Biosynthesis of Fluorescent Silver Nanoparticles From Leea Coccinea Leaves and Their Antibacterial Potentialities Against Xanthomonas Phaseoli Pv Phaseoli

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Abstract

The synthesis of Silver Nanoparticles (SNP) from plants is a simple, fast and environmentally safe route. In the present study, the aqueous extract of fresh leaves from *Leea coccinea* L. was evaluated as a possible source of reducing and stabilizing agents to obtain SNP. The synthesized SNP were characterized by spectroscopic techniques such as UV-Visible spectrophotometry and Fourier Transform Infrared spectroscopy (FTIR), Scanning Electron and Confocal microscopies and the antimicrobial activity was evaluated against to *Xanthomonas phaseoli* pv. *phaseoli* using the agar diffusion methods. The results showed that the evaluated extract was promising for the green synthesis of the SNP, which were visually identified by the formation of a dark brown complex and by the presence of a peak of maximum absorption at 470 nm in a UV-VIS spectrum. FTIR spectrum of SNP showed main characteristic signals of aromatic compounds, carboxylic group between others confirmed through phytochemical screening that evidenced the presence of flavonoids, phenols, leucoanthocyanidins, terpenes and steroids groups. Fluorescents SNP with high degree of agglomeration were visualized by microscopies technical used. A promissory antibacterial activity of SNP was demonstrated by a zone of inhibition of the microbial growing. These results suggest the need to go deeper in the physical-chemical characterization and kinetic studies and the biological evaluation to make possible the use of this vegetable source to future develop of antibacterial formulations to the bean seeds protection.

Introduction

Phytopathogenic bacteria cause a negative impact on agricultural production systems, decrease yields and increase economic losses (Pedroza et al. 2013). The genus *Xanthomonas* (Proteobacteria) is a diverse group of Gram negative bacteria that affects numerous crops (Pedroza et al. 2013; Corzo et al. 2015). The disease known as Common bacteriosis or Common blight, widely distributed in the world, is caused by *Xanthomonas phaseoli* pv. *phaseoli* (Smith) Vauterin y *Xanthomonas citri* subsp. *fuscans* (Burkholder) Starr & Burkholder (Corzo et al. 2015) and is the main biotic factor that affects the yields of *Phaseolusvulgaris* (common bean) crops because it causes losses in this crop ranging between 10-40%, depending on the susceptibility of the cultivar and environmental conditions (Pedroza et al. 2013; Corzo et al. 2015; Constantin et al. 2016; Francisco et al. 2013).

Currently, the management of this pest is complex and multifactorial, in which the control of the seed is essential. In this sense, efforts are made in the search for resistant varieties (Rodríguez et al. 2015), biological (Aguilar et al. 2019) and chemical controls (Ararsa et al. 2018) means that guarantee its quality. Numerous authors point out that the foliar application of copper-based bactericides, crop rotation, interleaving, among other actions, are successful if they are applied in an integrated management program (Ararsa et al. 2018). The use of chemical pesticides based on copper, either for the treatment of the seed, as for the systemic treatment, constitutes one of the most widespread alternatives to the control of this pest; however, numerous studies have shown the resistance of the bacteria (Ram et al. 2018).

On the other hand, previous studies suggest some types of nanoparticles (NP), specifically metallic ones (MNP), as promising alternatives in different fields of agricultural biotechnology for obtaining new antibacterial agents (Rajesh et al. 2012; Shende et al. 2016). The synthesis of silver nanoparticles (SNP) from plants constitutes a simple, fast and environmentally safe way of obtaining, in which numerous plant species are reported with positives results for this purpose (Gupta and Chauhan 2017).

Leea coccinea is between the 70 species of the genera Leea belong to Vitaceae family (before Leeaceae) (International Seed Testing Association 2013) and of wide global distribution (Australia, New Guinea, South and Southeast Asia, parts of Africa and the Americas). In Cuba, *L. coccinea* specie abounds in the western region and is used primarily as a living fence and as an ornamental plant because of the beauty of its foliage and inflorescences. Although other species of the genus have been studied (Emran et al. 2012; Joshi et al. 2016; Mahmud et al. 2017; Halder et al. 2018), in the literature reviewed there are hardly any reports on the chemical composition of this specie. The antimicrobial activity of Leea species has been demonstrated (Joshi et al. 2016; Tun et al. 2019). Recent reports have demonstrated the antinociceptive and anthelmintic activity of *Leea aequata* leaf extracts (Halder et al. 2018). Other authors have justified the antioxidant and antibacterial potential of root tubers of *Leea macrophylla* which is a potential tool in the treatment of disorders associated with oxidative stress and pathogenic infections (Joshi et al. 2016; Mahmud et al. 2017).

The phytochemical screening of ethanolic extract of root tubers of *Leea macrophylla* revealed that it is rich in phenolic compounds (Joshi et al. 2016; Mahmud et al. 2017); saponins (Joshi et al. 2016). Also this extract is rich in carbohydrates between others phytochemical (alkaloids and flavonoids) of high biological value (Joshi et al. 2016).

The aims of the current study were to evaluate the aqueous extract of the *Leea coccinea* leaves, as a possible source of reducing and stabilizing agents for obtaining SNP, as well as determining the potentialities as an antibacterial agent against *X. phaseoli* pv. *phaseoli*.

Materials And Methods

Plant material

Leea coccinea plants were located in the latitude 22,990173 North and longitude -82, 151213 West, at a height of 142 masl (Red point in Fig. 1). Leaves were collected and selected to remove soil contaminant. The authentication was carried out by the specialist in botanical from Agrarian University of Havana (UNAH) in Mayabeque, Cuba.

Preparation of plant extracts and biosynthesis of SNP

The leaves of *L. coccinea* were collected and thoroughly checked for the separation of any contaminant. The fresh leaves were triturated until a thick powder remains and 5 g were macerated with deionized water (1:8) (W/V). Then the mixture was heated to 90 ºC for 10 minutes and it was cooled and filtered through Whatman No. 1 paper.

The synthesis of the SNP was carried out by a typical procedure (Fig. 2). The solution of silver nitrate (2 mM) was added to the plant extract (9:1) (V/V). It was homogenized with a mechanical stirrer (IKA-VF2) and allowed to stand for 24 hours at room temperature (27±2 °C). The bio-reduction reaction was monitored by visual observation at different times (5 minutes, 1 hour and 24 hours). The occurrence of change of color to dark brown was indicative of the formation of SNP. The resulting suspension of SNP was centrifuged at 10000 rpm during 20 minutes and the pellet was washed with sterile water three times. SNP were stored at room temperature and in the dark until evaluation. The yield of SNP was determined by measuring of the dry weight of 1 mL of the reaction for triplicate on an infrared balance (Sartorious MA35) and calculated with relation to the initial mass of vegetable material. The results were expressed as the media valor in the range of the standard deviation.

Characterization of SNP

The biosynthesized SNP were characterized by various instrumental analyses. The pH determination of aqueous suspension of nanoparticles was carried out by Mettler Toledo instrument. The formation of SNP was demonstrated by UV–VIS spectrum (300-800 nm) through RAYLEIGH (VIS-723G) spectrophotometer and the spectrum of Fourier Transform Infrared spectroscopy (FTIR) in the range 4500- 200 nm using IR Prestige 21 equipment (SHIMADZU). The scanning electron microscopic (SEM) analysis was performed using the MIRA 3 LMU (TESCAN) SEM machine. Samples to SEM analysis were applied on carbon adhesive tapes and coated with gold (5 nm) with a Desk Sputter Coater DSR1. The films were dried during 24 hours. The EDX analysis of SNP was carried out on samples dried and drop coated on to carbon film using Energy Disperse Ray X (Oxford Instrument). Fluorescence properties were demonstrated with Confocal Microscopy (FICT- wide 479 nm Lent 20x). It was performed on samples sonicated previously for 10 minutes and applied on porous albumin slides covered with coverslips which were kept in a humid chamber until observation.

Phytochemical screening of aqueous extract of leaves of *L. coccinea* was carried out to identify the presence of secondary metabolites with reducing properties. Several qualitative tests were done following the internal protocol established in the Chemical- Ecology lab. and classics reactions such as Ninhydrine (free aminoacids), Ferric chloride (phenols), Gelatin (phenols), Lieber- Buchard (triterpens and steroids), Kedde (lactonic rings), Borntrager (quinone), Shinoda (flavonoids), Rosenheim (leucoanthocyanidins) and Mayer- Draggendorf- Hager- Wagner to alkaloids.

Evaluation of antimicrobial activity

Antimicrobial activity of SNP was evaluated to the nanoparticles precipitated by centrifugation (10000 rpm for 20 minutes) to remove excess of salt and others soluble compounds. The silver colloids were washed at least three times with deionized water. The antibacterial activity on *Xanthomonas phaseoli* pv. *phaseoli* was evaluated by the disks agar diffusion method (Clinical and Laboratory Standars Institute 2015). An inoculum was prepared at the concentration of 29 x 10^7 UFC. mL^{-1}, in sodium chloride solution (0,9 %), from a culture of 24 hours incubation at the temperature of 28 ºC in nutrient agar plates,

according to the McFarland scale. The strain used was Xap1, belonging to the cepary of the Laboratory of Plant Bacteriology of the National Center for animal and Plant Health in Cuba (Corzo et al. 2015). For application on disks, 10 uL of the precipitated SNP were applied in every disk. As positive controls, Erythromycin (5 µg) and Chloramphenicol (10 µg) disks were applied. Sterile water was used as a negative control. At least four replications of the treatment and controls were performed. Plates were incubated at 28 ºC for 24 hours. After this time, zone of inhibition (mm) of bacterial growth were measured with a graduated rule.
The data obtained were statistically processed by a simple variance analysis and the means were compared using the Duncan multiple range comparison test with a significance level of 5%, using the statistical package InfoStat/ L (Di Rienzo et al. 2016).

Results And Discussion

The reaction of bio-reduction between the Ag^+ cations and the phytochemical compounds of the aqueous extract of *L. coccinea* leaves occurred instantaneously (before of the five minutes), evidenced by the presence of a dark brown colored complex (Fig. 3A), indicative of the formation of nanoparticles (Vadlapudi and Amanchy 2017; Sarkar and Goutam 2017; Khan et al. 2018). UV-Visible spectroscopic absorbance (Fig. 3B) evidenced a signal with λ_{max} around of 470 nm due to strong surface Plasmon resonance, which reaffirms the formation of SNP (Travieso et al. 2018). The release of H^+ ions since the first minutes of the reaction was verified by the gradual decrease of the pH in the aqueous media (Fig. 3C). Authors had explained the oxidation process of Ag^+ by phytochemical compounds from plants, which are accompanied by the release of H^+ ions (Zuorro et al. 2019). Also, it has been proposed that tautomer transformations of other compounds from plants (Ex. flavonoids) from the enol form into the keto form can release reactive hydrogen atoms that reduce metal ions to coordination complex of Ag forming nanoparticles. Nevertheless, due to the complex composition of naturals extracts actually there is no enough proof about which specific phytochemicals are responsible for the formation of nanoparticles (Jeevanandam et al. 2016).

Phytochemical screening showed the presence of secondary metabolites such as phenols, triterpenes and steroids, flavonoids, free aminoacids and leucoantocianidine in the aqueous extract of leaves of *Leea coccinea* (Table 1). Some of these compound podrían reaccionar with Ag^+ to form the coordination complex so kinetic and chemical studies should be carrying out to the enlightenment of the reaction of bio-reduction due to the presence of several functional groups with reducing properties.

Currently, there are very few reports available on the phytochemistry of *L.* coccinea. Previous studies on chemical compositions of others species of Leea genera agreement with ours previous results. Flavonoids and flavonoid glycosides are found to be the major constituents of the genus (Lakornwong et al. 2014). Chemical studies of *Leea indica* reported six phenolic compounds, flavan-3-ols, flavonoids/flavonoid glycosides, dihydrochalcones and dimeric catechins (Singh et al. 2019). Similar studies based on NMR, MS, and ECD spectra reported one new lignan, one new lactam, five known lignans, four favonoid glycosides between other compounds were isolated from the ethanol extracts of the aerial parts of *Leea aequata* (Tun et al. 2019).

Table 1 Phytochemical screening of the aqueous extracts of *Leea coccinea* leaves

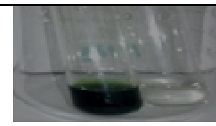
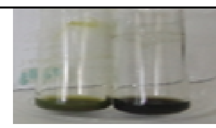
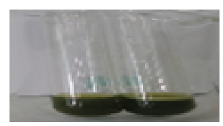
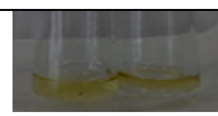
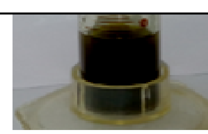
No.	Phytochemical	Test	Result	Qualitative reaction
1	Flavonoids	Shinode	+	
2	Triterpenes and steroids	Lieber- Buchard	+	
3	Saponins	Froth	-	-
4	Alkaloids	Mayer (M) Draggendorf (D) Hager (H) Wagner (W)	- - - -	
5	Phenols	Ferric chloride Gelatin	+ +	 
6	Lactonic rings	Kedde	-	
7	Aminoacids free	Nihhidrine	+	
8	Leucoanthocyanidins	Rosenheim	+	
9	Quinones	Borntrager	-	

Table Key: - Absent + Presence

FTIR spectrum confirmed the phytochemicals screening (Fig. 4). Measurements were carried out to identify the functional groups responsible for the reduction of the Ag+ ions and capping of the bio-reduced silver nanoparticles synthesized by *L. coccinea* leaf extract. FTIR spectrum of SNP shows the presence of signals such as strong peaks between 3083 and 3076 $cm^{-}1$ (peak 8) with a signal at 1538 cm^{-1} (peak 16) like aromatic with multiple substitutions(C=C stretching); other peak around 1616 cm^{-1} (peak 15) (C=O stretching) of carboxylic group. The absorption at about 1334 cm^{-1} (peak 18) is indicating residual amount of $(NO_3)^{-1}$ ion in the solution. Others signals appear at 1207 cm^{-1} (peak 19), between 3881 and 3831 cm^{-1} (peak 1), 3752 cm^{-1} (peak 2), 3565 cm^{-1} (peak 3), 3529 cm^{-1} (peak 4), between 3414 and 3335 cm^{-1} (peak 6) suggest polyphenolic OH group along with the peak around 880 cm^{-1} which represents the aromatic ring C-H vibrations. Others peaks around of 3270 cm^{-1}(peak 7), 3025 cm^{-1} (peak 9), between 2932 and 2925 cm^{-1} (peak 10), 2235 cm^{-1} (peak 11), 2171 cm^{-1}(peak 12), 1747 cm^{-1} (peak 13), 1688 cm^{-1} (peak 14), 1451 cm^{-1} (peak 17), 1156 cm^{-1} (peak 20) and between 1107 and 1035 cm^{-1} (peak 21) indicate the complexity of samples containing nanoparticles from natural sources and suggest the necessity of continuous studies about chemicals structures of compounds implicated in the bio-reduction.

The SEM images (Fig. 5 A y B) of SNP showed the presence of abundant nanoparticles with high grade of aggregation which was confirmed with confocal microscopy images where the emitted fluorescence was detected at 479 nm, demonstrating the occurrence of overlapping of smaller particles (Fig. 5 C). Numerous authors have reported agglomeration due to strong electrostatic interactions of metal ions. The fluorescent properties suggest to the presence of phytoconstituents or antioxidants with fluorophores groups. Others metal nanoparticles synthesized from plants have been reported with fluorescent properties (Talamond et al. 2015; Donaldson and Williams 2018).

The results of the qualitative analysis by EDX (Fig. 6) showed a high percentage of Ag evidenced in strong signals in the range of 2-4 keV, as well as the presence of carbon and oxygen indicative of hybrid NPs, with an organic component (Ex flavonoid) covalently bound to the metallic element. Likewise, the high compaction demonstrates its crystalline nature due to the reduction of Ag + cations by the reducing compounds present in the aqueous extract of *L. coccinea* leaves. (Singh et al. 2019; Kasithevar et al. 2017; Moodley et al. 2018 and Oluwasogo et al. 2019).

SNP showed promising activity against tested Gram negative bacterial strain of *X. phaseoli* pv *phaseoli* (Fig. 7). In previous studies in which the activity of AgNPs synthesized from the residual extract of the hydrodistillation of the essential oil of *Thymus vulgaris* was evaluated, the similar activity against this bacterium (Travieso et al. 2018) was demonstrated. However, in the present study in which the precipitated NPs were evaluated, the little diffusion of these nanostructures was found, so biological evaluation studies must be completed with the addition of stabilizing agents. On the other hand, the antimicrobial activity of AgNPs depends on parameters such as Ag concentration, as well as the size and shape of the nanostructures (Duval et al. 2019). However, in recent years other parameters have been demonstrated that influence antibacterial activity such as stabilization in the aqueous medium and the accessibility of the surface of NPs (Duval et al. 2019).

The formation of aggregates and agglomerates are the main destabilizing factor and reduce the accessible surface area in contact with the bacterial cell, and also inhibit the diffusion capacity in the *in vitro* tests (diffusion in agar), interfering with the results. Silver nanoparticles' diffusion coefficients are generally related to size and physicochemical characteristics (Xiaoxue et al. 2020). For this reason, diffusion methods are used in initial or screening studies, however they are not valid for the determination of Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (CMB), suggesting tests such as serial dilutions in those that, in addition, the liquid medium facilitates the interaction of the substances to be evaluated with the microorganism through agitation. Authors have addressed the limitations of the antimicrobial activity studies of AgNPs suggesting methods with a liquid medium (macrodilution and microdilution), the Kirby-Bauer method, prior knowledge of the diffusion coefficient of the sample, among other standardized ones.

Conclusions

In the present work were synthetizing fluorescent silver nanoparticles from *L. coccinea* leaves and the antimicrobial activity against to *X. phaseoli* pv *phaseoli* was demonstrated. Future research should be directed to chemical, biological and pharmaceutical studies for the future design of stable and effective formulations for plant protection.

Abbreviations

SNP: Silver nanoparticles; masl: Meters above sea level; FTIR: Fourier Transform Infrared spectroscopy; UV-VIS: Ultraviolet- Visible; nm: nanometer; NP: Nanoparticle; MNP: Metallic nanoparticle; W/V: Weight/ Volume; SEM: *Scanning electron microscopy*; EDX: Energy Dispersive X-ray; NMR: Nuclear magnetic resonance; MS: Mass spectrometry; ECD: Electronic circular dichroism

Declarations

Conflict of Interest

There are no conflicts of interest to be declared.

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González (from CENSA) and Dania, Ananayla and Ahmed (from CEA) were indispensable to successful ending of the study.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Ethics approval and consent to participate

All authors have read and agreed the ethics for publishing the manuscript.

Consent for publication

All authors approved the consent for publishing the manuscript to bioresources and bioprocessing.

Authors' contributions

Annie Rubio designed and performed the experiments, microbiological assays, statistical analysis, reagents/materials/analysis tools facilities, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

Beatriz Álvarez performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

Mylene Corzo designed and performed the microbiological experiments, reagents/ materials/ analysis tools facilities, authored or reviewed drafts of the paper, approved the final draft.

Lianet Díaz as associated student of the project performed the experiments of phytochemical screening, analyzed the data, contributed, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

Emilio Acosta performed the experiments of characterization of the nanoparticles, analyzed the confocal data, contributed reagents/materials/analysis tools, prepared photos, authored or reviewed drafts of the paper, approved the final draft.

Oriela Pino designed and supervised the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft, significant opportunities and applications of nanotechnology in plant protection.

María del Carmen Travieso designed and performed the experiments, processing of primary data, interpretation and integration of results, and written the document.

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Figures

Figure 1

Geo- localization of the vegetable material of Leea coccinea

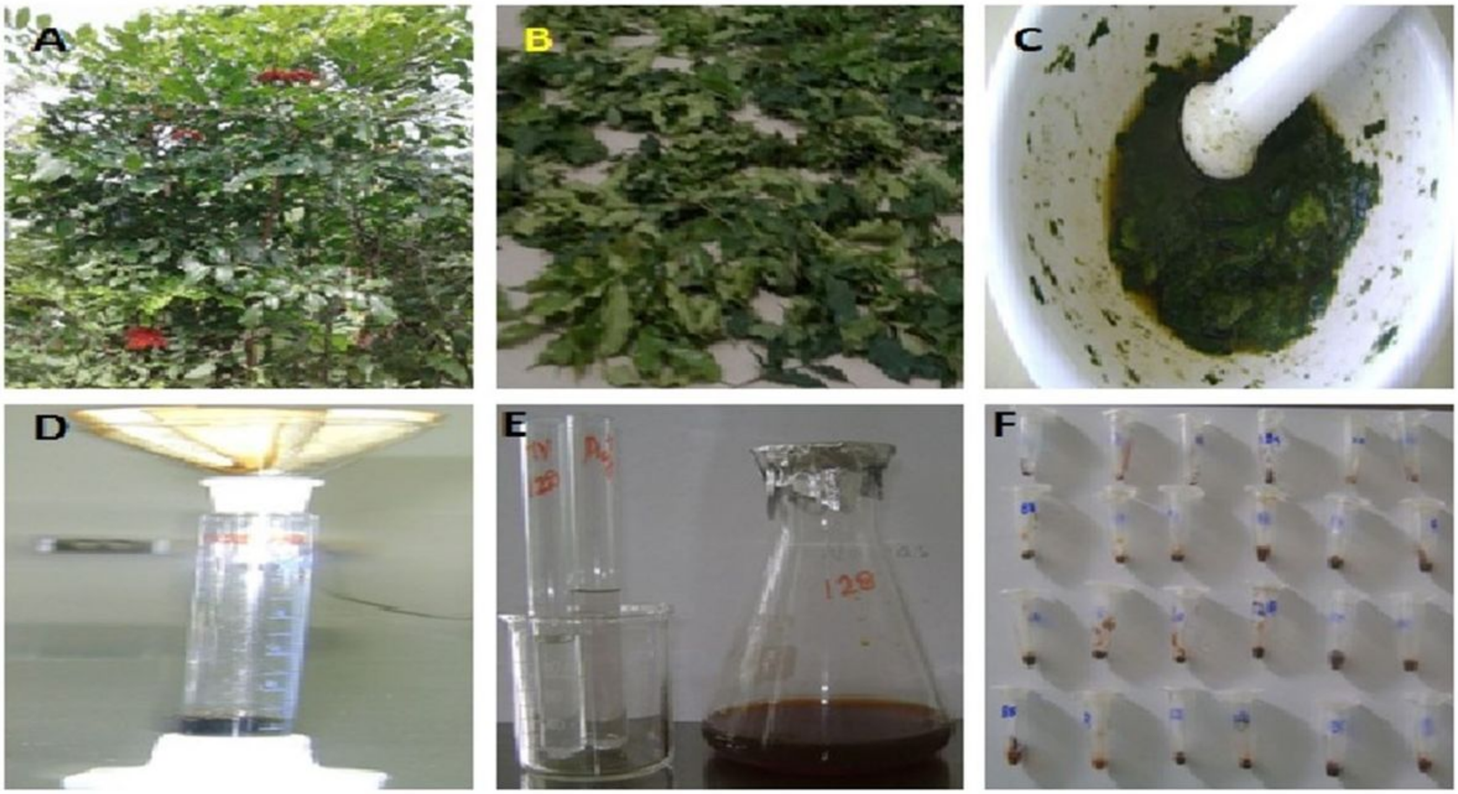


Figure 2

Biosynthesis of SNP from Leea coccinea (Lc) leaves. A: Shrubs of Lc, B: Leaves selection of Lc; C: Maceration; D: Filtration; E: Reaction of bio-reduction con Ag+; F: Precipitation

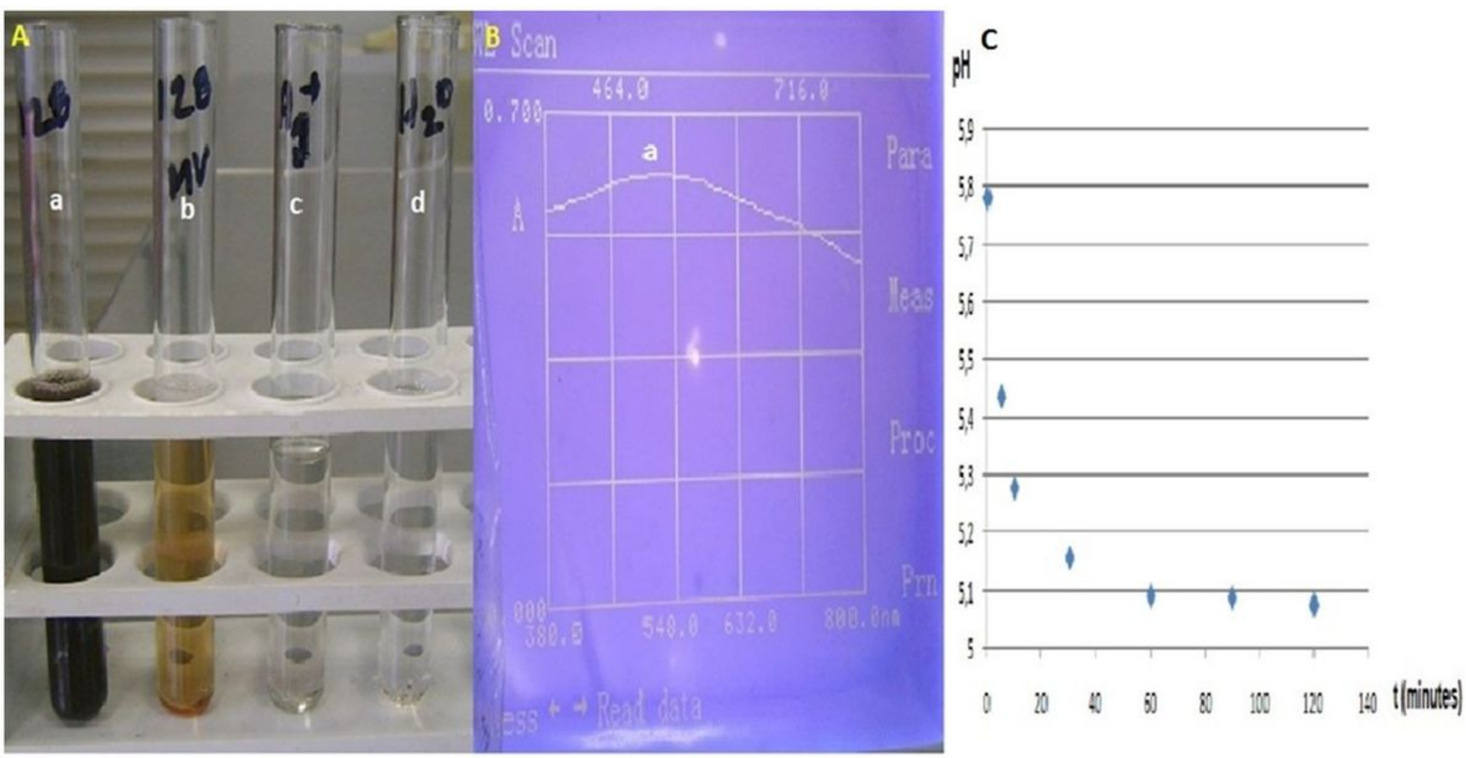


Figure 3

Identification of the formation of SNP from Leea coccinea. Visual (A), spectrophotometric (B) and pH variation during bio-reduction (C). a: positive reaction between aqueous extract of leaves (AEL): silver solution 2 mM (1:9) (V/V); b: negative reaction of AEL: distilled water (1:9); c: silver solution 2 mM: distilled water (9:1); d: distilled water

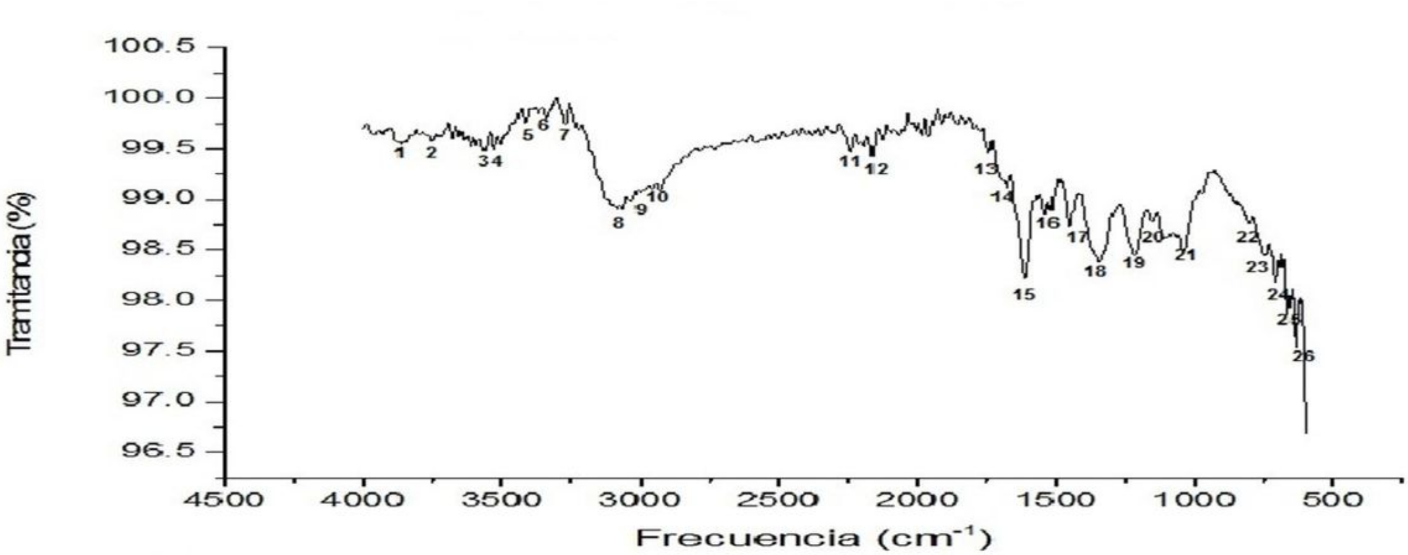


Figure 4

FT-IR spectra of SNP synthesized of L. coccinea leaf extract. Main peaks: 8 (16,18) (22-26): Aromatic (benzene with substitutions); 15(19): -COOH carboxylic (C=O); 17(10): C=H (methylene)

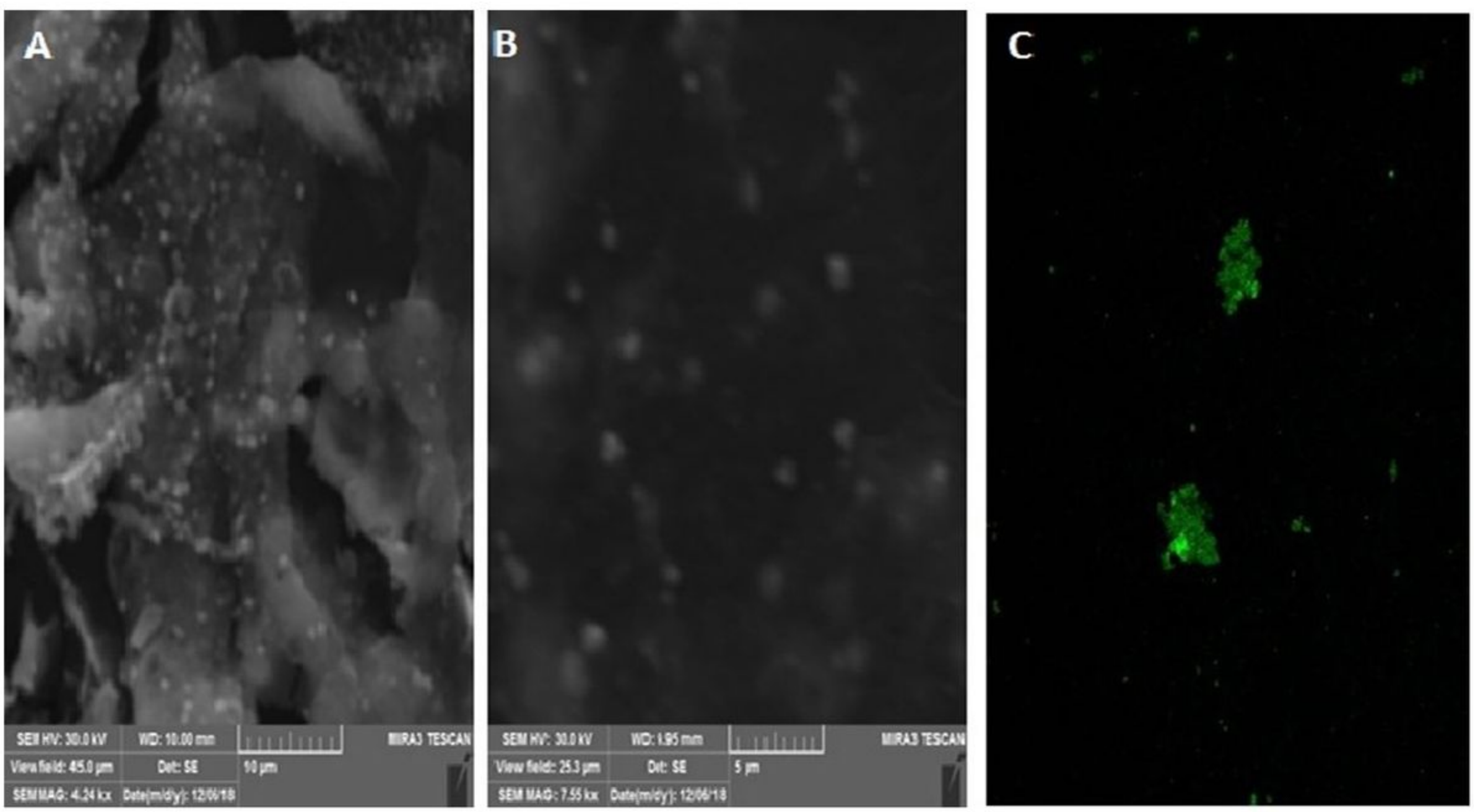


Figure 5

SEM (A,B) and confocal (C) images of the SNP synthesized from Leea coccinea leaf extract

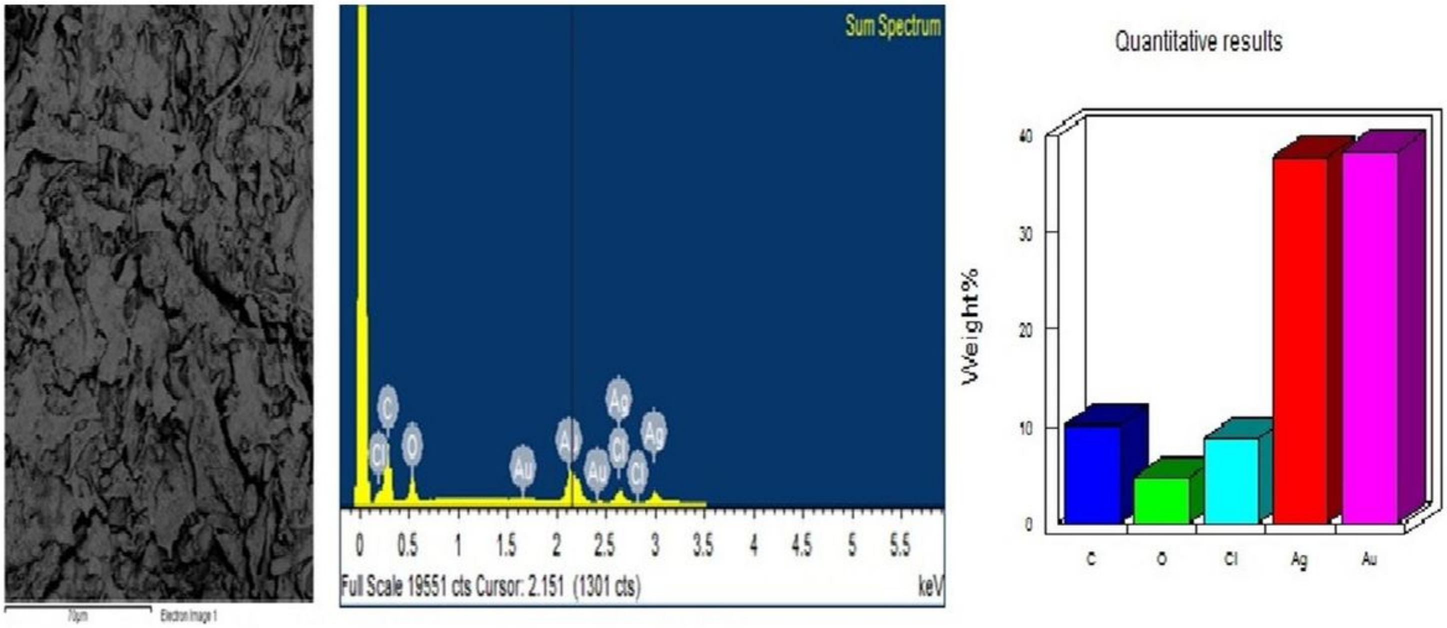
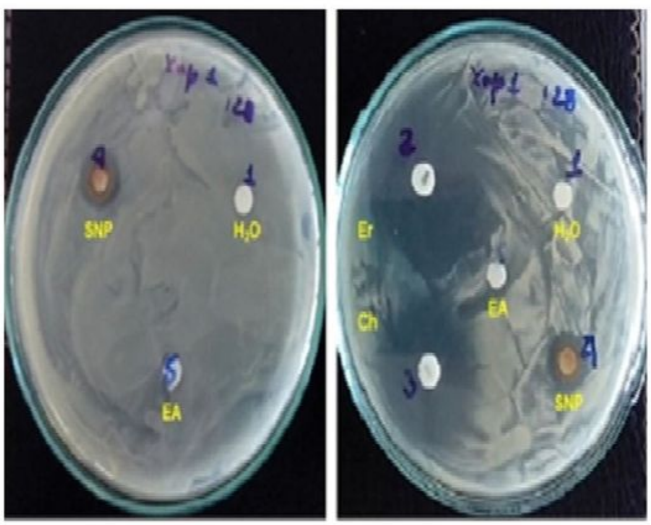


Figure 6

EDX of the SNP synthesized by Leea coccinea leaf extract



Number	Sample	n	Zone of inhibition (mm)
1	Sterile water	8	NI
2	Erithromycin (Er)	4	33,00 ± 1,41 a
3	Chloramphenicol (Ch)	4	29,00 ± 1,41 b
4	SNP	8	12,00 ± 0,00 c
5	Aqueous extract (AE)	4	NI

Figure 7

Antibacterial activity of SNP from Leea coccinea leaves against to Xanthomonas phaseoli pv phaseoli

Supplementary Files

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- GraphicalabstractArticleMCTravieso2020.jpg