaweeds	Spherical/2-17	*Aeromonas hydrophila, Escherichia coli, Salmonella* spp., *Serratia liquefaciens* [213]
Vitex negundo	Leaf	Spherical/26-39 [81]	-
Zingiber officinale	Root	Spherical/10-20	*Listeria* spp., *Staphylococcus* spp.[214]
Ziziphora tenuior	Leaf	Spherical/8-40 [215]	
Ziziphus jujube	Leaf	Different shapes/20-30	*Escherichia coli* [216]

Legends of Figures:

Figure 1. Mechanisms of nanoparticle synthesis (M+-metal ion).[15]

Figure 2. Possible mechanism of formation of silver nanoparticles.[18]

Figure 3. Schematic representation of various steps of AgNP synthesis, where enol form of ‘n’ number of polyphenolic groups of ‘n’ number of tannin present in AEC formed complex with Ag+ and further reduced n[Ag^{+}] to n[Ag^{0}], which simultaneously undergo nucleation, cluster formation, and AgNP growth. While in reduction, enol form changes into quinonoid form by releasing “n” number of electrons.[91]

Figure 4. Scheme of interaction between silver nanoparticles and human LoVo cell line.[74]

Figure 5. Nanomaterial-induced toxicity mediated by ROS Generation.[75]

Figure 1. Mechanisms of nanoparticle synthesis (M+-metal ion) [15].

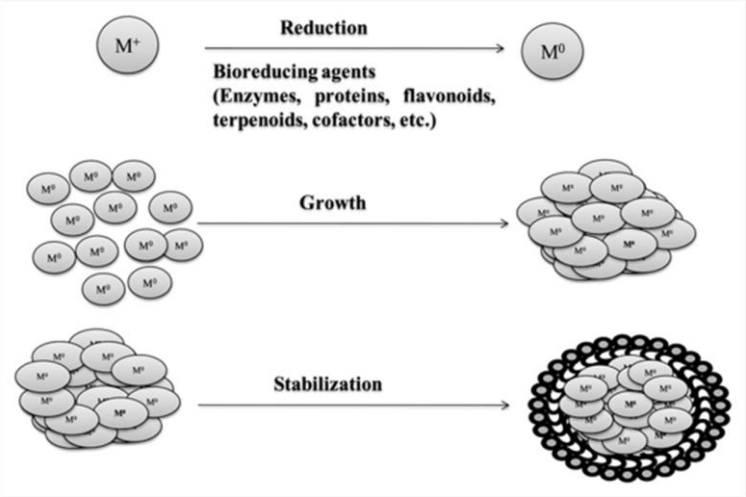


Figure 2. Possible mechanism of formation of silver nanoparticles [18].

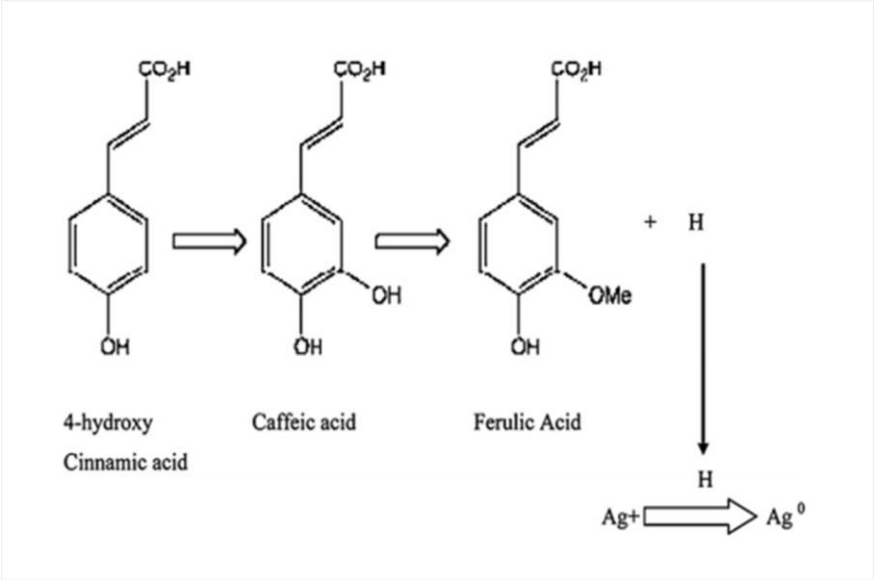


Figure 3. Schematic representation of various steps of AgNP synthesis, where enol form of ‘n’ number of polyphenolic groups of ‘n’ number of tannin present in AEC formed complex with Ag+ and further reduced n[Ag^{+}] to n[Ag^{0}], which simultaneously undergo nucleation, cluster formation, and AgNP growth. While in reduction, enol form changes into quinonoid form by releasing “n” number of electrons.[91]

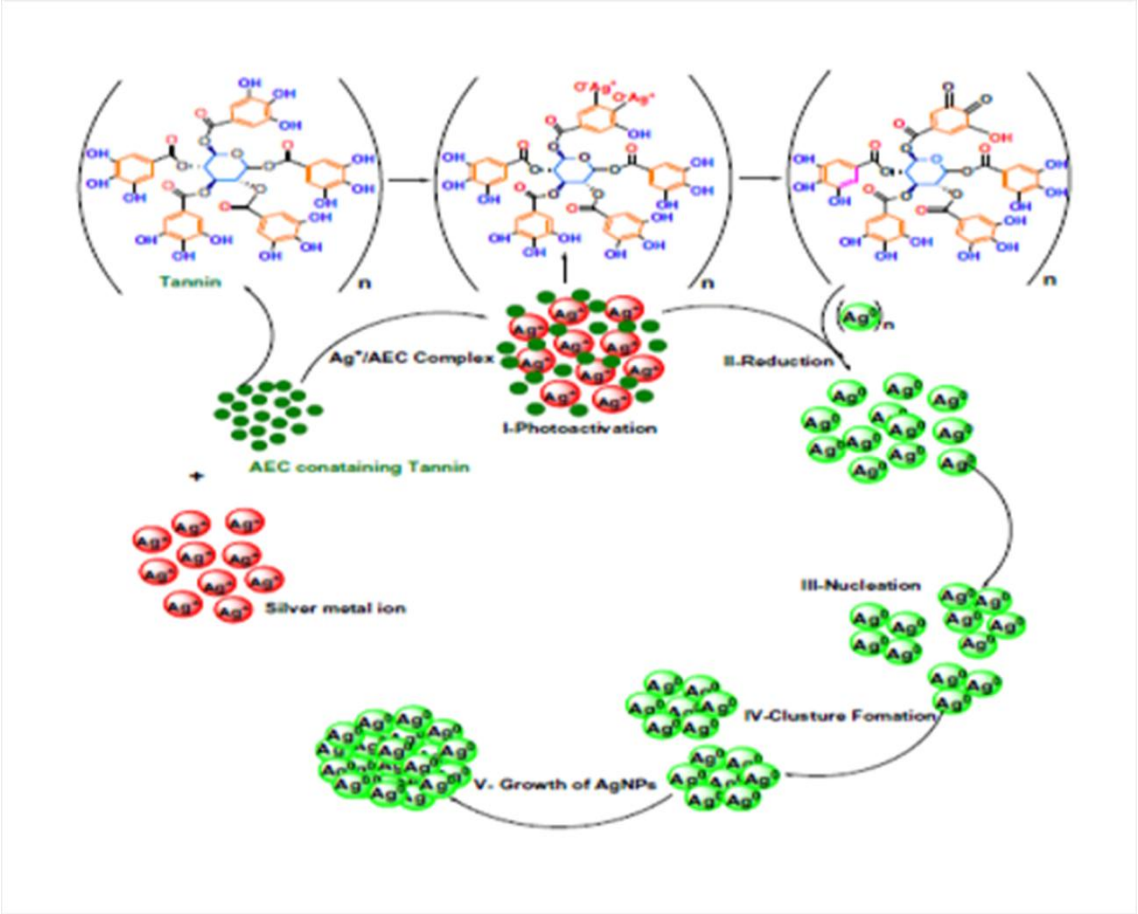


Figure 4. Scheme of interaction between silver nanoparticles and human LoVo cell line.[74]

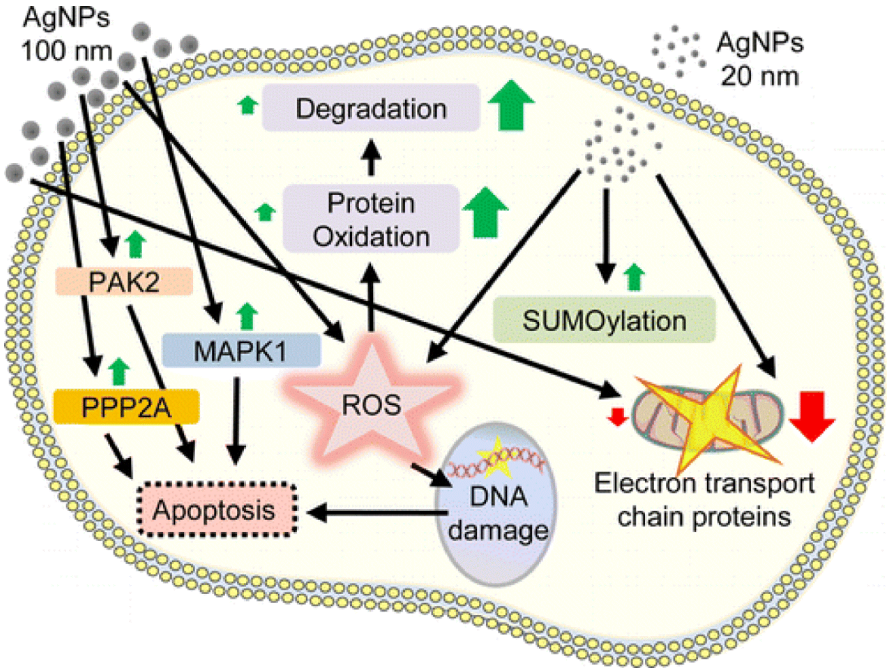
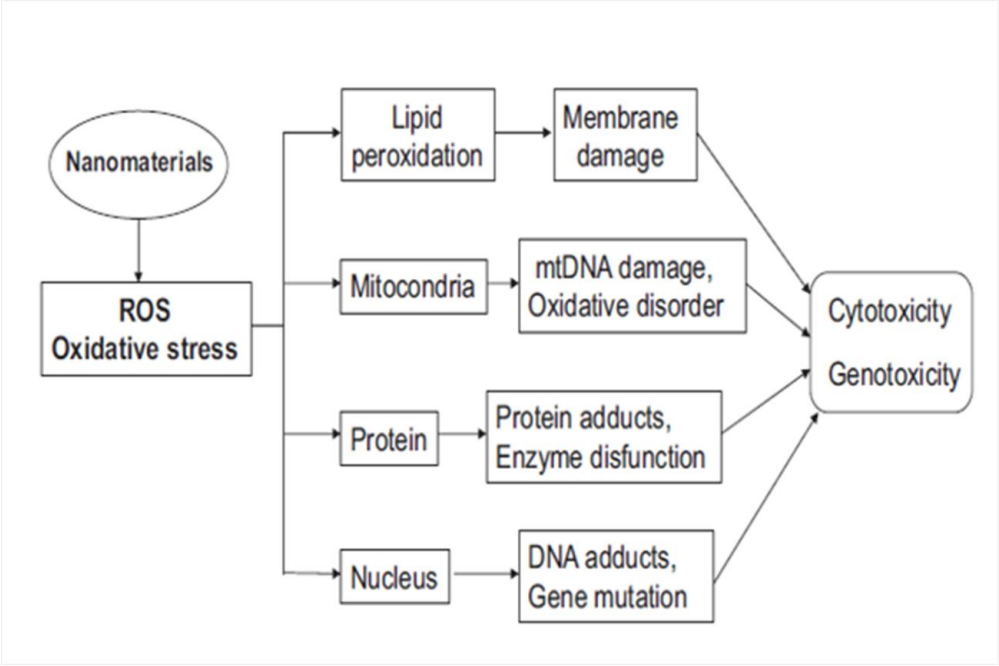


Figure 5. Nanomaterial-induced toxicity mediated by ROS generation.[75]



Capítulo 2 - Silver nanoparticles synthesized with pomegranate extract: extract reducing potential and antifungal effect of nanoparticles

*Artigo adequado às normas do periódico Biofouling

Silver nanoparticles synthesized with pomegranate extract: extract reducing potential and antifungal effect of nanoparticles

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Abstract

The biosynthesis of metal nanoparticles using plants has been receiving considerable attention as an alternative method of synthesis to using hazardous chemical and physical synthetic techniques. The aim of this study was to evaluate the potential of bioreduction of peel extract of pomegranate (*Punica granatum*) in the phythosynthesis of silver nanoparticles (AgNP) under different conditions of extract concentration and temperature of reaction. A specific electrode was used to calculate the remaining silver ions after the reactions, and the antifungal activity of the green-AgNP was verified against ATCC species of *Candida albicans* (10231) and *Candida glabrata* (90030) through microdilution method. The synthesized green-AgNP were confirmed by UV-Vis absorption spectroscopy, SEM analysis and EDS mapped 2D and dynamic light scattering pattern. The bioreduction potential of green-synthesis was dose dependent of pomegranate pell extract except at the highest temperature, and the lowest silver ions concentration was noted at 50ºC. Green-AgNP were more effective against *C. glabrata*, and the effectiveness against *C. albicans* seems to be strongly related to the remaining silver ions in the green-AgNP solution.

Keywords: silver, nanoparticles, G*ranatum*, *Candida*.

Introduction

The biosynthesis of metal nanoparticles using plants has been receiving considerable attention as an alternative to using hazardous chemical and physical synthetic techniques. Plants are being exploited for their unique metal tolerance and effective production of metal nanoparticles. A single plant contains a plethora of chemical elements including proteins, vitamins, enzymes, amino acids, polysaccharides and organic compounds that are according to Anand et al.(2017) "environmentally benign, yet chemically complex", and therefore would serve as ideal tools for enhanced medicinal applications. It is reported that phytochemicals such as terpenoids, polyols and flavones take part in the bio-reduction, stabilization and bio-capping mechanisms to form stable silver (AgNP).(Anibal, Peixoto et al. 2013, Anand, Tiloke et al. 2017)

Silver nanoparticles (AgNP) have been recognized as a significant antimicrobial for long (Bajaj, Pandey et al. 2017), becoming the last decade notably studied in combating against a wide range of bacterial and fungal pathogens (Bajaj, Pandey et al. 2017).

There is an incalculable variety of plants that can be used in the phytosynthesis. Pomegranate have been used for centuries to treat many diseases, and some studies using extracts of *Punica granatum* have shown high antioxidant (Bhaskar and Kumar 2012), anti-inflamatory(Lansky and Newman 2007), anticarcinogenic (Lansky and Newman 2007), apoptotic effects (Kiraz, Neergheen-Bhujun et al. 2016), anti-hemorrhagic activity (Goshtasebi, Mazari et al. 2015), antihyperglycemic (Bhaskar and Kumar 2012), anti-oxidant (Reddy, Gupta et al. 2007, Bhaskar and Kumar 2012), antimalarial (Reddy, Gupta et al. 2007), hypolipidemic (Bhaskar and Kumar 2012), and antimicrobial activities (Reddy, Gupta et al. 2007, Casaroto and Lara 2010, Prasad and

Kunnaiah 2014). When analysed by HPLC it were identified punicalin α and punicalin β as the major ellagitanins from the pomegranate (Machado, Leal et al. 2002).

Resistance of *Candida* species to azole antifungals is the most prevalent type of antifungal resistance (Sheehan, Hitchcock et al. 1999, Kontoyiannis and Lewis 2002, Nagayoshi, Miyazaki et al. 2017), an alarming increase in microorganisms resistant to triazoles has been reported recently (Ellepola and Samaranayake 2000), even more considering the growth of resistant microorganisms mainly in hospital environment (Morio, Jensen et al. 2017).

Punicalagin showed strong activity against *Candida albicans* and *Candida parapsilosis*, as well as in combination with fluconazole demonstrating synergistic interaction (Endo, Cortez et al. 2010). Besides that, *Punica granatum* as beneficial in reducing recurrent aphthous stomatitis pain and reducing the time of complete healing (Ghalayani, Zolfaghary et al. 2013) and as a preventive and therapeutic aid to periodontal disease (Hajimahmoodi, Shams-Ardakani et al. 2011, Prasad and Kunnaiah 2014), showed significant in vitro anti-*Helycobacter pylori* activity against the clinical isolates of *H. pylori*(Hajimahmoodi, Shams-Ardakani et al. 2011). Plants possibly use multiple strategies to deal with microorganisms that evolved over time (Abreu, Coqueiro et al. 2017), for this reason the secondary metabolites of plants represent a large library of compounds that could potentiate the effects of known antibiotics, so are important sources for new drugs or compounds suitable for further modification (Lu, Li et al. 2017).

This clinical problem arises in two settings: chronic refractory or recurring mucosal candidosis in AIDS patients (Fichtenbaum and Powderly 1998), and acute or subacute bloodstream candidosis in immunosuppressed or critically ill patients (Rex, Rinaldi et al. 1995). On the other hand, recurring vaginal candidosis in women without

predisposing disorders is rarely associated with resistant Candida spp (Cross, Park et al. 2000). *Candida* species were previously considered to be non-pathogenic fungi (Cuéllar-Cruz, Vega-González et al. 2012) or "opportunistic pathogens"(Williams and Lewis 2011) but are the most common causes of fungal infection (Turner and Butler 2014), because candidiasis frequently occurs in newborns, in immune-deficient people(Anwar, Malik et al. 2012), and in people being treated with broad spectrum antibiotics (Hani, Shivakumar et al. 2015). Approximately 90% of these infections are caused by five species, being *Candida albicans* the main pathological agent, followed by *Candida glabrata*, *Candida krusei*, *Candida parapsilosis* and *Candida tropicalis* (Cuéllar-Cruz, López-Romero et al. 2014, Turner and Butler 2014, Hani, Shivakumar et al. 2015). Invasive candidiasis is associated with high mortality and mortality in immunocompromised and hospitalized patients (Cuéllar-Cruz, López-Romero et al. 2014) specially in severely immunosuppressed patients with hematologic mallignancies (Zaoutis, Argon et al. 2005).

Therefore, the use of alternative therapies in associtaion or not with conventional ones could reduce the infections caused by Candida spp. Silver nanoparticles with low level of toxicity could be an approuch in treatments for instance recurring mucosal candidosis (Rodríguez-Tudela, Almirante et al. 2007) or even in the healing process of wounds (Gunasekaran, Nigusse et al. 2011). To cope whith this tissue, new efforts have been dedicated to discovering novel antimycotics.

The aim of this study was to evaluate the potential of bioreduction of peel extract of pomegranate (*Punica granatum*) in the phythosynthesis of silver nanoparticles under different conditions of extract concentration and temperature, as well as verified the antifungal activity of the resulting silver nanoparticles.

Materials and Methods

Obtaining the crude extract of pomegranate (Punica granatum) peels

Pomegranates were harvested in May 2015 season from Setuo Nomizo farm located at Eixo 21° 08’ 01’’. S, 51° 06’ 06’’ W (Mirandópolis, São Paulo, Brazil). Pomegranate peels were separated and stove-dried at 45°C for aproximatelly 72 hours, and then grounded in a processor at 18,000 rpm for approximately 30 seconds (TE-102, Tecnal Indústria, Comércio, Importação e Exportação de Equipamentos para Laboratório LTDA, Piracicaba, SP). The crushed peel was sieved using two sieves (Granutest, Telastem, São Paulo, SP) with granulations of 0.59 and 0.25 mm. The organic extract was then prepared using 150 g of the powder obtained from the peel (Bakkiyaraj, Nandhini et al. 2013) and etanol 70% by the maceration method followed by percolation proccess (Aslani, Zolfaghari et al. 2016). The extract was dehydrated (Bakkiyaraj, Nandhini et al. 2013) by rotary evaporator and then the crude extract was stored in ambar flask until be used. It was previously characterized in relation of pH, dry mass, ellagic acid and total phenolics expressed as gallic acid.

Determination of ellagic acid and total phenols

The ellagic acid content was determined by High Performance Liquid Chromatography (HPLC) (Shimadzu, Shimpack ODS C 18 reverse phase column, 100 mm x 2.6 mm). As mobile phase, HPLC grade methanol in 2% aqueous acetic acid gradient elution (0-7 min, 20-72.5% v/v methanol, 7-7.5 min, 72.5-95% v/v methanol, 7.5-8.5 min 95% v/v methanol, 8.5-9 min 95-20% v/v methanol, 9-10 min 20% v/v methanol) was added to a stream of 1.0 mL/min. The separation was achieved at 25° C. The injection volume was 5 μL and the wavelength used was 254 nm. The standard ellagic acid curve was prepared at concentrations between 6.25 and 100 μg / mL from a

stock solution at a concentration of 200 µg / mL in methanol (Panichayupakaranant, Itsuriya & Sirikatitham, 2010).

The total phenols were quantified in the crude extract by the spectrophotometric method with the results expressed in gallic acid. In the 50 mL of deionized water, the extract was homogenized using vortex and ultrasonic bath for 30 min. The standard curve was prepared from a stock aquous solution of gallic acid (24.97 mg/100 mL) in aliquots of 0.8, 0.9, 1.0, 1.1 and 1.2 mL, added with 2.5 mL of Folin-Denis reagent and 5.0 mL of 29% sodium carbonate, read in UV-Vis spectrophotometer at 760 nm (Zielinski and Kozlowska, 2000). The samples were analyzed in triplicate.

Phytosynthesis of silver nanoparticles using pomegranate peel extract and phisico-chemical characterization

Colloidal silver nanoparticles were synthesized by the reduction of silver ions (Ag^{+}) of $AgNO_3$ (Merck KGaA, Darmstadt, Hesse, Germany) by the alcohollic extract of pomegranate obtained previously as described by Fernandes (2016).

Inicially, 100 mL of $AgNO_3$ aqueous solution (289 $mmol \cdot L^{-1}$) was heated in diferents temperatures, 25, 50, 75 and 95° C with diferents concentration of pomegranate extract, 7, 14 and 28 $mg \cdot mL^{-1}$, in which the pH was maintained between 8.0 and 8.5. The mixture was maintained under magnetic stirring and heating for 10 minutes until the appearance of the dark brown color, which qualitatively demonstrates the formation of silver nanoparticles. Silver nanoparticles were also prepared at 95ºC using 28 mg mL^{-1} of extract during with different times of reaction, 5, 10 and 15 minutes. The reaction time was varied at this temperature, in order to verify if the higher temperature would accelerate the reaction and if reducing the time for 5 minutes or increasing for 15 minutes would lead to a greater degradation of the phytochemical

components, and, hence would impair the antimicrobial and stabilizing effect of the green silver nanoparticles.

After the synthesis process of silver nanoparticles, the particles were analised by absorption spectroscopy in the Ultraviolet/Visible (UV/Vis) (Jasco V-660) in the region of 300–800 nm, using a commercial quartz cuvette. The samples of green-silver nanoparticles were qualitatively characterized by scanning electron microscopy (SEM) on a Zeiss Supra 35VP microscope with field emission gun electron effect (FEG-SEM, 10 kV). Energy Dispersive X-Ray Detector (EDX) analyzes with mapping in 2D images were constructed by analyzing energy released from the emission C Kα, Ag Lα1 and O Kα.

X-ray diffraction (XRD) analysis was done in the Shimadzu XRD diffractometer with a Cu Kα radiation operating at 30 kV and 30 mA and 2θ range from 35 to 85° with step scan of 0.02° and scan speed of 0.2° min^{-1}. To collect AgNPs patterns, nanoparticles were deposited on the surface of a silicon substrate (Si) by dripping the aqueous colloidal dispersion on the substrate at a room temperature, and the solvent was evaporated.

Dinamic light scattering experiments were performed at room temperature and at a fixed angle of 173° on a Malvern Zetasizer Nano ZS equipped with 50 mW 533 nm laser and a digital auto correlator. In this method, the number-average values obtained were compared to the size distributions of the AgNP.

Quantification of silver ions

The amount of free silver ions present in each sample of green-silver nanoparticles at different extract concentrations (7, 14 and 28 mg mL-1) and temperatures (25, 50, 75 and 95° C) was calculated by a specific electrode (Orion 9616BNWP, Thermo Scientific, Beverly, MA, USA), in the measurement ranging from

0.01 to 107.9 µg Ag/mL. The combined electrode was calibrated with standards containing 54 to 60 to achieve equivalent silver concentrations in the samples (ISA reading Cat .No. 940011) which provides a constant background ionic strength (1 ml of each sample/standard for 20 µL of ISA).

Planktonic cell susceptibility test: determination of Minimal Inhibitory Concentration (MIC) to green-AgNP

The minimum inhibitory concentration (MIC) of each sample of green-AgNP were obtained using the microdilution method following the Clinical Laboratory Standards Institute (CLSI guidelines, document M27-A2). Briefly, the samples were diluted in deionized water in geometric progression from 2 to 1024 times, and then, diluted at 1:5 in RPMI 1640 medium (Sigma-Aldrich, Chemical Co, St Louis, Missouri, USA). Reference strains (American Type Culture Collection (ATCC)) of *Candida albicans* (10231) and *Candida glabrata* (90030) were stored as frozen stocks at -70º C in glycerol 20% until required. After, they were subcultered on Sabouraud dextrose agar medium (SDA, Difco, Le Pont de Claix, France) at 37 °C for 24 h. *Candida* cells were adjusted to a turbidity equivalent to a 0.5 McFarland Standard, and then they were diluted (1:5) in saline (0.85% NaCl) and subsequently diluted (1:20) in RPMI 1640medium (Sigma-Aldrich, Chemical Co, St Louis, Missouri, USA). Each inoculum (100 µL) was added to the respective well of 96-microtiter plates containing 100 µl of each specific concentration of green-AgNP solution. The plates were incubated at 37°C, and the MIC values were determined visually as the lowest concentration of green-AgNP with no microorganism growth after 48 h. The results were confirmed by plating the content of each well on Sabouraud Dextrose Agar (SDA, Difco). The assays were done in triplicate on three different occasions.

Results

Synthesis and characterization of green-AgNP

UV-Vis absorption spectroscopy showed silver in nanosized dimensions in all phythosinthesis of AgNP by peel extract of pomegranate, regardless of the reducing agent used. It was demonstrated by the presence of an intense absorption peak, denominated plasmonic band, well-defined plasmon band centered at 375 nm, characteristic of nanosized silver (Figure1) (Lok, Ho et al. 2006). It characterizes noble metal nanoparticles, with strong absorption band observed in the visible region. The concentrations of ellagic acid and total phenols expressed in galic acid of 4.21 and 158.61 mg mL^{-1}. All the different conditions of this synhtesis promoted the formation of nanoparticles.

SEM analyses showed silver nanoparticles with a definite shape, in all synthesis at different temperature and concentration (Figure 2). The images shows differents forms of particles, in which, it was observed spherical, pentagonal and triangular shapes. It was observed that the decrease of the particles size with the increase of the temperature and concentration of extract (Figure 2). The 2D images were constructed by analyzing the energy released from the issuance Si Kα, C Kα and Ag Kα, and indicated the distribution of these elements on the demarcated area in the micrograph (Figure 3). The elements C is characteristics of alcoholic extract that actua as reductor agent and Ag element characteristic of formation of silver nanoparticles.

Dinamic light scattering graphics (Figure 4) show the distribution of colloidal green-AgNP, with increasing of the aglomeratation of the particles in the highest temperature of synthesis.

Minimum inhibitory concentration (MIC)

The results showed that the SN were fungicidal against the tested yeasts at very low concentrations and the fungicidal activity was dependent on the yeast species tested (Table 1). These results were confirmed by plating the content of each well on SDA, and there was no growth for any of the strains resultant from the MIC point. The lowest MIC (2.09 μg mL^{-1}) were obtained with green-AgNP produced with 15 mg mL^{-1} of peel extract at 95º C against both Candida ssp (Table 1). Nanoparticles produced above 50ºC were more effective against both species of *Candida*, independent of the concentration of extract and the remmaining Ag^{+} ions. Although the reducing potential of the reaction had been higher at 50ºC and at 28 mg mL^{-1} extract (lower concentration of Ag^{+} ions), the antifungal activity of green-AgNP, specially against C. glabrata,was increased when synthesized above 70ºC independent of the concentration of extract used in the reaction.

The Ag^{+} ions concentration was dose dependent of the peel extract concentration used in the phythosynthesis reaction (Table 1). The lowest and the highest concentration of ions in the syntheses with 28 mg mL^{-1} of extract at 50° C (6.9 μg$Ag^{+}\cdot mL^{-1}$) and 7 mg mL^{-1} at 95° C (907.3 μgAg^{+} mL^{-1}).

Discussion

In general, the concentration of free ions increased with the increase of the temperature and decreased with the increase of the extract concentration, and also, when increasing the temperature above 70°C the concentration of free ions doubled. However, at the extract concentration of 7 mg mL^{-1} the heating at 50°C favored the synthesis by lowering free ions from 452.19 to 269.99 μg Ag^{+} mL^{-1}. The probable explanation for the better results with heating its because the levels of ellagic acid might have increased, as it was demontrated by Szwajgier et al.(2014) (Szwajgier, Halinowski et al. 2014) when their samples were heated for 30-60 minutes.

The concentration of ellagic acid in the peel extract of pomegranate was 4.21 mg mL-1, and total phenols expressed in galic acid was 158.61 mg mL-1. Ellagic tannins are glucose esters with hexahydrolxydiphenic acid that, once hydrolyzed, release ellagic acid as active principle (Falsaperla, Morgia et al. 2005). Ellagic acid may occur in its free form or, much more commonly, as a breakdown product of hydrolyzable tannins, specifically, ellagitannins which is a dimer of gallic acid (Lansky 2006). It presents antioxidant properties, and it is one of the most atractive compounds amongst the numerous natural substances with proapoptotic action that have been experimentally investigated in vitro (Festa, Aglitti et al. 2001, Wedge, Meepagala et al. 2001, Falsaperla, Morgia et al. 2005).

Phenolic compounds contained antioxidant properties and high tendency to chelate metals. Phenolic compounds possess hydroxyl and carboxyl groups. For instance, these compounds may inactivate metal ions by chelating and additionally suppressing the superoxide-driven Fenton reaction, which is believed to be the most important source of reactive oxygen species (ROS) (Iravani and Zolfaghari 2013).

Increasing the extract concentration from 7 to 15 mg•mL-1 there was an expressive decrease in the amount of free ions at the temperatures of 25, 50 and 70°C. Nevertheless, at the highest temperature (95°C) the reduction of silver ions was lower possibly due to the degradation of the phytochemical components in the extract which present the property of reducing silver ions and stabilizing the nanoparticles(Mittal, Chisti et al. 2013). Also, when the extract concentration increased from 15 mg to 30 mg mL^{-1}, the decrease of ions was notable at all temperatures.

The amount of free ions clearly interefered in the MIC values specially against *Candida albicans* strain. MIC results were lower with higher ions concentrations, a fact compatible with several previous studies (Chappell and Greville 1954, Schreurs and

Rosenberg 1982, El Badawy, Silva et al. 2011), where free ions provide higher toxicity to the cell and to the microorganism in comparision with stable silver nanoparticles (El Badawy, Silva et al. 2011). Acoording El Badawy et al.(2011) the toxicity is decreased by removing Ag^+ from the AgNP suspension. Even after purification, the dissolution of AgNP to Ag^+ can be a source of toxicity (El Badawy, Silva et al. 2011). Moreover, Lok et al. (2006) observed that AgNP led to a rapid collapse of proton motive force, inducing a massive loss of intracellular potassium and decreasing the cellular ATP levels, apparently caused by the collapse of membrane potential(Lok, Ho et al. 2006), in mitochondria silver ions stimulate adenosine triphosphatase activity of mitochondria obtained from rabbit cerebral córtex (Chappell and Greville 1954) and in *Escherichia coli* silver ions inhibited phosphate uptake and exchange causing efflux of accumulated phosphate (Schreurs and Rosenberg 1982).

In the present study, samples produced with lower extract concentrations (7 and 14 mg mL-1) the silver ions were dramatically decreased from 452.19 to 32.07 μg Ag^+/mL at 25^{o}C and from 269.99 to 13.44 μg Ag^+/mL at 50^{o}C. On the other hand, all green-AgNP produced at 70^{o}C had antifungal activity against both strains of *Candida*, regardless of the silver ions remmaining in the solution. The increase in temperature promotes the breakdown of complex compounds into ellagic acid (Szwajgier, Halinowski et al. 2014), as a breakdown product of hydrolyzable tannins, specifically, ellagitannins (Lansky 2006) thus ellagic acid becoming more concentrated in the AgNP solution synthesized above 70°C, then contributes to the antifungal effect of AgNP, because current research seems to indicate that the most therapeutically beneficial pomegranate constituents are ellagitannins (including ellagic acid) (Quideau and Feldman 1996, Bakkiyaraj, Nandhini et al. 2013, Usta, Ozdemir et al. 2013). Pinelli

reported that the total amount of (poly)phenolic compounds identified by HPLC increased after heat treatment (Pinelli et al. 2007).

Candida glabrata is more susceptible than *Candida albicans* being all samples of nanoparticles tested effective at very low concentrations. These results could have occured due to the different strategies of defense of each specie of *Candida* (Silva, Negri et al. 2011). For instance when in the host, *C. albicans* follows an agressive strategy to subvert the immune response and to obtain nutrients for its survival enzymes (Silva, Negri et al. 2011), forms hyphae and aggressively destroys tissue (Jayatilake, Samaranayake et al. 2006), eliciting a strong immune response (Polke, Hube et al. 2015). Whereas, regardless of many aspects of pathogenicity are still unknown, *C. glabrata* seems to have a strategy based on stealth, evasion and persistence (Brunke and Hube 2013).

Although the correlation between the effectiveness and the amount of free μg Ag^{+}/mL is evident, when the concentration of Ag^{+} increased from 568 to 907 μg/mL there was no reduction in the values of MIC agaisnt both *Candida* strains. As it ensues with some antimicrobials, there might have a level of saturation of the passage of these free ions through the fungi that could have limited its antifungal effect (Brajtburg, Elberg et al. 1984, Brajtburg and Bolard 1996). In a study with Zn^{+2}, (Failla, Benedict et al. 1976) it was observed two distinct processes of ions accumulation in *Candida utilis*. First, it would occur a rapid and limited energy and temperature independent binding to the cell surface. The second process would be slower, but sustained energy and temperature dependent translocation across the cell membrane. Energy dependent Zn^{+2} uptake is a highly specif process that exhibits saturation kinetics (Failla, Benedict et al. 1976), and it could also happen with Ag^{+}, where the membrane would exhibit saturation over a specific concentration of μg Ag^{+} mL^{-1}.

Moreover, the charge of silver nanoparticle also influences the antimicrobial activity (Abbaszadegan, Ghahramani et al. 2015), positively charged AgNP leading to a atraction of a negatively charged head group (Löffler, Einsele et al. 2000) of phosphatydylinositol and phosphatidylserine presents in the composition of plasma membrane of *C. albicans.*

The synthesis process and the coating agents used interfere with the surface loading of AgNP, which reflects the charge on the AgNPs surface, varied in type (negative/positive) as well as the magnitude based on the capping agent or the reactants used during the synthesis process (El Badawy, Silva et al. 2011) it is well documented that phytochemicals capped the nanoparticle (Das and Brar 2013) however, litle is known about the capacity of interfering or not with the charge of the nanoparticle.

So, the obtained results from the synthesized AgNP, will lead bring awareness toward the application of green-nanomaterials as an alternative to be used as antifungal agent ecofriendly produced and suitable for developing a biological process for large-scale production. However, it is necessary to explore if the antioxidant, antiinflamatory, antiapoptotic, antimicrobial properties presented in the *Punica granatum* are added in the green-AgNP nanoparticle when compared to conventional produced-ones, since the phytochemicals covering the nanoparticles can still contain the benefitial pharmaceutical properties of that plant. Also, further studies are recommended on the mechanism of action of the phytosynthesized AgNP and the role of the silver ions at molecular level on the microorganisms.

Conclusion

Pomegranate peel extract successfully produced AgNP, and the bioreduction potential was proportional of the concentration used in the reaction. Besides, the lowest

and the highest concentration of silver ions were found at 50ºC and 95ºC. The temperature of 70 ° C favored the reductor potential of the extract, and produced silver nanoparticles with antifungal activity with low MIC values. In general, green-AgNP produced antifungal activity at very low concentration against both *Candida* strains. Also, *Candida albicans* seems to be more susceptible to the remaining silver ions in the phytosynthesized AgNP.

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Legend of Tables and Figures:

Table 1. Temperature, time and concentration of pomegranate peel extract used in the phythosynthesis reaction, remmanining silver ions in the green-AgNP solution and MIC of each sample of green-AgNP against *Candida* spp.

Figure 1. UV–Vis absorption spectrum of green-AgNP in differents concentrations.

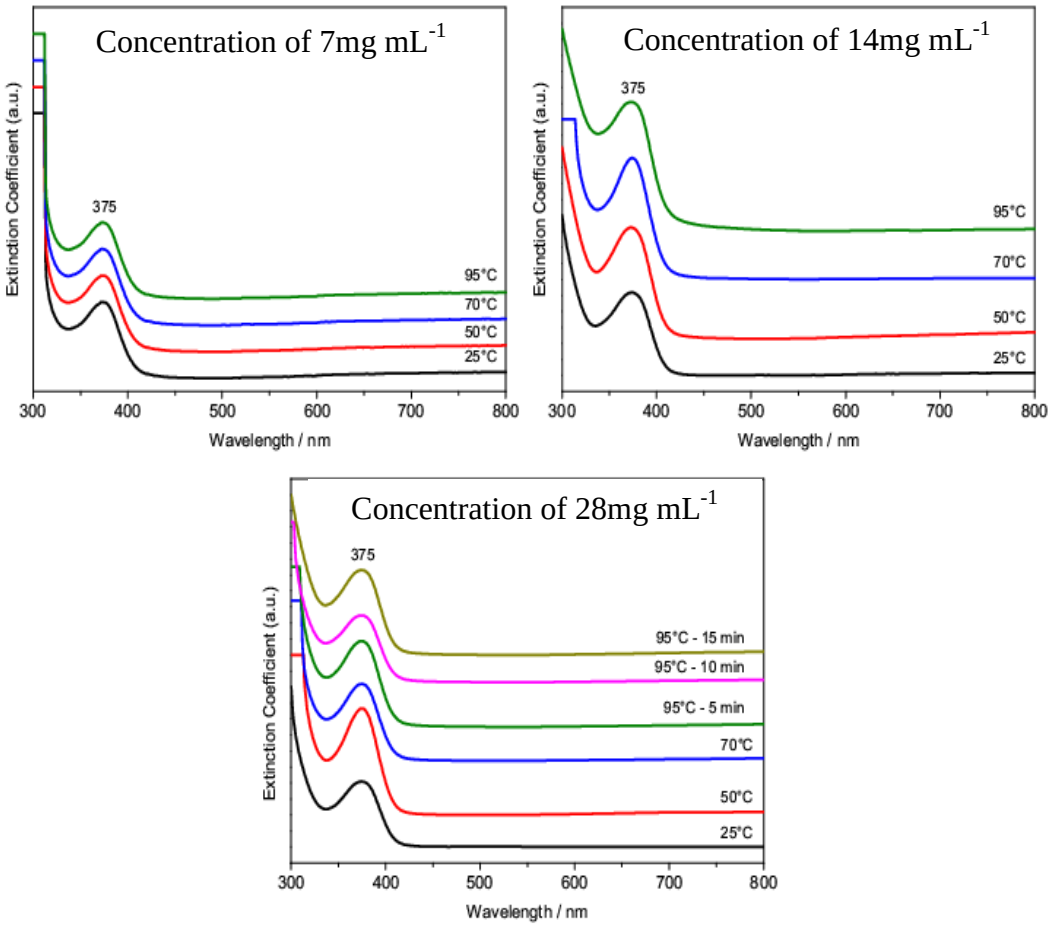
Figure 2. SEM micropraphs of the silver nanoparticle. A) 25°C – 7 mg mL^{-1}; B) 25°C – 14 mg mL^{-1}; C) 25°C – 28 mg mL^{-1}; D) 50°C – 7 mg mL^{-1}; E) 50°C – 14 mg mL^{-1}; F) 50°C – 28 mg mL^{-1}.

Figure 3. SEM and EDS mapped in 2D elements issuance Si Kα, C Kα and Ag Kα false color. Analysis of the distribution of silver nanoparticles on the sample: a) SEM image; b) chemical mapping of silicon element present in the substrate, where the electron beam was focused directly on the substrate and is showed in red color; c) chemical mapping of carbon element present in the alcoholic extract in green color; d) chemical mapping of silver element, in blue color.

Figure 4. Dinamic light scattering pattern of AgNP phytosynthesized at different conditions.

Table 1. Temperature, time and concentration of pomegranate peel extract used in the phythosynthesis reaction, remmanining silver ions in the green-AgNP solution and MIC of each sample of green-AgNP against *Candida* spp.

Temperature (ºC)	Time (min)	Peel extract (mg mL^{-1})	Silver ions (ug Ag^{+} mL^{-1})	MIC (ug mL^{-1})	
				C. albicans	*C. glabrata*
25	10	7	452.19	4.19	4.19
		14	32.07	-	4.19 - 8.38
		28	9.19	-	4.19 - 8.38
50	10	7	269.99	8,38	8.38
		14	13.44	-	4.19 - 8.38
		28	6.90	-	4.19 - 8.38
70	10	7	568.90	4.19	2.09
		14	82.18	4.19 - 8.38	2.09
		28	14.22	16.75	2.09 - 8.38
95	10	7	907.30	4.19	2.09
		14	816.54	2.09 - 4.19	2.09
	5	28	22.17	-	4.19 - 8.38
	10		31.35	8.38	4.19 - 8.38
	15		35.77	-	4.19 - 8.38



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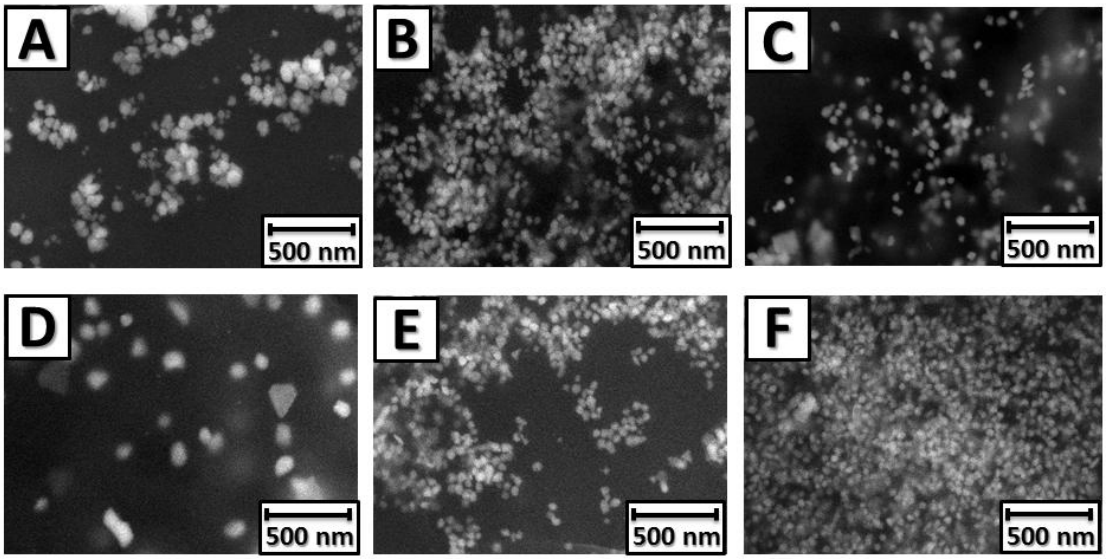

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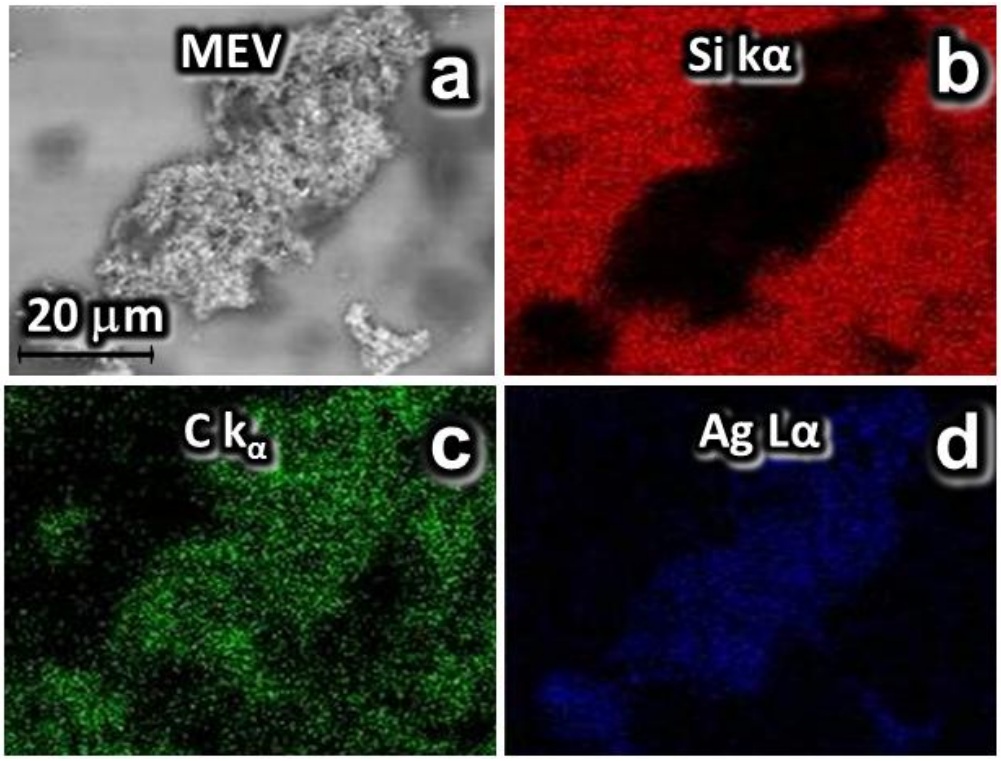

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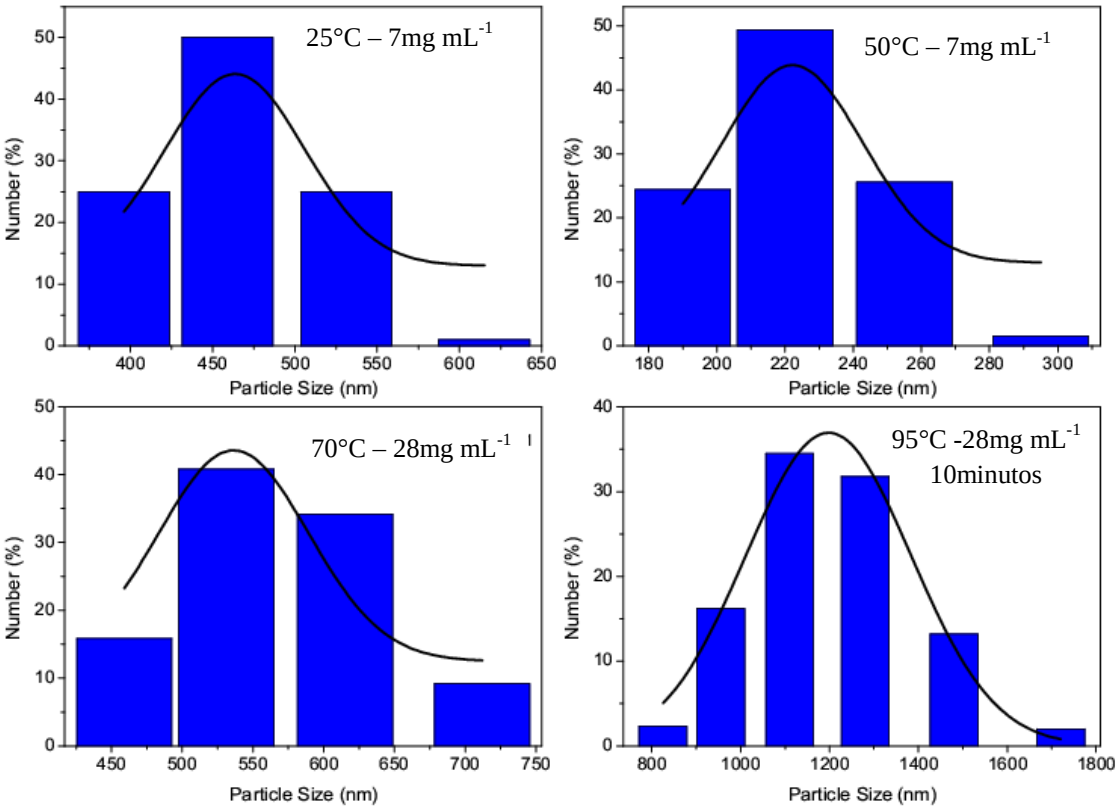

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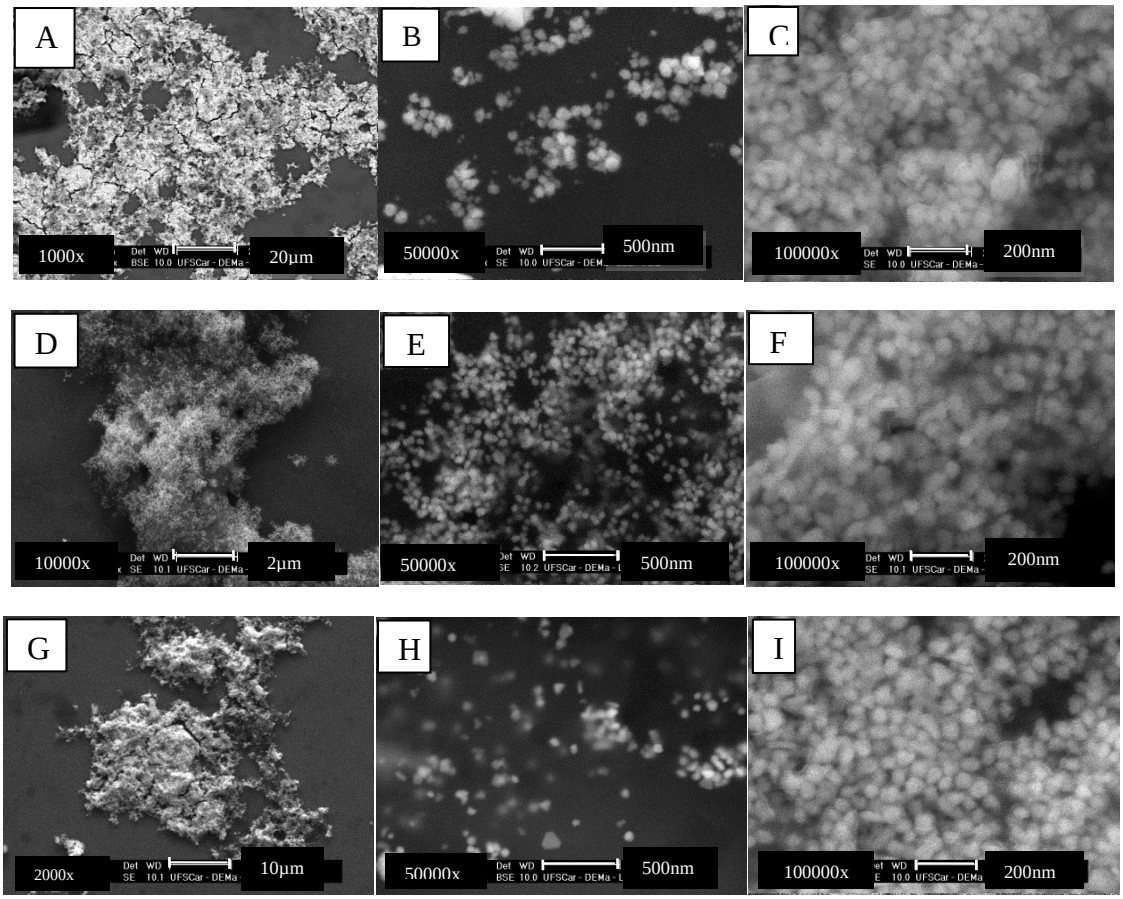
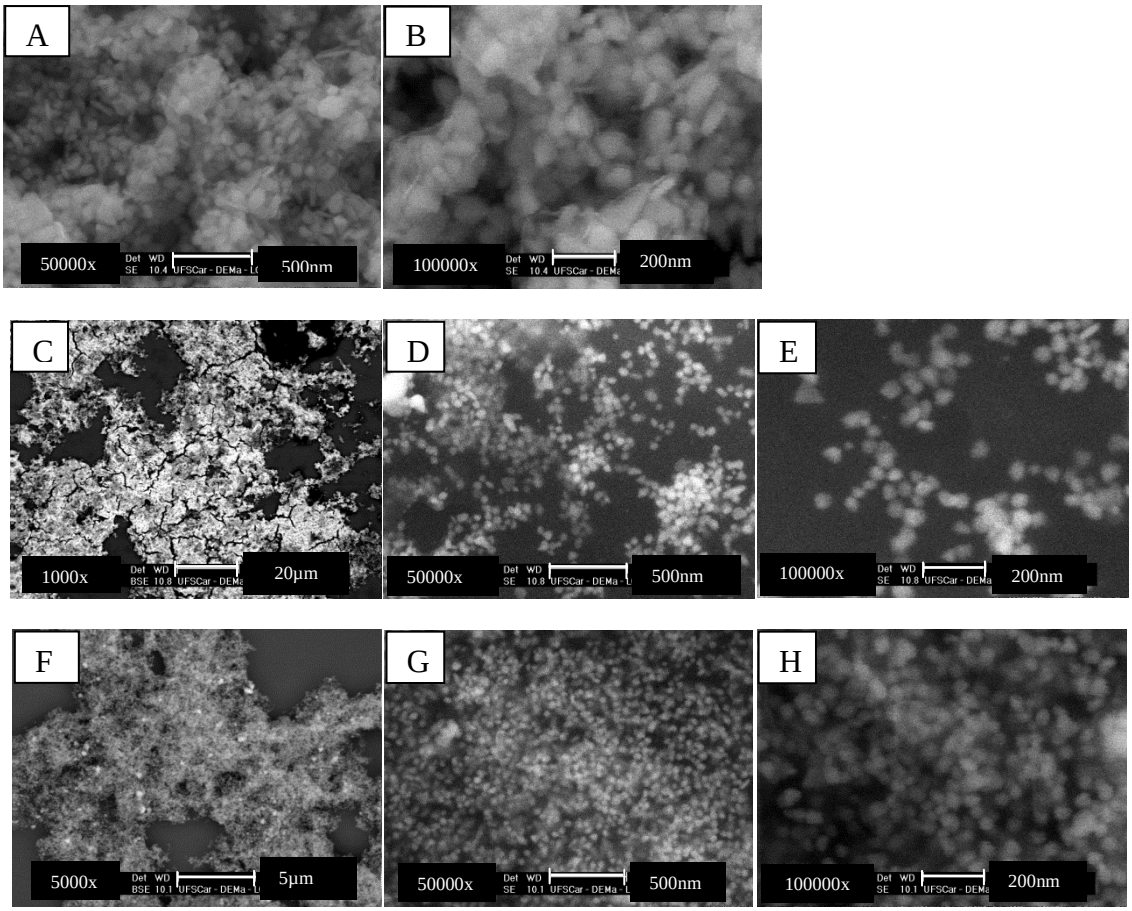
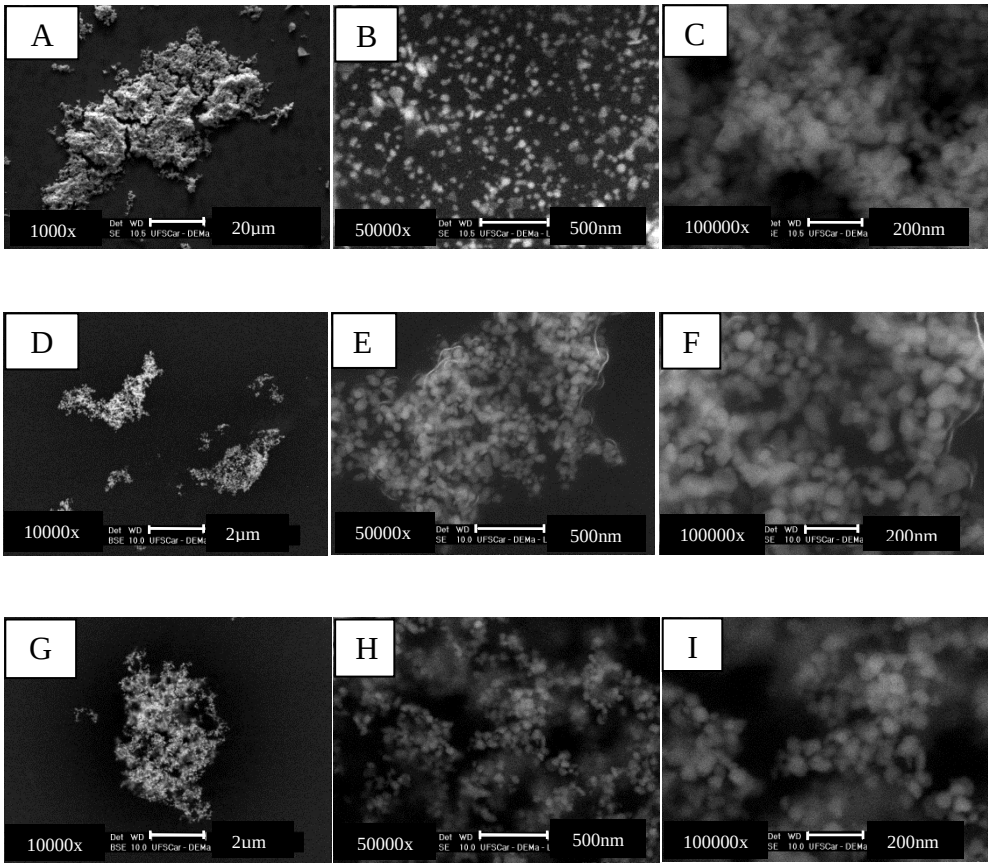
Anexos



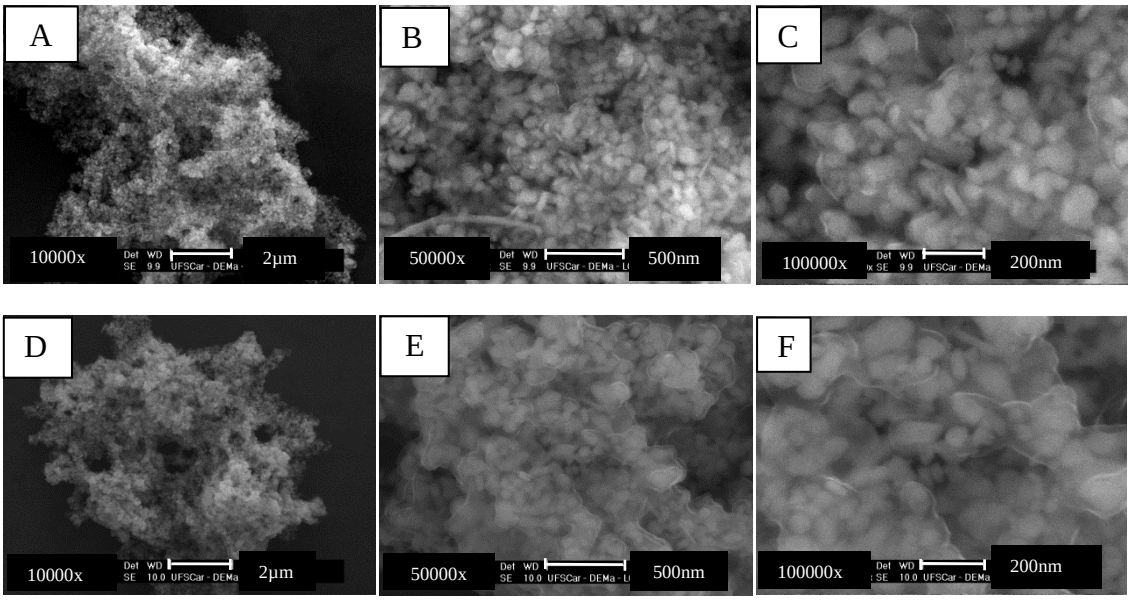
MEV microscopias da nanopartícula de prata. A)25°C - 7mg mL^{-1}; B) 25°C - 7mg mL^{-1}; C) 25°C - 7mg mL^{-1}; D) 25°C - 14mg mL^{-1}; E) 25°C - 14 mg mL^{-1}; F)25°C - 14mg mL^{-1}; G)25°C - 28mg mL^{-1}; H) 25°C - 28mg mL^{-1}; I)25°C - 28mg mL^{-1}.



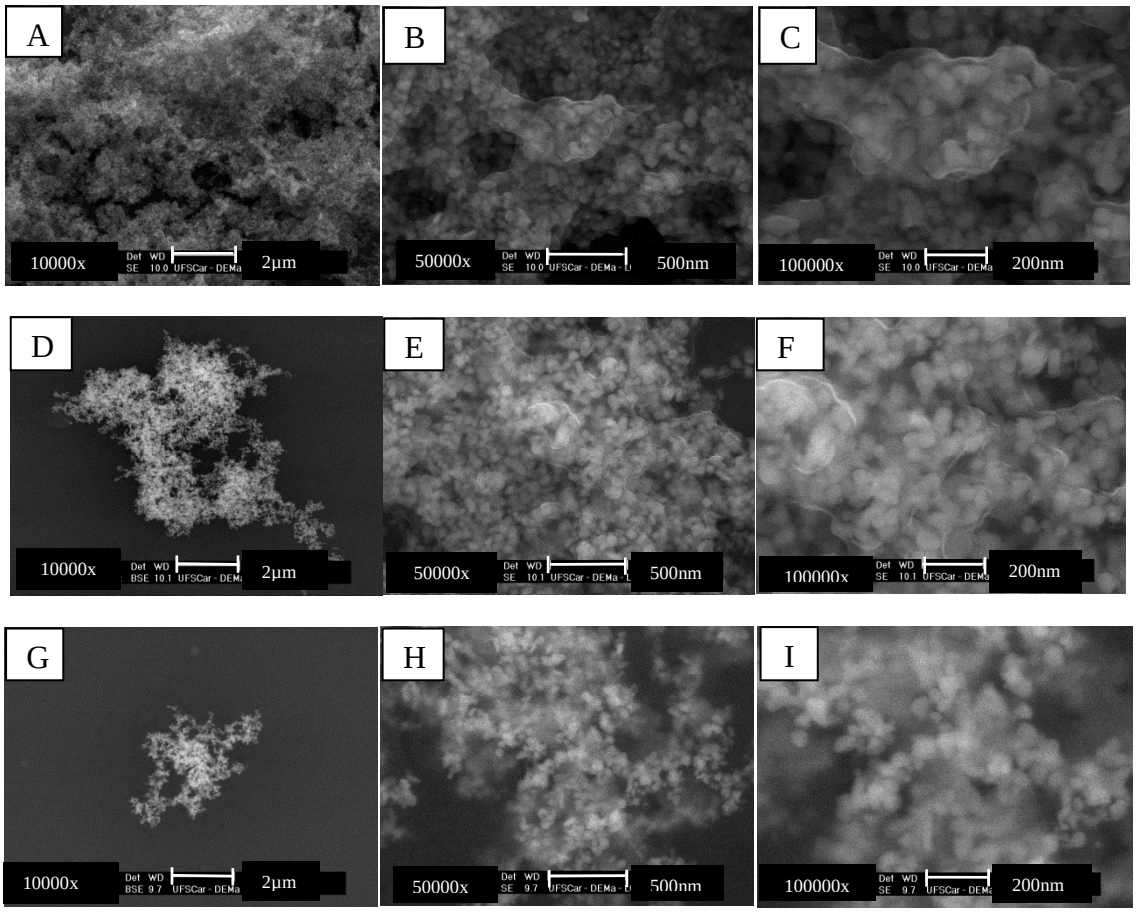
SEM micropraphs of the silver nanoparticle.A) 50°C - 7mg mL^{-1}; B)50°C - 7mg mL^{-1}; C) 50°C - 14mg mL^{-1}; D)50°C - 14mg mL^{-1}; E)50°C - 14mg mL^{-1}; F)50°C - 28mg mL^{-1}; G)50°C - 28mg mL^{-1}; H) 50°C - 28mg mL^{-1}.



SEM micropraphs of the silver nanoparticle. A) 70°C - 7mg mL^{-1}; B)70°C - 7mg mL^{-1}; C) 70°C - 7mg mL^{-1}; D) 70°C - 14mg mL^{-1}; E)70°C - 14mg mL^{-1}; F)70°C - 14mg mL^{-1}; G)70°C - 28mg mL^{-1}; H)70°C - 28mg mL^{-1}; I) 70°C - 28mg mL^{-1}.

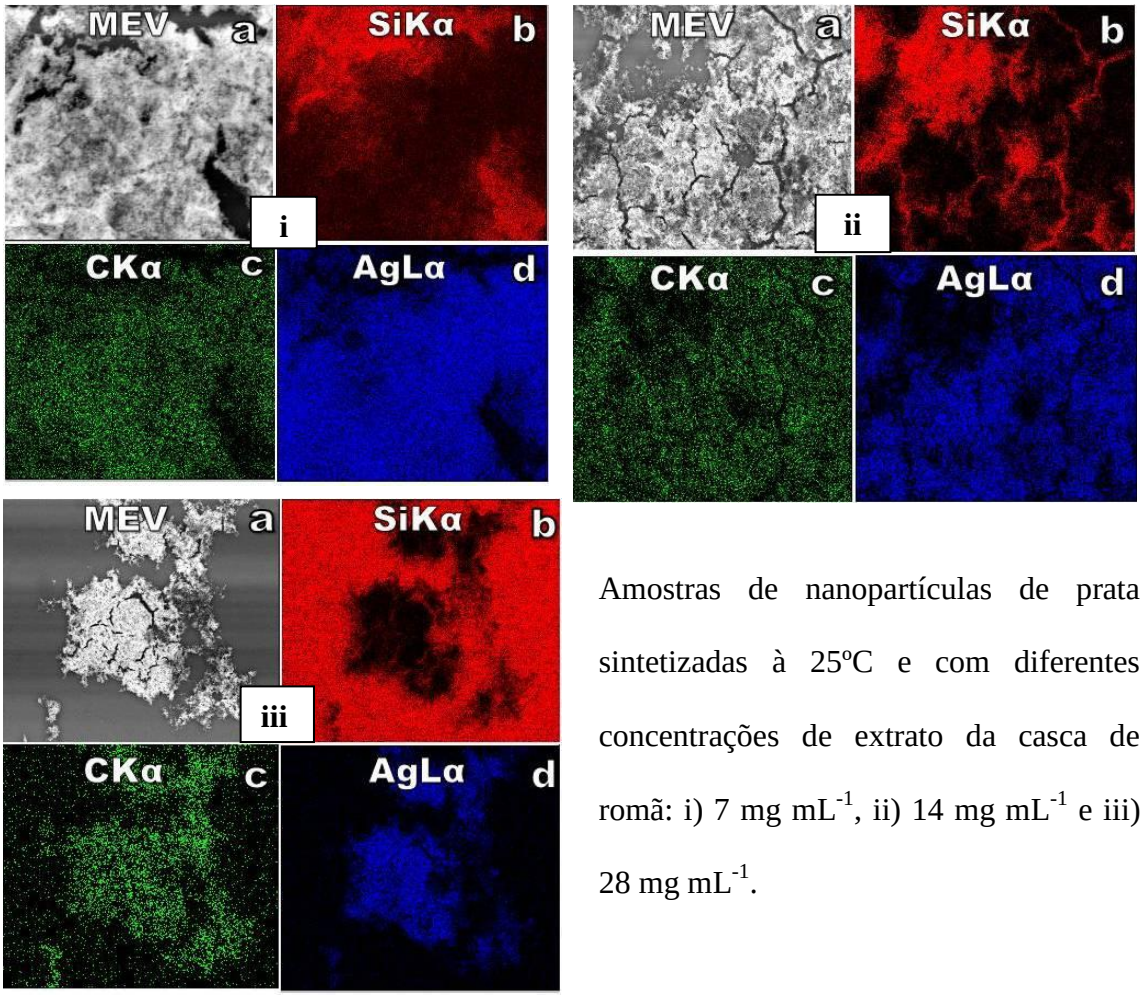


SEM micropraphs of the silver nanoparticle. A)95°C - 7mg mL^{-1}; B) 95°C - 7mg mL^{-1}; C) 95°C - 7mg mL^{-1}; D) 95°C - 14mg mL^{-1}; E) 95°C - 14mg mL^{-1}; F) 95°C - 14mg mL^{-1}.



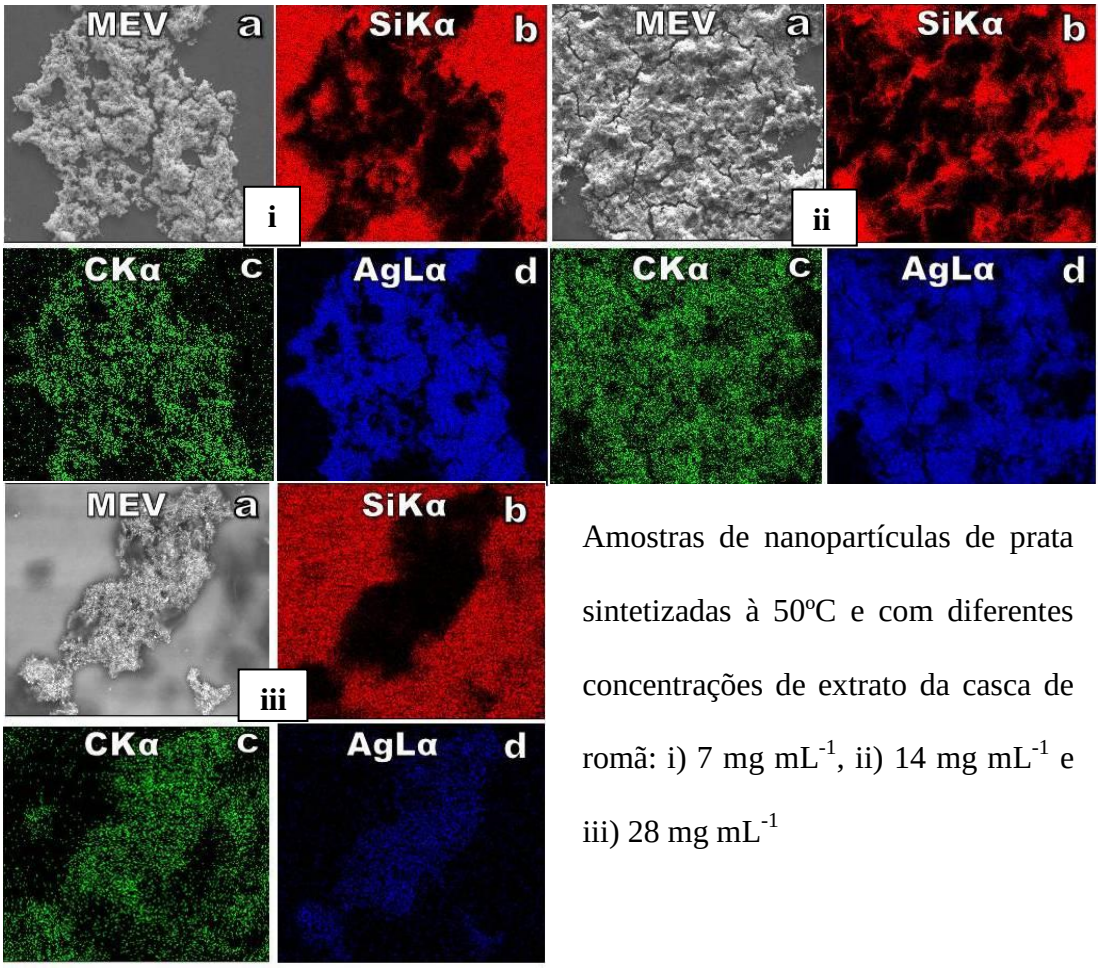
A) 95°C 28mg mL^{-1}- -5 minutos; B) 95°C 28mg mL^{-1} - 5 minutos; C)95°C 28mg mL^{-1} - 5 minutos; D) 95°C 28mg mL^{-1} -10 minutos; E) 95°C 28mg mL^{-1} -10 minutos; F) 95°C 28mg mL^{-1} -10 minutos; G) 95°C 28mg mL^{-1} -15 minutos; H) 95°C 28mg mL^{-1} -15 minutos; I) 95°C 28mg mL^{-1} -15 minutos.

MEV e EDS mapeados na emissão de elementos 2D Si Kα, C Kα e Ag Kα cor falso. Análise da distribuição de nanopartículas de prata na amostra 25 ° C - 7mg mL^{-1}, 14mg mL^{-1} e 28mg mL^{-1}. a) Imagem SEM; b) mapeamento químico do elemento silício presente no extrato alcoólico em cor vermelho;c) mapeamento químico do elemento de carbono presente no substrato, onde o feixe de elétrons foi focado diretamente no substrato e é mostrado em cor verde; d) mapeamento químico do elemento prata, em cor azul.



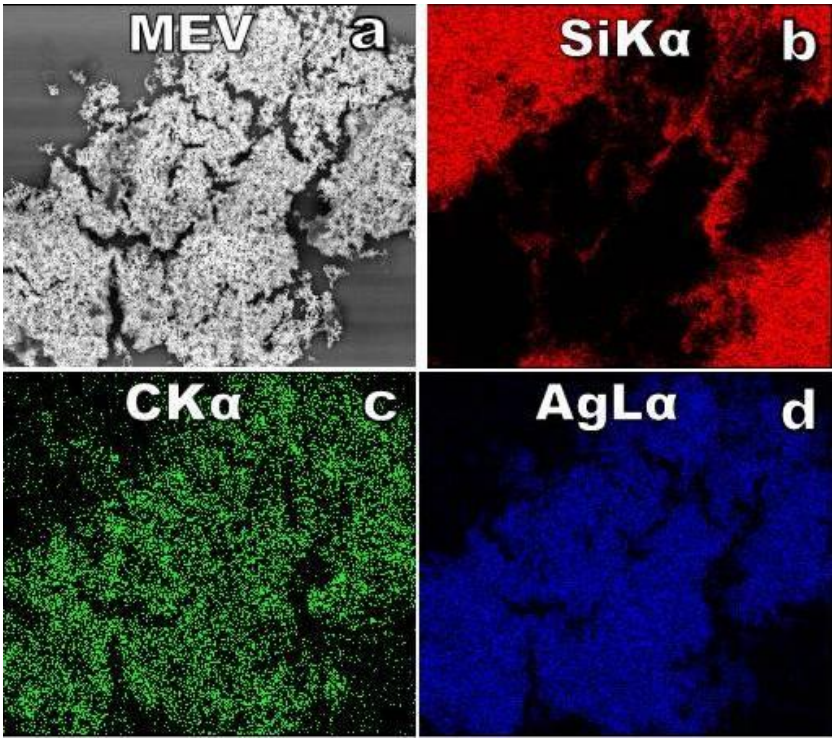
Amostras de nanopartículas de prata sintetizadas à 25ºC e com diferentes concentrações de extrato da casca de romã: i) 7 mg mL^{-1}, ii) 14 mg mL^{-1} e iii) 28 mg mL^{-1}.

MEV e EDS mapeados na emissão de elementos 2D Si Kα, C Kα e Ag Kα cor falso. Análise da distribuição de nanopartículas de prata na amostra 50 ° C - 7mg mL^{-1}, 14mg mL^{-1} e 28mg mL^{-1}. a) Imagem SEM; b) mapeamento químico do elemento silício presente no extrato alcoólico em cor vermelho; c) mapeamento químico do elemento de carbono presente no substrato, onde o feixe de elétrons foi focado diretamente no substrato e é mostrado em cor verde; d) mapeamento químico do elemento prata, em cor azul.

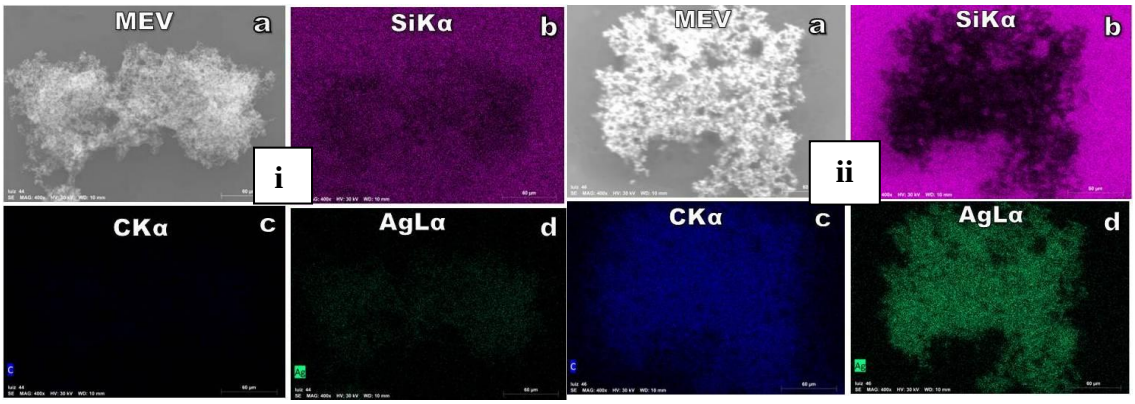


Amostras de nanopartículas de prata sintetizadas à 50ºC e com diferentes concentrações de extrato da casca de romã: i) 7 mg mL^{-1}, ii) 14 mg mL^{-1} e iii) 28 mg mL^{-1}

MEV e EDS mapeados na emissão de elementos 2D Si Kα, C Kα e Ag Kα cor falso. Análise da distribuição de nanopartículas e prata na amostra 70 ° C - 7mg mL^{-1}. a) Imagem SEM; b) mapeamento químico do elemento silício presente no extrato alcoólico em cor vermelho; c) mapeamento químico do elemento de carbono presente no substrato, onde o feixe de elétrons foi focado diretamente no substrato e é mostrado em cor verde; d) mapeamento químico do elemento prata, em cor azul.

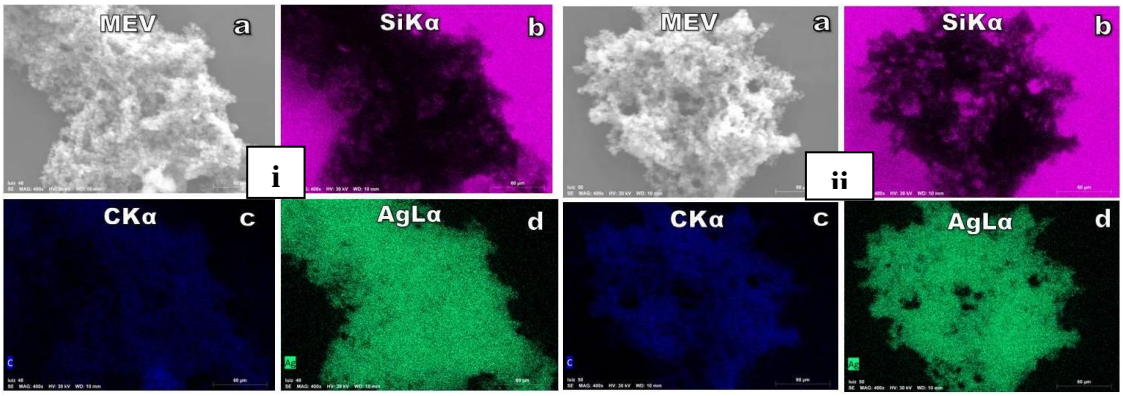


MEV e EDS mapeados na emissão de elementos 2D Si Kα, C Kα e Ag Kα cor falso. Análise da distribuição de nanopartículas e prata na amostra 70 ° C - 14mg mL^{-1}e 28mg mL^{-1}. a) Imagem SEM; b) mapeamento químico do elemento de silício presente no substrato, onde o feixe de elétrons foi focado diretamente no substrato e é mostrado em cor lilás; c) mapeamento químico do elemento carbono presente no extrato alcoólico em cor azul; d) mapeamento químico do elemento prata, em cor verde.



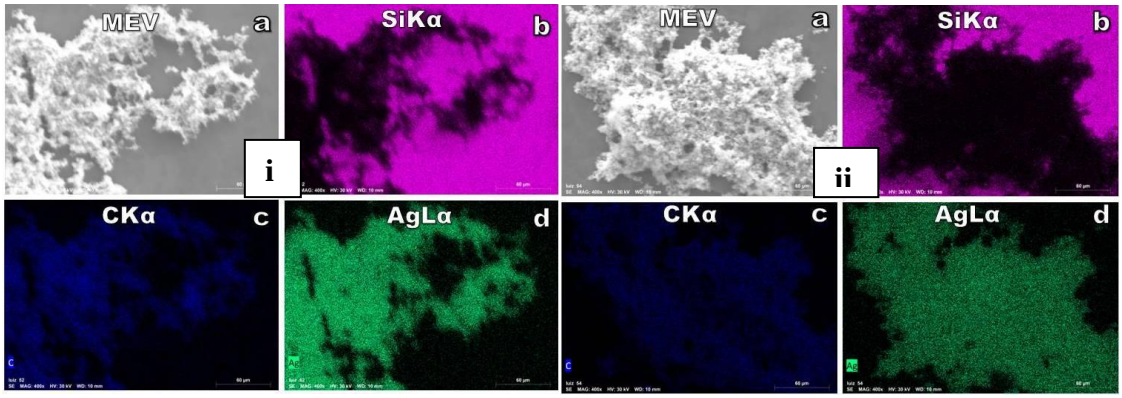
Amostras de nanopartículas de prata sintetizadas à 70ºC e com diferentes concentrações de extrato da casca de romã: i) 14 mg mL^{-1} e ii) 28 mg mL^{-1}.

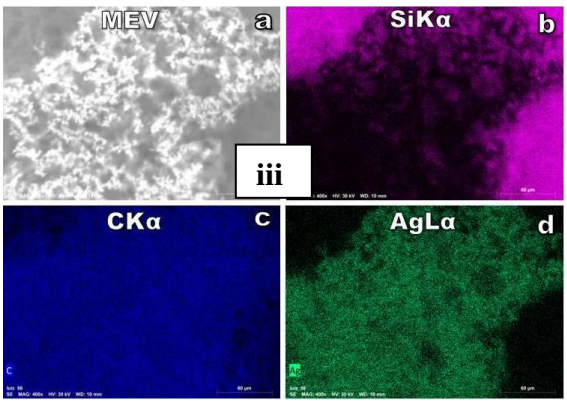
MEV e EDS mapeados na emissão de elementos 2D Si Kα, C Kα e Ag Kα cor falso. Análise da distribuição de nanopartículas e prata na amostra 95 ° C - 7mg mL^{-1}e 14mg mL^{-1}. a) Imagem SEM; b) mapeamento químico do elemento de silício presente no substrato, onde o feixe de elétrons foi focado diretamente no substrato e é mostrado em cor lilás; c) mapeamento químico do elemento carbono presente no extrato alcoólico em cor azul; d) mapeamento químico do elemento prata, em cor verde.



Amostras de nanopartículas de prata sintetizadas à 95ºC por 10 min nas concentrações de extrato da casca de romã de: i) 7 mg mL^{-1} e ii) 14 mg mL^{-1}.

MEV e EDS mapeados na emissão de elementos 2D Si Kα, C Kα e Ag Kα cor falso. Análise da distribuição de nanopartículas e prata na amostra 95 ° C - 28mg mL^{-1}por 5, 10 e 15 minutos. a) Imagem SEM; b) mapeamento químico do elemento de silício presente no substrato, onde o feixe de elétrons foi focado diretamente no substrato e é mostrado em cor lilás; c) mapeamento químico do elemento carbono presente no extrato alcoólico em cor azul; d) mapeamento químico do elemento prata, em cor verde.





Amostras de nanopartículas de prata sintetizadas com 28 mg mL^{-1} de extrato da casca de romã, à 95ºC por: i) 5 min, ii) 10 min e iii) 15 min.

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A) Norma do periódico Environmental Science & Technology - Capítulo I

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ES&T consists of two sections: research and front matter. The research section includes Research Articles, Policy Analyses, Critical Reviews, Perspectives, Correspondence/Rebuttals, and Additions and Corrections. Research section manuscripts are reviewed initially by the assigned editor and then, if appropriate, by other scientists who assess the significance, originality, and validity of the work. The Editor-in-Chief and associate editors, listed in the masthead, make final decisions about all research material published in *ES&T*. The front matter section consists of Features, Viewpoints, and Letters to the Editor, along with Comments. The Managing Editor and Editor-in-Chief handle all front matter.

Research Article (length limit: 7,000 word-equivalents). These papers concern the scientific understanding of natural environments and engineered environmental systems and technologies. *ES&T* has a focus on environmental processes, particularly those affected by human activities. This includes chemical, biological, and physical phenomena as well as mathematical and computational methods that are directly relevant to the understanding and management of the environment and pollution. The journal also publishes articles that describe significant scientific advances or novel technologies for pollution remediation, control, and prevention.

ES&T strives to publish novel research of scientific significance and environmental importance. Novelty is defined as new experimental data, new interpretations of existing data, or new analyses of environmental phenomena. Significance is judged with respect to the breadth of impact of the reported findings. Manuscripts that report data of a routine nature or that address topics that are already well understood will not be considered. Whenever possible, research on new measurement technologies should include results with authentic environmental samples, and evaluations should be performed under environmentally realistic conditions.

Manuscripts that report on initial findings of an urgent nature may be submitted to *Environmental Science & Technology Letters* (*ES&TL*). Manuscripts that are declined by *ES&T* should not be submitted to *ES&TL* without permission of the associate editor who handled the manuscript. Likewise, manuscripts declined by *ES&TL* should not be submitted to *ES&T* without the permission of the responsible *ES&TL* editor.

Policy Analysis (length limit: 7,000 word-equivalents). These manuscripts typically focus on the interface of science and engineering with public policy and provide new insight for understanding and managing human–environmental systems. Topics of particular interest include risk assessment, critical evaluations of environmental regulations, and life-cycle analysis.

Critical Review (length limit: 10,000 word-equivalents). These manuscripts are thoroughly documented assessments of particular areas in the environmental science and technology research domain. Critical Reviews should increase readers' knowledge through discriminating analysis and insightful organization of the material. Critical Reviews are not intended to consist of catalogues of prior research. Factors considered when evaluating Critical Reviews include the current and likely future importance of the field under review, thoroughness of the literature coverage, clarity of the presentation, and identification of future research needs.

Perspective (length limit: 4,000 word-equivalents). These contributions are personal reviews of a field or area, and they are focused, rather than comprehensive. Perspective authors are asked to assess the current status of a chosen field with an emphasis toward identifying key progress being made and research that is needed to advance a subdiscipline, theory, or technology. Although Perspectives are primarily invited, they may be considered without invitation. Prospective authors for unsolicited Perspectives should contact the Managing Editor (a_grostern@acs.org) prior to submission.

Correspondence/Rebuttal (length limit: 1,000 word-equivalents). These manuscripts provide scholarly comment on papers appearing in the research section (Research Articles, Policy Analyses, Critical Reviews, and Perspectives). Correspondence should be submitted within six months of the publication date of the original paper. They must raise substantive scientific or technical questions; Correspondence that consists mainly of opinion will not be considered. The author(s) of the original paper will be given an opportunity to respond. Correspondence on previously published Correspondence will not be considered, and personal invective will not be tolerated. Correspondence may undergo peer review under the direction of the assigned editor.

Additions and Corrections (Errata). These contributions may be used by the authors of a paper to correct errors and omissions of consequence that are identified after publication. Readers who detect errors of consequence in the work of others should contact the corresponding author of the paper in question. All Additions and Corrections are subject to editorial approval, and corrections of minor errors or omissions will not be published. Additions and Corrections must be approved by all coauthors before submission.

Feature (length limit: 5,000 word-equivalents). These manuscripts provide a balanced examination of significant developments and issues affecting the environmental community. The assessment of timely topics from multiple perspectives—scientific, regulatory, technical—provides readers with an authoritative and up-to-date understanding of the subject. Feature articles are written in a magazine or journalistic style rather than as a scientific article. Features undergo peer review, with reviewers providing comments on the factual accuracy, clarity, and significance of the contribution. Prospective authors should contact the Managing Editor (a_grostern@acs.org) prior to submission of a Feature.

Viewpoint (length limit: 1,000 words + author affiliations + 5 references + 1 single-frame figure with 50-word caption OR a 350-word table). These contributions, written in the style of an opinion piece in a newspaper or magazine, provide authors

with a venue to comment on an issue of pressing importance to *ES&T*'s readership. They should be neither wholly political nor summary in nature. Viewpoint articles should express an opinion of a clear scientific nature, based on rigorous scientific research in an environmental discipline. Viewpoints generally are not peer reviewed but are subject to editorial approval.

Letter to the Editor (length limit: 500 words + author affiliations + 250 words of references). These contributions provide comments on articles published in the front matter (Features, Viewpoints, and Comments). Letters to the Editor should be submitted within two months of the publication date of the original material. The author(s) of the original material will be given an opportunity to reply. If you wish to comment on research content, as opposed to front matter, please submit a Correspondence (see above).

Manuscript eligibility

ACS Ethical Guidelines

Scientific ethics are taken very seriously by *ES&T* and its publisher, the American Chemical Society (ACS). Authors must adhere to the ACS Ethical Guidelines to Publication of Chemical Research (ACS Ethical Guidelines).

ES&T and ACS are committed to deterring plagiarism (including self-plagiarism). Submissions that include text, figures, tables, or other elements that have already been published will be rejected unless permission to reuse the material has been granted by the copyright holder(s). Multiple violations of the journal's ethical policies may result in authors becoming ineligible for future submission. *ES&T* uses CrossCheck's iThenticate software to screen submitted manuscripts for similarity to published material.

Prior publication policy

ES&T considers for publication original work that has not been previously published and is not under consideration for publication elsewhere. Related work under consideration for publication in any medium must be cited in the manuscript and the Editor-in-Chief informed at the time of submission. In addition, an author must inform the Editor-in-Chief of prior dissemination of the content in print or electronic formats in the cover letter. Posting of pre-prints to a pre-print server is considered acceptable but requires citing of the pre-print. Please note the use of a pre-print server in the cover letter, and as appropriate, state how the manuscript has been adjusted/updated between the pre-print version and the version submitted to *ES&T*. Failure to alert *ES&T* in your cover letter to any prior publication of your submission may be viewed as an ethical violation. Upon publication in *ES&T*, authors are advised to add a link from the pre-print to the published paper via the Digital Object Identifier (DOI).

For further information on application of the prior publication policy to theses/dissertations, conference/symposia proceedings, websites, and publicly available reports, see FAQ #6, below.

Resubmission of previously rejected manuscripts

If your manuscript is declined (i.e., rejected), read the decision letter carefully. Manuscripts are often declined because the editor determines that the subject matter is not appropriate for *ES&T* or that the novelty or significance of the manuscript is insufficient. Of course, editors sometimes make mistakes. For this reason, *ES&T* has a process for appealing decisions on manuscripts, which is described in the Appeal Process section of this Guide, below. **If you wish to submit a revised version of a declined manuscript to *ES&T*, you must first contact the associate editor who handled your original submission to request permission to resubmit.**

If you receive permission to resubmit, indicate in your cover letter that it is an authorized revision of a previously submitted manuscript, provide the original manuscript number, and state how the manuscript has changed. If the manuscript was reviewed, submit a detailed, point-by-point list of your responses to each of the comments of the reviewers or provide convincing reasons for declining to do so. The manuscript should be submitted online (see the Manuscript Submission section of this Guide, below), where it will receive a new manuscript number. During the submission process, mark "Yes" when asked if the manuscript has been previously submitted "in whole or in part."

Manuscripts that editors judge to be resubmissions, in whole or in part, of previously submitted manuscripts that do not comply with these rules will not be considered for publication. Moreover, failure to alert *ES&T* to a resubmission, even in part, is an ethical violation.

Manuscript preparation

Formatting requirements

Length limits. The length limits are listed in the Article Types section, above. For all submissions except Viewpoints and Letters to the Editor, to determine the length, count all text, excluding title page, references, and figure/table captions. Next, add 300 words for each small figure, scheme, or table. Large multipart figures (more than three panels) or extensive tables (taking up a page or more) should be counted as 600 words. At the discretion of the assigned editor, some figures or tables may be counted as more than 600 words.

Manuscripts that exceed the length limit will be unsubmitted (returned to the Draft section in Paragon Plus) with a request to shorten, or they may be immediately rejected. One possible way to reduce the length is to make the Introduction and Discussion sections more concise. A second way to shorten manuscripts is to make appropriate use of Supporting Information (SI; see below), which is available to readers on the *ES&T* website.

Authors who believe that exceeding the length limit is essential must include a compelling argument in their cover letters. Ultimately, however, the decision about whether a manuscript that exceeds the recommended length is appropriate for review is made by the assigned editor.

Line numbers. The text of all manuscripts should be double spaced in a single column with the lines numbered consecutively in a separate column at the margin.

General style

Consult a recent issue of *ES&T* for general style. Assume your readers to be professionals who are not necessarily experts in your particular field; spell out all acronyms on first use in the abstract and in the body of the article. *ES&T* does not allow footnotes, with the exception of an author information footnote on the title page and table detail/definition footnotes.

After the Abstract, divide the article into sections; three are often sufficient, especially for Research Articles:

Introduction. Clearly state the purpose and significance of the research and put it into the context of earlier work in the area. Do not attempt a complete survey of the literature. Introduction sections are typically around 500 words in length.

Materials and Methods. Describe pertinent and critical factors involved in the experimental work but avoid excessive description. Details not essential for understanding the paper can be placed in SI. If you have already published the procedures used, properly cite to previous publications and expand only on differences in the current work.

Results and Discussion. Discuss your findings, postulate explanations for data, elucidate models, and compare your results with those of others. Be complete but concise. Avoid irrelevant comparisons or contrasts, speculations unsupported by the new information presented in the paper, and verbose discussion.

Do not include a conclusion or summary section or paragraph in Research Articles. Incorporate all major findings and implications in the Results and Discussion section. There is no need to repeat or summarize information presented earlier in the paper.

Elements of a manuscript

Title. Use brief, specific, and informative titles. If trade names are used, give generic names in parentheses. Key words in titles assist in effective literature retrieval.

Authorship. List the first name, middle initial(s), and last name of each author. Omit professional and official titles. An author's affiliation should be based on where they were when the work was performed. When the present address of an author is different, include the new information in a footnote. In a paper with more than one author, the name of the corresponding author, to whom post-publication inquiries should be addressed, carries an asterisk (*). Provide an email address for the corresponding author.

Abstract. A 150–200-word abstract must accompany Research Articles, Policy Analyses, Critical Reviews, and Perspectives. Describe the purpose, methods or procedures, significant new results, and implications. Do not break the abstract into sections or separate paragraphs. Define any abbreviations used in the abstract. Include major quantitative data if they can be stated briefly, but do not include background material. Do not include reference numbers in the abstract. For Features, include a 3–5 sentence synopsis titled "Abstract," written at a level comprehensible to the scientifically literate general public.

Table of Contents (TOC)/Abstract Art. This graphic, required for Research Articles, Policy Analyses, Critical Reviews, Perspectives, and Features, appears next to the abstract online and in all versions of the article; it is also used in other situations in which a representative graphic is needed. The selected image should give readers a quick visual representation of the essence of the paper. It should be simple and relatively free of text and technical characters, and make use of color for visual impact. Abstract art may include a photograph of a field site or a schematic portraying the central findings of the paper. Please consult a recent issue of the journal for examples. **All portions of the TOC graphic must have been created by the authors of the paper. Material not actually created by the authors cannot appear in TOC graphics even if the copyright owner of the material does not want credit.**

Size requirement = 240-point width by 135-point height (3.33" x 1.875"; 4.76 cm x 8.47 cm)

Additional specifications:

- Images must be original (not previously published) and created by one of the authors of the paper.
- No copyright, credit, permission, or attribution statements are allowed.
- No captions or legends are permitted.
- Photographs may not show any identifiable individuals unless a model release is provided for ALL identifiable individuals. Any photographs must have been taken by an author of the paper.
- No copyrighted, public domain, Creative Commons license, ClipArt, or stock photo material may be used.

- No postage stamps, currency, or trademarked items (company or institutional logos, images, and products) may appear in the graphic.
- No maps may be used.
- TOC art is subject to final approval by the assigned editor.
- **Authors must certify in their cover letters that they have complied with this TOC art policy and confirm that the submitted image was created by an author and has never been published.**

Tables. Tables should be furnished with appropriate titles of one phrase or sentence; details or definitions should be placed at the bottom as footnotes. Tables should be numbered consecutively with Arabic numbers (i.e., 1, 2, …). Double-space them with wide margins, ensure that each data entry is placed in its own cell, and prepare tables in a consistent format, preferably using a word processor's table format feature.

Graphics. Graphics must meet the journal's minimum quality standards and will be returned to authors for improvement if necessary. Graphics should be numbered consecutively with Arabic numbers (i.e., 1, 2, …) and accompanied by a caption. They should have good resolution; be clear, concise, and complete; and use legible font. Colors may be used to enhance graphics.

Formulas and equations. Chemical formulas should correspond to the ACS Style Guide. Chemical equations should be balanced and numbered consecutively along with mathematical equations. Mathematical arguments should be as brief as possible.

Safety. Authors must emphasize any unexpected, new, and/or significant hazards or risks associated with the reported work. This information should be in the Methods and Materials section of the manuscript.

Acknowledgments. Include essential credits in an Acknowledgment section at the end of the text. As noted below, sources of financial support must be acknowledged.
References. Literature references in *ES&T* must be numbered in order of appearance. The accuracy of the References is the responsibility of the authors, who are encouraged to avoid references to works that have not been peer reviewed. Any references in publications that would be difficult for most reviewers to obtain or are unpublished should be uploaded as Information for Review Only. DOI numbers are helpful but not mandatory unless they are the only identifying information available (e.g., for recently published articles).

For additional information on References, including formatting, please consult the ACS Style Guide. Some examples of reference formats are shown here.

Disclosures

The corresponding author must reveal any potential and/or relevant competing financial or other interest (of all authors) that might be affected by publication of the results contained in the manuscript. Potential conflicts of interest and sources of funding of the research reported must be clearly stated at the time of manuscript submission and included in the Acknowledgements. If no potential for a conflict of interest is declared, the following statement will be published in the article: "The authors declare no competing financial interest." See the ACS Ethical Guidelines for additional details.

Supporting Information

Authors are encouraged to shorten the text of research manuscripts by using the SI to present ancillary data and material of interest mainly to specialists. In particular, the Materials and Methods section should provide the detail needed for the reader to determine whether the interpretations are supported by the data, but experimental

details, including schematics of apparatus and maps of study sites, should be reported in SI, as should detailed calculations, derivations, databases, and data that are not essential to understanding the Results and Discussion (e.g., reference spectra).

SI should be formatted with a cover sheet listing authors; manuscript title; and the number of pages, figures, and tables. SI pages must be numbered consecutively, starting with page S1. Tables and figures should be numbered Table S1 and Figure S1.
If the manuscript is accompanied by any SI files for publication, a brief description of each file is required. This paragraph of descriptions should be placed at the end of the manuscript before the References. The appropriate format is:

Supporting Information. Brief descriptions in nonsentence format listing the contents of the files supplied as Supporting Information.
SI is not permitted for Correspondence/Rebuttal, Corrections and Additions, Features, Viewpoints, or Letters to the Editor.
English language assistance

The English in all submissions must meet the journal's minimum standards for publication. Manuscripts containing numerous errors in grammar and word choice can frustrate reviewers and make the review process challenging. A number of providers, including ACS ChemWorx, offer professional editing by native English speakers. Although using such services does not guarantee acceptance of a manuscript, they may help in clarifying the significance of your research.

Manuscript submission

All submissions, including new manuscripts, revisions, corrections, and comments, must be made electronically via the ACS Paragon Plus Environment. Complete instructions and an overview of the online submission process are available on the Paragon Plus website.
Submission requirements
1. Manuscript file

Details regarding layout of your manuscript can be found in the Manuscript Preparation section of this Guide, above. The preferred format for manuscript files is an MS Word document with the text and all graphics, including TOC art, embedded within a single file. The SI should be included as a separate file.
Manuscript templates, which are helpful but not required, are available here. Complete instructions for submission of manuscripts and SI, including the platforms and word processing packages supported, are available here.
2. Graphics file(s)

As noted above, graphics should be embedded within the manuscript when possible. It is also acceptable to submit separate TIFF, PDF, EPS (vector artwork), or CDX (ChemDraw file) files. If separate graphic files are submitted, they should be named in a manner clearly identifying their function (e.g., Scheme 1, Figure 1, Table 1). Each separate graphic file must also include the caption for the respective graphic in the manuscript itself.

Special requirements for EPS and TIFF files graphics (both when embedded in a Word file and when submitted separately):
- EPS files: All fonts must be converted to outlines or embedded in the graphic file. The document settings should be in RGB mode. Although EPS files are accepted, the vector-based graphics will be rasterized for production.
- TIFF files: Black & white line art must have a resolution of 1200 dpi; grayscale art (a monochromatic image containing shades of gray) must have a resolution of 600 dpi; and color art (RGB color mode) must have a resolution of 300 dpi.

3. Supporting information (optional)

Details regarding SI can be found in the Manuscript Preparation section of this Guide, above.

A list of acceptable electronic file types for SI is available here, with a list of viewers available here. All SI files of the same type should be submitted as a single file (rather than as a series of files containing individual images or structures). For example, all SI available as PDF files should be contained in one PDF file, if possible, and all CIFs should be submitted as a single file. Whenever possible, SI should be consolidated into a single word-processing file with graphics embedded.

4. Cover letter

A cover letter must accompany every submission. The cover letter should list the authors and their affiliations, give the manuscript title, and provide complete contact information for all authors. It should also include a rationale for consideration by *ES&T*. If you have a non-preferred editor, you may explain your reason for making the request in your cover letter.

Rationale: Please read this Guide carefully and explain why your manuscript is appropriate for publication in *ES&T*. A substantial fraction of submissions to *ES&T* are not sent out for review because an editor concludes that the manuscript does not meet the journal's standards for novelty, scientific merit, or environmental importance. The cover letter is your opportunity to convince the editor that this is not the case.

Attestations:

- Authorship: Affirm that all authors are aware of and accept responsibility for the manuscript.
- Prior publication: Please note any prior dissemination of the content in print or electronic formats in the cover letter. As appropriate, state how the manuscript has been adjusted/updated between the prior disseminated version and the version submitted to ES&T.
- TOC art: Confirm that all photos and images were author generated and comply with the requirements listed in the TOC Art section of this Guide, above.
- Other graphics: For any figures that appeared in a previous publication, confirm that you have obtained the appropriate permission from the publisher and included any required attribution.

5. Funding sources

Authors are required to report ALL funding sources and grant/award numbers relevant to their manuscript. To meet this requirement, the submitting author must enter all sources of funding for ALL authors relevant to the submission in BOTH the Open Funder Registry tool in ACS Paragon Plus and in the manuscript. See here for complete instructions.

6. Unpublished research cited in your manuscript

If the References section of your manuscript includes any unpublished research, you must upload a copy as Supporting Information for Review Only.

7. Author list

During manuscript submission, the person who submits the manuscript must provide contact information—full name, email address (institutional email address preferred), institutional affiliation, and mailing address—for all coauthors. The submitting author will also need to provide his or her personal, validated ORCID iD at original submission or revision. Because all of the author names are automatically

imported into the electronic Journal Publishing Agreement (eJPA; see the Post-Submission Processes section of this Guide, below), the names must be entered into Paragon Plus in the same sequence as they appear on the first page of the manuscript.

The submitting author accepts the responsibility for notifying coauthors that the manuscript is being submitted. Deletion of an author after the manuscript has been submitted requires a confirming email to the handling editor from the author whose name is being deleted. For more information on ethical responsibilities of authors, see the ACS Ethical Guidelines. For more information on ORCID iDs and institution identification, see FAQs #10 and #11 below.

8. Contact and post-publication corresponding authors

At submission, one author must be designated as the contact author in the Paragon Plus system. During the review process and prior to publication, all email notifications will be sent to the contact author. Only one author may be designated as the contact author; this author receives and may sign the eJPA. After publication, the author marked on the manuscript with an asterisk (*) is designated as the corresponding author—that is, the person whom readers contact after publication of the paper. The contact and corresponding author may be, but are not required to be, the same person. Complete, current contact information for the corresponding author on the title page of the manuscript is required. Please note that you may designate more than one corresponding author on the manuscript.

9. Reviewer list

Authors are required to provide the names and contact information (affiliation and email address) of at least four experts in the field as possible reviewers for the manuscript. You may also include the names of people you do not want to review the paper due to potential bias. Do not suggest as a potential reviewer anyone affiliated with your institution or with whom you (or your coauthors) served as coauthor or have a close personal or professional relationship (including former research supervisors, advisers, and colleagues with whom one of the authors has had, within the past five years, a professional relationship (e.g., collaborator, coauthor)). Providing more than four qualified reviewers may expedite and improve the review process.

10. Editor preference

Although it is not required, you may specify one or more preferred editors during the Paragon Plus submission process. Do not suggest as potential editors anyone who would not qualify as a reviewer for the reasons listed above. Please note that assignment of your manuscript to your preferred editor is not guaranteed.

If there is an *ES&T* editor whom you feel should not handle your manuscript, please explain your reasons in the cover letter. Generally, you should specify a non-preferred editor when he or she would be disqualified as a reviewer for any of the reasons listed above.

After a manuscript has been assigned to an editor, all correspondence should be directed to that editor. The editor’s contact information will be provided in the assignment email.

Response to reviewers

If you are submitting a revised manuscript or an authorized resubmission of a manuscript that was already peer reviewed, you must submit point-by-point responses to each of the comments of the reviewers. We recommend that you copy the reviewer’s

comment into the text immediately prior to your response. You should also upload, as Information for Review Only, a version of the manuscript with changes highlighted to allow the editor to easily discern the revisions that have been made.

Post-submission processes

Journal publishing agreement (JPA)

Copyright of articles published in *ES&T* is transferred to ACS by the author(s). A signed JPA must be submitted prior to acceptance of a manuscript. The electronic JPA (eJPA) form becomes available on the "My Authoring Activity" tab of the contact author's home page in Paragon Plus after the manuscript has been assigned to an editor for handling. A PDF version of the JPA is also available, but **authors are strongly encouraged to use the eJPA**. If the PDF version is used, all pages of the signed document must be submitted. If the contact author cannot or should not complete either the electronic or PDF version for any reason (e.g., he or she is prohibited from doing so by an employer), another author should complete and sign the form. Forms and complete instructions are available here. Should you need further guidance on completing the JPA, please contact the assigned editor's office (his or her email address is provided in the manuscript assignment notification email).

Review process

Due to the large volume of submissions, a significant fraction of manuscripts submitted to *ES&T* are declined after review by an editor and are not sent for external review. Common reasons for declining manuscripts without external review include insufficient novelty, lack of sufficient environmental relevance, failure to place the rationale for the study or the results in the context of existing literature, insufficient quality of the data, and problems with manuscript presentation (e.g., adherence to length limits, inadequate English, or figure quality). For more information, see *ES&T*'s 2015 editorial on the topic.

Manuscripts that meet editorial expectations are sent for external review to experts in the field. Manuscripts are typically evaluated by three or more experts, but in exceptional cases, editors may rely upon fewer reviews. Editors make decisions about whether or not to request revisions on the basis of the reviews and their own insights into the research.

Manuscript transfer process

If your submission is declined for publication by *ES&T*, the assigned editor might deem your work to be better suited for another ACS Publications journal and suggest that you consider transferring the submission. For more information on the manuscript transfer service, see FAQ #12, below.

Appeal process

If your manuscript is declined (rejected) and you feel the decision is not justified, you may appeal the decision directly to the editor who made it, providing a clear explanation for why you believe he or she should reconsider the decision. If the editor upholds the rejection, you may appeal the decision to the Editor-in-Chief (eic@est.acs.org). When outlining your appeal to the Editor-in-Chief, please include confirmation that you first asked the handling editor to reconsider the decision and then provide a clear explanation as to why you believe that the associate editor's decision is unreasonable. The Editor-in-Chief's decisions on appeals are final.

This same process should be used for appealing an editor's denial of your request to resubmit a previously submitted manuscript.

Publication process

Patent activities and intellectual property issues. Authors are responsible for ensuring that all patent activities and intellectual property issues, **including permissions to reprint figures**, are satisfactorily resolved prior to first publication (*Just Accepted*, ASAP, or in issue). Acceptance and publication will not be delayed for pending or unresolved issues of this nature.

Just Accepted manuscripts. *Just Accepted* manuscripts are peer-reviewed, accepted manuscripts that are posted on the *ES&T* website prior to technical editing, formatting for publication, and author proofing—usually within 24 hours of acceptance by the editorial office. During the manuscript submission process, authors can choose to have their manuscript posted online as a *Just Accepted* manuscript. To ensure rapid delivery of the accepted manuscript to the Internet, authors must adhere carefully to all requirements in this Guide. For further information, please refer to the *Just Accepted* FAQ, accessible here. Note that publishing a manuscript as *Just Accepted* is not a means of complying with public access mandates from organizations such as the US National Institutes of Health. Also, if the authors are planning to prepare a press release to coincide with publication of their manuscript, they should see FAQ #16.

Contact post-acceptance. After a manuscript has been accepted for publication, all correspondence should be directed to proofs@acs.org or American Chemical Society, Journal Publications, 2540 Olentangy River Rd., Box 3330, Columbus, OH 43210; phone 1-614-447-3665; fax 1-614-447-3745.

Proofs. The contact author of an accepted manuscript will receive e-mail notification and complete instructions when page proofs are available for review via a secure website. Authors will access the secure site through ACS ChemWorx and will need an ACS ID. To obtain an ACS ID or to reset your password, go to www.acschemworx.org.

Page proofs should be checked carefully against the manuscript, paying particular attention to tables, graphics, captions, equations, and formulas. Corrections should be returned as soon as possible. No paper is released for ASAP publication until author approval has been received. All corrections, revisions, and additions must be listed with their location in the proof clearly identified.

Authors are expected to finalize the manuscript during the review process. At the page proof stage, alterations are restricted to minor typographical or grammatical corrections, such as fixing editorial and spelling mistakes. Substantive changes in interpretation or corrections of data, or other important changes, including changes to the title or the list of authors, are subject to editorial review and may delay or cancel publication.

ASAP publication. Accepted manuscripts will be published on the "Articles ASAP" page on the *ES&T* website as soon as page proofs are corrected and all author concerns are resolved. Publication on the Internet usually occurs within 1–4 working days of receipt of page proof corrections, and this can be well in advance of the cover date of the printed issue. The date on which the document is posted on the Internet is the official date of publication, as the article becomes completely citable using the DOI (10.1021/acs.est.XXXXXXX).

E-prints. Under the ACS Articles on Request policy, ACS will provide (free of charge) to all contributing authors a unique URL that they may email to colleagues or post on external websites. These author-directed links are designed to facilitate distribution of an author's published work to interested colleagues in lieu of direct distribution of the PDF file by the author. The ACS Articles on Request policy allows

50 downloads within the first year after Internet publication and unlimited access via the same author-directed links 12 months after Internet publication.

ACS AuthorChoice Open Access. ACS AuthorChoice options establish fee-based mechanisms for authors or their research funding agencies to sponsor the open availability of their articles on the Internet. ACS AuthorChoice offers authors a wide range of open access license options, Creative Commons licenses, and provisions for immediate or 12-month embargoed open access, and includes ACS Certified Deposit. Authors will find useful information about compliance with open access policies here and FAQs here.

After acceptance, authors will be presented with the opportunity to purchase an ACS AuthorChoice option, and authors who do so will be presented with the appropriate license at that time. For a review of all license options available, see here. For questions or further assistance with ACS AuthorChoice, please contact support@services.acs.org.

Additions and Corrections

Once an article has been published online as an ASAP article or formally assigned to an issue, whichever occurs first, changes may be made only through a new Additions and Corrections submission (see Article Types section of this Guide, above) in Paragon Plus. Please do not email requested edits to the office of the handling editor or Editor-in-Chief. As noted above, only omissions or errors of consequence can be addressed after the proof has been approved.

Retraction

Articles may be retracted for scientific or ethical reasons. Articles that contain seriously flawed or erroneous data such that their findings and conclusions cannot be relied upon may be retracted in order to correct the scientific record. Retractions may be requested by the article author(s) or by the editor(s) but are ultimately published at the discretion of the Editor-in-Chief. When an article is retracted, a Notice of Retraction will be published with information about the original article title, author list, and the reason for retraction. Retracted articles will be accompanied by the related Notice of Retraction and will be marked as "Retracted." The originally published article will remain on the Internet except in extraordinary circumstances (e.g., when deemed legally necessary or if the availability of the published content poses public health risks). ACS follows guidance from the Committee on Publication Ethics (COPE) when considering Retractions.

If you believe you may need to retract your published *ES&T* article, please email the Editor-in-Chief (eic@est.acs.org), copying the Managing Editor (a_grostern@acs.org), with an explanation of the reason(s) for retraction.

B) Norma do Periódico Biofouling:

Biofouling is an international, peer-reviewed journal publishing high-quality, original research. All submitted manuscripts are subject to initial appraisal by the editor, and, if found suitable for further consideration, to peer review by independent, anonymous, expert referees. Authors who would like their papers double-blind peer reviewed should submit the title page separately. Submission is online via ScholarOne Manuscripts.

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