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Original Article

Antimicrobial evaluation of silver nanoparticles using extracts of *Crescentia cujete* L.

Avaliação antimicrobiana de nanopartículas de prata usando extratos de *Crescentia cujete* L.

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Abstract

New natural reducing agents with a lower negative impact on the environment and with a high antimicrobial potential are required for the process of obtaining silver nanoparticles through the chemical reduction method. The use of plant extracts can be a fast track in the formation of nanoparticles. In this case, organic compounds such as terpenes, flavonoids, enzymes, proteins, and cofactors present in plants act as reducing agents for nanomaterials. This research evaluated the antimicrobial property of silver nanoparticles from extracts of *Crescentia cujete* L. The presence of quercetin (flavonoid) was determined by high-performance liquid chromatography (HPLC); the production of silver nanoparticles (AgNPs) was established by green synthesis; the size and morphology of the nanomaterials were evaluated by scanning electron microscope (SEM). The antimicrobial capacity was studied by two analysis methods: modified culture medium and surface seeding. The presence of quercetin (26.55 mg L^{-1}) in the crude extract of *Crescentia cujete* L., identified by HPLC, was evidenced. Nanoparticle formation was spherical, with an average size of 250 ± 3 and 460 ± 6 nm. Microbiological cultures with treatment showed 94% microbial inhibition. It was concluded that the *Crescentia cujete* L., leaves shoed an acceptable concentration of quercetin to be used as a useful adjuvant to enhance the reduction of NPs synthesis. The nanoparticles produced by green synthesis proved to have a positive effect to combat pathogenic microorganisms.

Keywords: HPLC, quercetin, nanomaterial, green synthesis, antimicrobial agent.

Resumo

Novos agentes redutores naturais com menor impacto negativo ao meio ambiente e com alto potencial antimicrobiano são necessários para o processo de obtenção de nanopartículas de prata, pelo método de redução química. O uso de extratos vegetais pode ser um caminho rápido na formação de nanopartículas. Nesse caso, compostos orgânicos como terpenos, flavonoides, enzimas, proteínas e cofatores presentes nas plantas atuam como agentes redutores dos nanomateriais. Esta pesquisa avaliou a propriedade antimicrobiana de nanopartículas de prata de extratos de *Crescentia cujete* L. A presença de quercetina (flavonoide) foi determinada por cromatografia líquida de alta eficiência (HPLC); a produção de nanopartículas de prata (AgNPs) foi estabelecida por síntese verde; o tamanho e a morfologia dos nanomateriais foram avaliadas dos por microscópio eletrônico de varredura (SEM). A capacidade antimicrobiana foi estudada por dois métodos de análise: meio de cultura modificado e semeadura em superfície. Foi evidenciada a presença de quercetina (26,55 mg L^{-1}) no extrato bruto de *Crescentia cujete* L., identificada por HPLC. A formação das nanopartículas foi esférica, com tamanho médio de 250 ± 3 e 460 ± 6 nm. Culturas microbiológicas com tratamento mostraram 94% de inibição microbiana. Assim, concluiu-se que as folhas de *Crescentia cujete* L. apresentam uma concentração aceitável de quercetina para ser utilizada como um adjuvante útil para potencializar a redução da síntese de NPs. As nanopartículas produzidas pela síntese verde provaram ter um efeito positivo no combate a microrganismos patogênicos.

Palavras-chave: HPLC, quercetina, nanomaterial, síntese verde, agente antimicrobiano.

1. Introduction

The presence of biological microorganisms in the environment can be influenced by various factors such as anthropogenic activities and different environmental conditions (temperature, relative humidity, among others) (Rai et al., 2021), which allow these microorganisms to adapt to the surrounding environment, remaining in the

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air for long periods of time. In the usual microbiota of the air, it is possible to find algae, viruses, protozoa, fungi, and bacteria which fulfill different biological roles (Mandrioli, 2002). For example, fungi cause alterations in organic and inorganic compounds as well as diseases in animals and humans (Borrego et al., 2008). As for bacterial growth in closed environments, it can cause symptoms and diseases related to allergies, poisoning, and pathogenic infections (An et al., 2006).

In this context, different organic compounds have been used to combat them; however, microbial resistance has been reaching a critical level. For this reason, attention has turned to the use of metal nanoparticles (NPS) because they have greater durability, greater stability and greater antimicrobial efficiency (Stankic et al., 2016). However, current methodologies in the elaboration of these nanomaterials use various reducing agents such as sodium borohydride, hydroxylamine, tetrakis (hydroxymethyl) phosphonium chloride (THPC), and N, N-dimethylformaldehyde (Kumar et al., 2013). These chemicals are usually expensive and/or harmful to the environment. As an alternative to the above, "green synthesis" has been used and has shown to be environment friendly, since it involves the use of yeasts, plant extracts, or microorganisms (bacteria and fungi) in the production of nanoparticles that have antibacterial/antifungal properties (Sastry et al., 2003). These nanoparticles can be used to mitigate the pathogenic load of numerous compounds and surfaces (Graily-Moradi et al., 2020).

The use of plant extracts can be a fast track for the produce of NPs. In this case, organic compounds such as terpenes, flavonoids, enzymes, proteins, and cofactors which are present in plants act as reducing agents to promote the formation of metallic nanoparticles (Naranjo-Herrera et al., 2017). Several studies affirm that plants with high flavonoid contents are responsible for the stabilization of nanoparticles (Correa et al., 2016; Raghunandan et al., 2010; Zheng et al., 2013). A study carried out by Levchenko et al. (2011) reported that the concentration of bioflavonoids, used as reducing agents, especially quercetin, assists in the production of metallic NPs.

Considering the above, AgNPs have gained importance as inhibitor of microbial growth. For example, Ramamurthy et al. (2013) reported the synthesis of AuNPs using the aqueous extract of *Solanum torvum* and obtaining effective antimicrobial activity. Likewise, Aritonang et al. (2019) evaluated the inhibition of bacterial growth using aqueous extracts of fresh leaves of I. *balsamina* and L. *camara*, particularly against the growth of *S. aureus* and *E. coli*, which are human pathogens.

These investigations demonstrated that plant extracts possess a diversity of compounds and properties used in the green synthesis of nanomaterials. Considering that Colombia is a diverse country in floristic resources, it is important to study the potential of some Colombian endemic plants as reducing and stabilizing agents in the green synthesis processes of nanoparticles. Extracts from plants such as *Crescentia cujete* L., have been shown in numerous studies to have properties that have been used in ancestral medicine (Balogun and Sabiu, 2021).

For this reason, the main objective of this research is to evaluate the antimicrobial potential of silver nanoparticles synthesized by *Crescentia cujete* L, in order to be implemented as an inhibitor of microbial growth that affect human health.

2. Materials and Methods

2.1. Preparation of plant extract

Fresh leaves of *C. cujete*, were collected in the municipality of Villa del Rosario in the department of Norte de Santander-Colombia. The plant material was analyzed at the Bionanotechnology Laboratory of the Servicio Nacional de Aprendizaje (SENA for its initials in Spanish) at the Cúcuta branch. The leaves were cleaned using distilled water and then dried in the shade at room temperature for five days. Subsequently, the dry material was ground in a POLYMIX® mill with a 0.2 mm sieve, obtaining a pulverized product.

2.2. Identification and quantification of flavonoids by high pressure liquid chromatography (HPLC)

To evaluate the flavonoid content obtained from the species, a plant/solvent ratio of 1:10 (m/v) was set, using 10 g of the plant previously pulverized in 100 mL of methanol (CAS 67-56-1, Sigma®) with constant stirring for two hours at 40 °C. The extract was then centrifuged at 3000 rpm for five minutes, followed by gravity filtration using Whatman No.4 paper. The samples were taken to a rotary evaporator (Hei-VAP Expert) at a temperature not higher than 40 °C, with a vacuum of 42 and 218 bar. The rotation speed of the flask was 250 rpm, and the execution time was 15 minutes, recovering a final volume of approximately 40 mL. The product obtained was stored in amber bottles for subsequent analysis.

For the identification and quantification analysis, the HPLC equipment (Jasco 4000 Series) was used; this was equipped with a Chromelon 7.2 processing system, a binary pump (PU-4180), an automatic injector (AS-4050), a diode detector (DAD), and a PrincetonCORE-100 column (100 mm × 4.6 mm × 5 µm) at 40 °C. Wavelength detection was performed at 254 nm-300 nm. Prior to analysis, all solutions (sample vs. standard) were filtered through 0.45 µm pore membranes (Nylon Syringe Filter). The following parameters were considered for evaluation: injection volume of 50 µL (sample/standard) with an analysis time of 70 min and the mobile phase made up of two solvents: solvent A (0.1% phosphoric acid solution) and solvent B (acetonitrile); in gradient elution: 0 min (100% A), 50 min (40% A and 60% B), 60 min (40% A and 60% B), 70 min (100% A), at a flow rate of 1.0 mL/min.

The identification of the flavonoid was carried out by comparing the standard vs. sample retention times. Quantification was performed using a calibration curve built with five quercetin concentration levels (10–50 mg L^{-1}) (CAS 117-117-39-5, Sigma®), 1000 mg L^{-1} standard solutions were prepared, dissolving 10 mg of quercetin in 10 mL of methanol. All analyzes were performed in triplicate.

2.3. Biosynthesis of silver nanoparticles

Ten grams of *C. cujete*, leaf were taken and were crushed together with 200 mL of Milli-Q water using a commercial blender. The homogeneous mixture was brought to a temperature between 60 °C and 70 °C, remaining in that range for 15-20 minutes under constant stirring at 300 rpm. Next, the mixture was left to cool at room temperature. Then, the extract was stored hermetically in polyethylene bottles at 4 °C for one day. Afterwards, the samples were purified by gravity filtration using Whatman No.4 paper.

The synthesis reaction was carried out using silver nitrate ($AgNO_3$) (CAS: 7761-88-8) with a concentration of 2mM. To do so, a heating plate was used with constant stirring at 300 rpm until it reached a temperature between 55 °C to 60 °C. Then, 5 mL of the extract previously prepared was added slowly, leaving 2.42 mg L^{-1} of concentration. Photographs of color change were taken every 30 min from start to end (0-90 min). The reaction was quenched rapidly to stop the growth of the nanoparticles. Finally, the product obtained was stored at 4 °C in polyethylene bottles. The process comprised three biological repetitions and three techniques.

2.4. Characterization

After synthesis, the final aqueous solution was analyzed using Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Spectroscopy (EDX) (COXEM CX 200). Samples were prepared by immersing a Cu grid in 15 µL of the synthesis of nanoparticles for 5 min, working in high vacuum mode at an acceleration voltage of 200 kV. For the elemental analysis of the AgNPs, EDX was used at an acceleration voltage of 20KeV. The observations generated provided qualitative and semi-quantitative information on the chemical elements present in the aqueous extracts of the leaves used. All experiments were performed at room temperature in triplicate.

2.5. Antimicrobial activity

The antimicrobial activity of the AgNPs against microorganisms (bacteria and fungi) present in the Tecnoacademia environment was tested. To do so, two procedures were carried out. The first one involved a modified culture medium (method A), which consisted in incorporating 500 µL of the nanoparticle solution into 20 mL nutrient agar and potato dextrose agar, leaving it to solidify at room temperature. The second method consisted in adding 500 µL of the AgNPs solution to the surface of the already solidified media (method B), spreading it evenly with the help of a sterile microbiological rake. As a negative control, sterile water was used in each of the aforementioned methods (Supplementary Material, Figure S1). Subsequently, all the culture media that were prepared (with and without treatment) were exposed to the environment for 15 minutes. The Petri dishes were incubated at 37 °C for 48 hours for bacteria and at 28 °C for seven days for fungi. The methodology was conducted in triplicate.

The identification of microorganisms took place using conventional methods based on their macroscopic and microscopic characteristics. For bacteria, Gram stain was used, and for the identification of fungi, the "impression on transparent adhesive tape" technique was applied, using lactophenol blue solution as dye. Each isolated microorganism was visualized by optical microscopy, using the Euromex Microscopen BV microscope.

3. Results

3.1. Identification and quantification of flavonoids by HPLC

The identification of the chemical components was made considering the results of separation, identification, retention time (RT) and the UV absorption profile. Figure 1A corresponds to the chromatographic peak of the standard sample, showing a RT of 49,917 min. Figure 1B corresponds to the *C. cujete*, sample (50,033 min), representing a variation of ± 0.12 minutes.

For the quantification of flavonoids, a calibration curve was made, showing good linearity at concentrations of 10 to 50 mg L^{-1} of quercetin (y=12279x + 4E+06, with R^2 = 0.9648). With these data, the concentration of the samples was calculated by substituting Y as the absorbance value. Table 1 summarizes the RT values and the calculation of quercetin concentration for the *C. cujete*, extract.

3.2. Biosynthesis and characterization of silver nanoparticles

The first result obtained during nanoparticle synthesis was color change in the colloidal substance. It was seen that when adding the plant extract to the solution containing the $AgNO_3$ metal salts, a gradual color change occurred from light yellow to terracotta, accentuating between 30 and 90 min (Figure 2A).

It was possible to see the morphology and size of the AgNPs obtained by green synthesis 24 hours after their reaction; these were somewhat spherical (Figure 2B). The average size of the nanoparticles formed ranged from 250 ± 3 to 460 ± 6 nm. Similarly, the EDX analysis revealed the presence of metallic silver in bioreduced form with a percentage of 93.10% (data not shown).

Table 1. Parameters of the calibration curve for quercetin concentration.

Compound	Retention time [min]	Linear range of concentration (mg L^{-1})	β_0	β_1	R^2	Concentration (mg L^{-1})
Quercetin	50.033 ± 0.12	10 to 50	12.279	4.000,000	0.9648	26.55

β_0: intercept; β_1: slope; R^2: coefficient of determination.

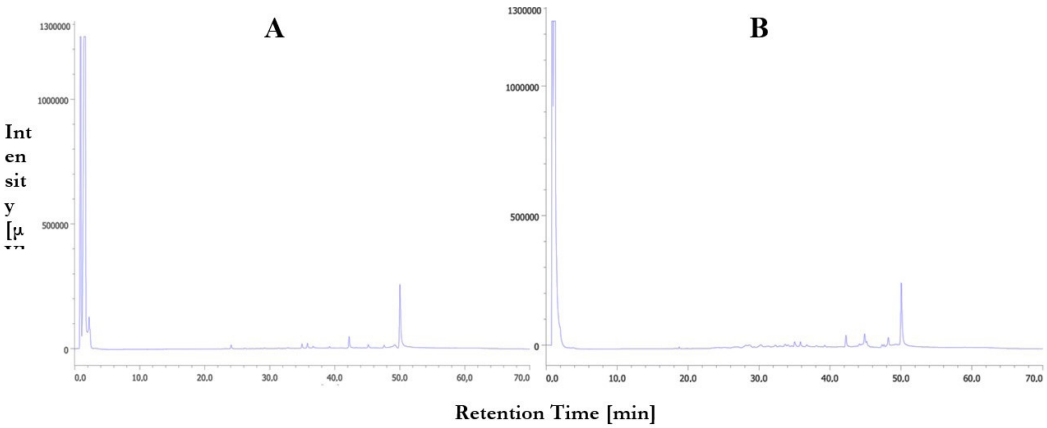

Figure 1. Chromatographic profiles. (A) Standard profile of quercetin; (B) Profile of *C. cujete*, extracts.

Figure 2. Biosynthesis and micrograph of AgNPs formation. (A) Record of color change; (B) SEM image of silver nanoparticles synthesized with *C. cujete*, leaves.

3.3. Antimicrobial activity

The microflora in the control culture media (without treatment) were identified according to their macroscopic characteristics. This observation allowed the characterization of 26 white, yellow, and orange bacterial colonies with creamy morphology and irregular borders two days after exposure. Regarding fungal identification, growth was observed eight days after the environmental sampling. The fungi were white with black and brown center, flat, and cottony (Figure 3A). The ability of the nanoparticle-containing methods to inhibit the growth of microorganisms described above is shown in Figure 3B and 3C. Method A showed high antimicrobial potential, significantly inhibiting the appearance of bacteria and fungi 9 and 14 days after application (daa). The second analysis, using method B, supports the results obtained in the previous test, showing little microbial growth.

4. Discussion

The suggested extraction method using temperature, agitation, and methanol as a solvent was ideal to determine the concentration of the flavonoid (quercetin) contained in the *C. cujete*, leaves. The use of temperature as a factor that improves the extraction of flavonoids has been widely referred to in the literature, mainly because this factor helps the diffusion and solubilization of the compound (Bassani et al., 2014; Liu et al., 2015). In this context, flavonoids, including quercetin, are secondary metabolites synthesized by higher plants, mosses, and ferns to protect them from different abiotic and biotic factors (Takahashi and Ohnishi, 2004). Likewise, quercetin has been reported as one of the chemical constituents responsible for the reduction of nanoparticles, mainly because it has five hydroxyls with hydrogens, which are capable of acting as reducing agents in an oxidation-reduction reaction (Dubey et al., 2010). In accordance with the above, Levchenko et al. (2011) reported that the concentration of bioflavonoids (rutin and quercetin) used as reducing agents allowed the formation of metallic nanoparticles. Other investigations indicate that silver ions could be reduced to nanoparticles using plant leaf extracts (Huang et al., 2007), attributing their reduction to phenolic compounds, terpenoids, polysaccharides and flavones present in plant material. In the evaluation of the phenolic profiles in *C. cujete* leaves, Das et al. (2014)

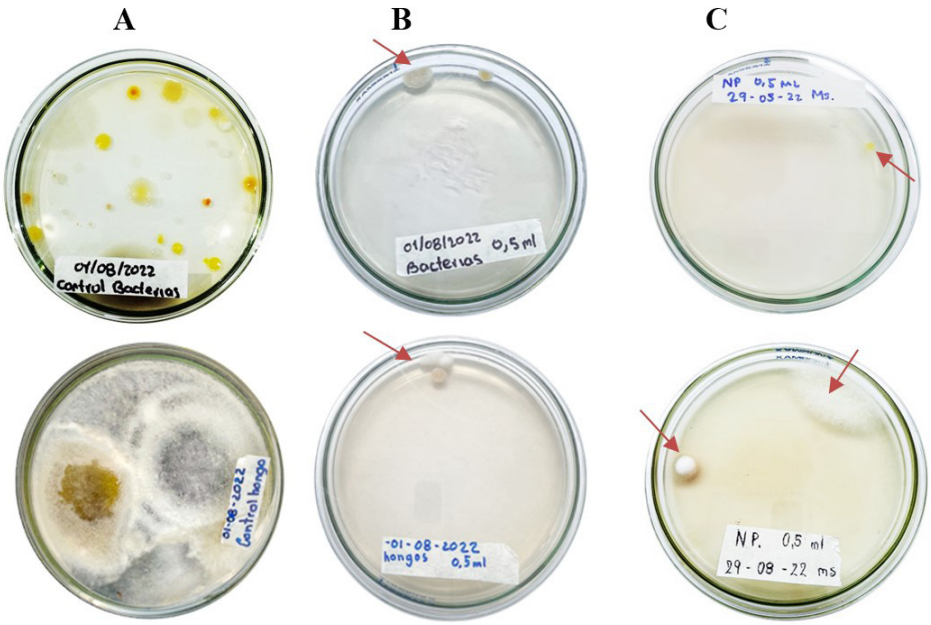

Figure 3. Bioassay 1 and 2. (A) Controls (without nanoparticles); (B) Modified medium for bacterial (figure above) and fungal (figure below) growth; (C) Surface seeded for bacterial (figure above) and fungal (figure below) growth. Red arrows indicate presence of microorganisms.

found important contents of polyphenols and flavonoids expressed in 16.04 ± 3.23 mg L^{-1} of quercetin, a result that resembles that obtained in this research (26.55 mg L^{-1}). This finding suggests that the extract has acceptable amounts of this compound, which could act as a precursor in the synthesis of AgNPs. It is important to clarify that the results of quercetin in *C. cujete*, leaves obtained by chromatography was an initial criterion in the search for substances that inhibit oxidation reactions acting as reducing agents of green synthesis. It must be taken into account that the chemical composition of *C. cujete*, is quite complex and includes other substances in addition to flavonoids, which could have a significant role as reducing agents. These circumstances make additional characterization tests necessary to confirm the findings.

To continue with the analysis of nanoparticles, it can be argued that color change in the synthesis is an initial parameter of the formation of these nanomaterials. This is the result of the reduction of the metal salts of $AgNO_3$ to aqueous precursors of metal ions thanks to the action of secondary metabolites present in the plant extract (Singh et al., 2016). This observation is consistent with the study reported by Khan et al. (2013), who under similar conditions, reported color change during synthesis when using *P. glutinosa* extract. Likewise, the characterization of the shape and size of metallic silver is of great importance to ensure that these nanomaterials are homogeneous in diameter and without agglomerations. Our work is consistent with the results reported by Chandran et al., (2006) who obtained metallic nanoparticles of approximately 400 to 500 nm from the synthesis of *Aloe Vera* leaf extract. Concluding that the shape and size of these nanomaterials could be attributed to the amount of green extract applied, which leads to conclude that the larger the volume of the reducing agent, the smaller the size of the nanoparticles.

The antimicrobial property of our treatment with nanoparticles had a positive effect by minimizing the microbial load. Multiple studies have reported antifungal and antibacterial activity using plant extracts as a reducing agent in the manufacture of AgNPs (Ahmad et al., 2011; Bankar et al., 2010; Singhal et al., 2011). Factors such as shape, concentration, and size of the NPs lead to an increase in osmotic pressure (affecting the transport of organic ions) and irreparable damage to DNA condensation (inhibition of proteins), preventing microorganisms from multiplying (Castro Batioja, 2018; Menéndez, 2004). If the size of the NPs is too large (500-900 nm), they enter the pathogen cell by phagocytosis, which triggers cell death.

The results also show that some microbial agents carried out their metabolic processes in the treatment with nanoparticles, revealing different bacterial groups such as: bacilli and cocci, being gram-positive bacteria the most frequent. Likewise, fungal forms were observed, with a predominance of filamentous fungi of the genera *Penicillium sp* and *Aspergillus sp*. Several studies report the presence of fungi and bacterial colonies in each of their treatments when using silver nanoparticles synthesized from plant extracts in their assays, a result that suggests little protective activity against these microorganisms (Anbukkarasi et al., 2015; Correa et al., 2016; Periasamy et al., 2022). This phenomenon can be explained mainly because the peptidoglycan of gram-positive bacteria is in charge of giving rigidity to the cell

wall and protecting the microorganism from osmotic lysis (Pírez and Mota, 2006). This fact probably gives the microorganism greater adaptability by minimizing the antimicrobial capacity of NPs. As for fungi, they have a cell wall made up of of glucans, proteins, chitin, and other glycoproteins (Adams, 2004). It must be taken into account that since these structures are the most difficult for particles of nanometric size to go through, fungal spores can last several years in a suspended state and germinate quickly when they find the right environment for their development.

Additional studies such as the evaluation of the maximum and minimum concentrations of AgNPs, exposure time, and whether environmental conditions minimize the effectiveness of nanoparticles must be carried out before using these nanomaterials in the field. However, the findings show that our approach can be successfully used to produce interesting amounts of nanoparticles that effectively trigger protection against pathogenic microorganisms. Furthermore, we strongly believe that our laboratory protocol produces high-quality AgNPs that exhibit significant yield and are cost-effective; therefore, they can be used as an antimicrobial agent.

5. Conclusions

The result of the analysis by HPLC chromatography conducted with the leaves of *C. cujete*, allows us to conclude that one of the reducing agents (quercetin) is present in green synthesis. The SEM characterization showed that it is possible to obtain nanoparticles with stable shape and size by green synthesis. In general, little microbial growth was evidenced in the treatments with nanoparticles, which indicates that it is indeed possible to obtain biotechnologically sintered metallic nanoparticles using plant extracts as a redacting agent. This is a cost effective, easy-to-scale, and environmentally friendly alternative with great potential to be used in industry in general.

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Supplementary Material

Supplementary material accompanies this paper.

Figure S1. Experimental design of the bioassay.

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