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Evaluation of the Biotechnological Potential of *Monteverdia salicifolia* (Mart ex. Reissek) Biral

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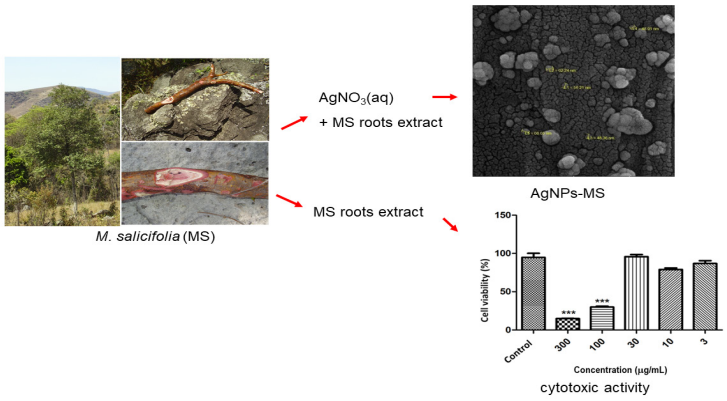
Plant extracts are a good alternative of reducing agents in the synthesis of metal nanoparticles. In this paper, we report the evaluation of the cytotoxic activity against T3 cell lines of the ethanolic extract of *Monteverdia salicifolia* (Mart ex. Reissek) Biral roots (MS) as well as a green one-pot route of synthesis of silver nanoparticles (AgNPs) using that extract as reducing and stabilizing agent. The extract exhibited dose dependent activity. The smallest particle size (48.01 nm) was achieved in just 25 minutes by employing a temperature of 65 °C and $AgNO_3$ and MS concentrations equal to 0.9 mmol.L^{-1} and 0.67 mg.mL^{-1}, respectively. The AgNPs-MS nanocomposite was characterized by UV–vis spectroscopy, FEG microscope and zeta potential, which proved that MS was effective at reducing and capping the AgNPs. In order to emphasize the advantage of the methodology applied in this synthesis, it was compared to a usual procedure using $NaBH_4$ as a reducing agent and the greenness analysis was also carried out, using the Green Star.

Graphical abstract



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1. Introduction

Monteverdia (syn. *Maytenus*) genus is the most representative of the Celastraceae family. The most known species is *Monteverdia iilcifolia*, popularly known as "espinheira santa", which presents proven efficacy against stomach disorders [1]. The common secondary metabolites found in that genus are pentacyclic triterpenes, quinone methides and flavonoids. They exhibit different biological activities, such as anticancer, anti-inflammatory and antiviral [2]. *Monteverdia salicifolia* (Mart ex. Reissek) Biral occurs in Southeast of Brazil and is popularly known as *cafezinho*. Its leaves are used in the traditional medicine for treatment of gastric ulcers and skin allergies [3]. This species is a source

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of different compounds, including tingenone, lupeol, pinostrobin and friedeline, which show antitumor [2,3] and cardioprotective [4] activities.

Nanotechnology is the science used to synthesize and characterize in nanometric scale (1 a 100 nm) [5]. These materials show advantages due to their big superficial area related to the volume, which contributes to their physical and chemical properties, making possible their application in different fields. Research developed in biomedicine has shown the use of nanostructured systems, mainly for diagnostics and treatment of cancer [6,7], development of antimicrobials [8,9], controlled drug release [10], and catalysis [11].

Silver nanoparticles (AgNPs) are extensively reported worldwide, once they show a particularly good performance in pharmacology, due to their high permeability in different cell lines, such as skin, lung, brain, and liver, which evidences their bioactivity [12]. Moreover, products containing AgNPs show antimicrobial, antifungal and antiviral properties [10,12].

There are many methods to obtain AgNPs [13, 14]. However, the conventional methodology relies on hazardous chemicals and solvents, presenting high energetic demand and high cost. Thus, aiming to reduce the environmental impact and costs during the process, an eco-friendly methodology was developed. The green synthesis uses environmental resources to produce nanoparticles, such as plant, bacteria or fungal [14], followed by reactants with low toxicity or non-toxic, in order to reduce harmful waste.

The use of plant extracts in green synthesis shows many advantages such as speed in the process and formation of stable nanoparticles with high yield. Many studies report the use of plant extracts in the synthesis of AgNPs and their bioactivity. For example, extracts of the leaves of *Xanthium strumerium* L [15], *Ocimum sanctum* [16] and *Spondias tuberosa* [17] were able to reduce silver ions in the reaction with $AgNO_3$, commonly used in this process.

Considering the importance of the development of green methodologies, this study was aimed to evaluate the potential of *M. salicifolia* root extract in the synthesis of AgNPs and its cytotoxic activity against 3T3 cell lines. In order to emphasize the positive points of this eco-friendly phytosynthesis, a comparison was carried out with the traditional synthesis of AgNPs, using $NaBH_4$ as the reducing agent, based on the 12 principles of the Green Chemistry [18].

2. Results and Discussion

This investigation revealed dose-dependent cytotoxic activity from the ethanolic extract of *M. salicifolia* roots. In concentrations higher than 30 µg/mL (100 and 300 µg/mL) the extract is cytotoxic and cell viability was less than 50%, that is, more than half of the cells did not survive the exposure to higher concentrations of the extract. In lower concentrations the cells were viable, with no statistically significant difference (Figure 1). Consequently, concentrations below 30 µg/mL of ethanolic extract from *M. salicifolia* roots could be safely used in the preparation of nanoparticles. It seems relevant to highlight that the biological potential of a plant can be different, depending on which part of the species is used, mainly in the extraction process. In our previous work, compounds extracted from the roots of this same plant, using the chloroform extract, were able to inhibit the growth of HeLa and A-549 cell lines [19].

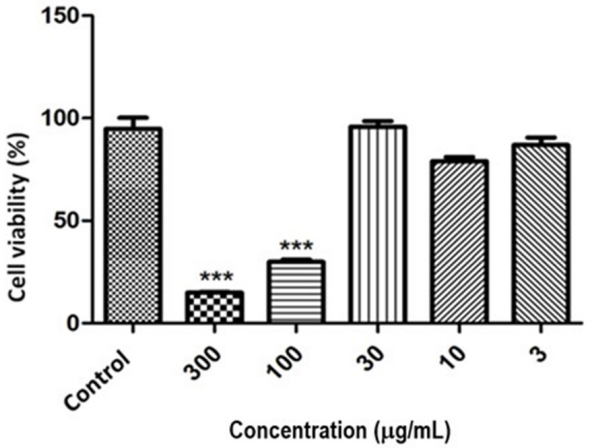


Fig. 1. Cell viability of 3T3 fibroblasts after 48 h incubation with ethanolic extract from *M. salicifolia* roots at concentrations ranging from 3 to 300 µg/mL. Data are expressed as percentage of viability compared to control (untreated cells). Mean ± SD from 3 independent experiments. ***Represents a value of $p < 0.001$ that was considered to be highly significant when compared to the control.

The AgNPs-MS were successfully synthesized using the ethanolic extract of *M. salicifolia* roots as a green source of reducing agent owing to the presence of antioxidant compounds such as proanthocyanidin and 4'-O-methylepigallocatechin [3]. The formation of AgNPs-MS was initially detected based on the visual change in color from colorless to colloidal yellow after 25 minutes of heating and sitting. This occurs due to the excitation vibrations of the surface plasmon resonance (SPR) in AgNPs, indicating the transition of silver ion (Ag^+) into silver nanoparticle (Ag^0) [16] and confirming the biosynthesis of AgNPs-MS. In this method, the extract acts simultaneously as a reducing and stabilizing agent. The formation of those nanomaterials was analytically proven by UV−visible spectroscopy. UV−visible peak was obtained at 420 nm, characteristic band of Ag [12], (Figure 2) showing the formation of well-stabilized AgNPs. Similar results were observed earlier with another species of this genus, *M. senegalensis* hexane leaves extract, peaking at 439 nm [20]. This difference can be due to the different compounds of that extract. The zeta potential observed for the AgNPs-MS was -28.4 mV (Figure 3). The result obtained indicates that the particles are stable, due to the association to the ZP absolute value near to 30 mV and -30 mV [21]. The negative value confirms the negative charge on the surface of the nanoparticles, which contributes to the prevention of cluster formation, being also an important point for the stability of the AgNPs that show biological activity. The analysis of the images obtained by FEG microscope (Figure 4) revealed that the biosynthesized nanoparticles showed spherical shape and sizes in the range of 48.01nm to 82.72nm. During the synthesis of the AgNPs-MS, a negative influence of the sunlight was observed. When the process occurred in a sunny environment, the band observed in the UV-vis spectrum became larger and in a range higher than 420 nm, which indicates the formation of bigger and unstable nanoparticles and clusters [20]. This change of behavior can be explained due to the fact that phenolic compounds, which are the major metabolites in the extract studied, can be degraded under light and higher temperatures [22]. Consequently, the reducing potential of the extract can be decreased. The importance of phytosynthesis of metal nanoparticles is widely reported in the literature. Secondary metabolites guarantee the specific properties of these

nanomaterials, improving their relevance in science. Other plant extracts from *Monteverdia* species had already been used successfully in silver nanoparticles, such as *M. royleanus* [23] and *M. senegalensis* [20]. Moreover, due to the complex structures, shapes, morphologies and biological functions, these natural materials play an important role in controlling the structures, morphologies, biological activities as well as many other aspects of nanomaterials, which are irreplaceable and cannot be substituted with chemical and physical synthesis.

The greenness analysis of the conventional synthesis of AgNPs using $NaBH_4$ as reducing agent [24] and the phytosynthesis proposed in this work were carried out, considering the 12 principles of Green Chemistry [18]. Observing the green stars (Figure 5), it was possible to verify that the methodology where the extract is used, indeed, showed a higher area corresponding to greenness. It seems relevant to observe that both procedures were carried out in just one step, and they do not need catalysts. In addition, the reactions do not need derivatization. Then, the principles P8, P9 and P10 [25] are performed in these methodologies. The synthesis using the extract show advantages regarding principles P1, P2, P7 and P12, which correspond to not generating innocuous waste (P1); there were no excess reactants or coproducts (P2); renewable reactants were used (P7) and just one reactant ($AgNO_3$) presented health hazards (P12). This methodology only showed the disadvantage of using heating, in contrast with the conventional synthesis. The green star was a good tool for a detailed analysis of synthesis protocols, being sensitive enough to verify negative and positive points in different procedures. The observation of the application of the Green Chemistry principles in synthetic process evidences the importance of search and development of increasingly sustainable methods.

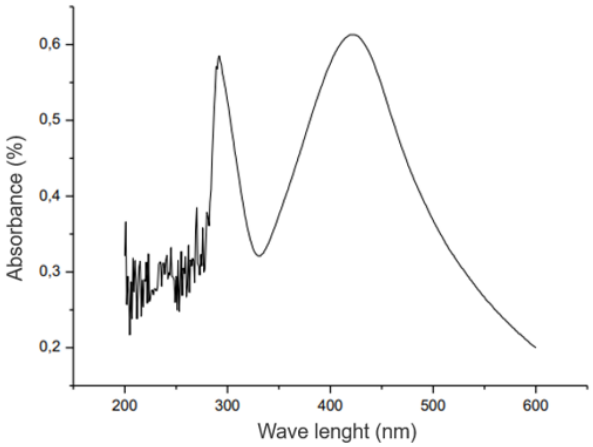


Fig. 2. UV-vis spectrum of AgNPs-MS.

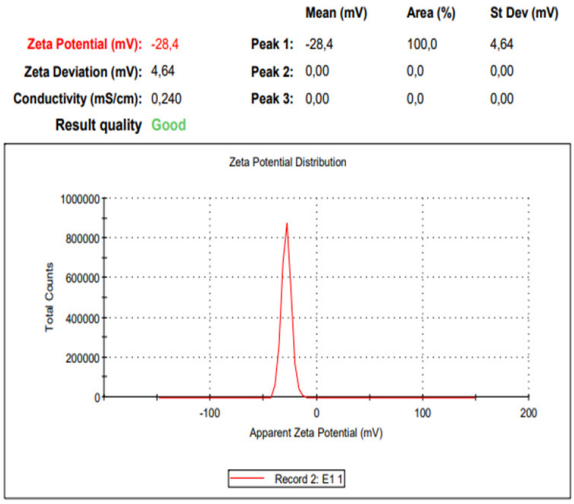
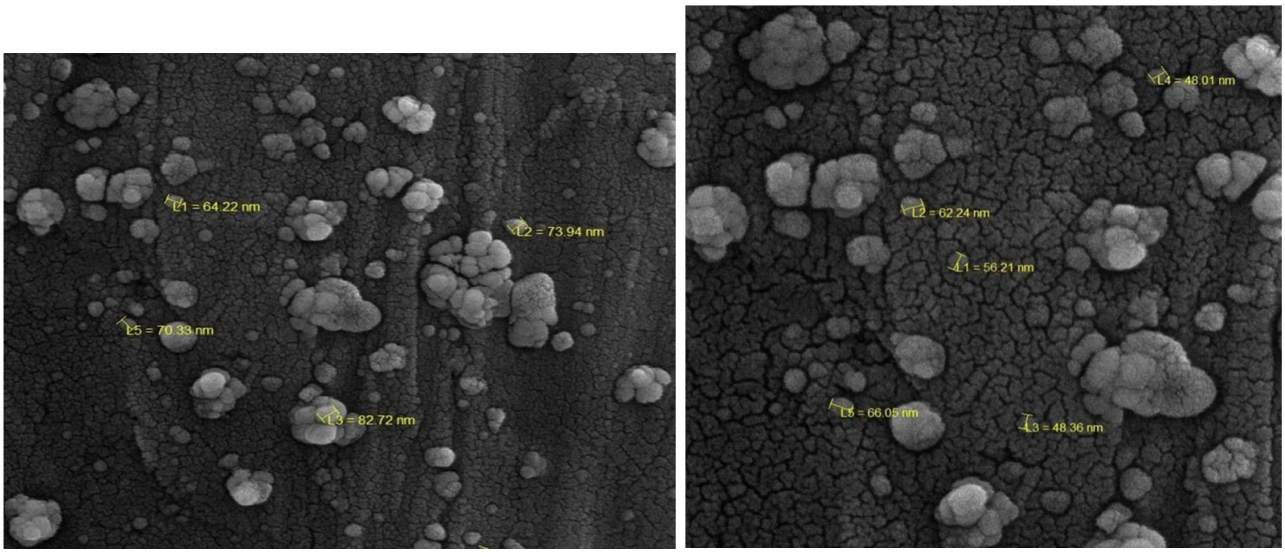


Fig. 3. Zeta Potential of AgNPs-MS.



A **B**

Fig. 4. FEG microscope images of AgNPs-MS, which show size in the range of 64.22 nm – 82.72 nm (A)and 48.01 nm – 66.05 nm (B).

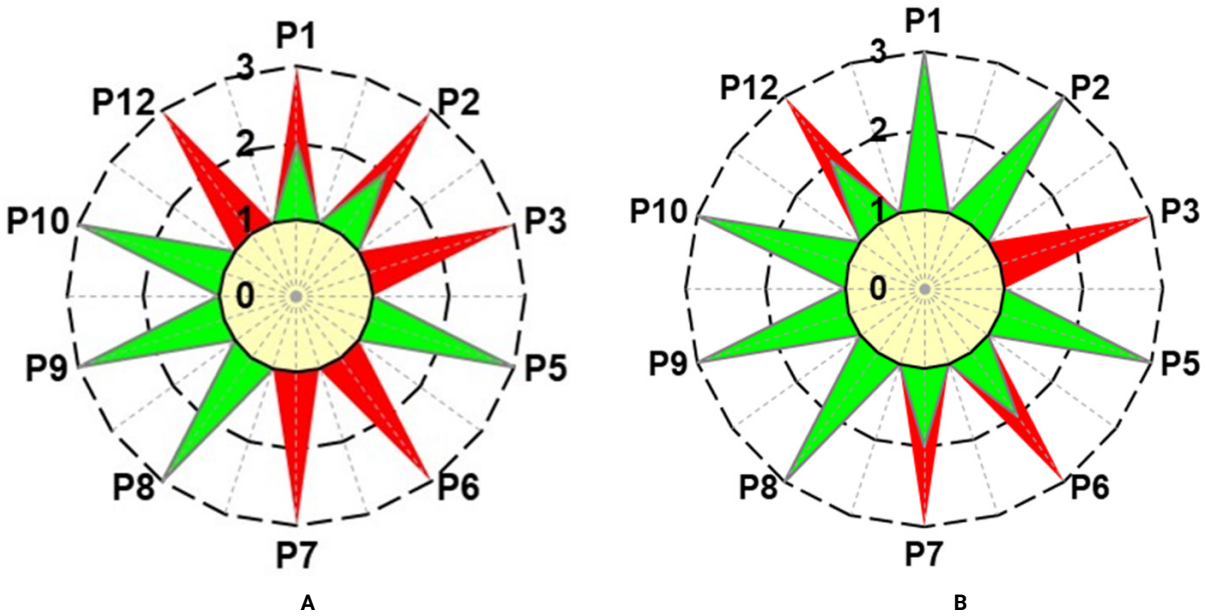


Fig. 5. Comparison between the Green stars of the conventional procedure (**A**), with ISF*=50% and the phytosynthesis (**B**), with ISF = 75% for the synthesis of AgNPs. (*ISF = index of star fill). Green color indicates agreement with the Green Chemistry principles; red color indicates disagreement with the Green Chemistry principles.

3. Material and Methods

3.1. Plant extract

The ethanolic extract from *M. salicifolia* roots (Mart ex. Reissek) Biral was previously prepared according to Magalhães et al. [3].

3.2. In vitro cytotoxicity test of ethanolic extract from *M. salicifolia* roots

Fibroblasts (3T3-Swiss albino) were obtained from the Rio de Janeiro Cell Bank. Cells were routinely cultured in Dulbecco's modified Eagle's Medium, DMEM (Sigma). This medium was supplemented with 10% fetal bovine serum (FBS, Life Technologies) containing 0.1% of antibiotic mix: 10,000 units penicillin and 10 mg streptomycin per mL (Sigma). Sodium bicarbonate (2mg/mL) was also added. Cultures were kept at 37 °C in a humidified 5% CO_2 incubator. Cells were in an exponential growth phase at the time of testing. These cells were subcultured every 2-3 days. The viability of the cells exceeded 95% as determined by the trypan blue (0.4% trypan blue solution, Sigma) dye exclusion method.

The cytotoxicity was carried out using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) assay to investigate changes in mitochondrial/non-mitochondrial dehydrogenase activity [26]. In brief, fibroblasts were seeded on 96-well plates (2 x 10^4 cells/mL) and cultured in DMEM containing 10% FBS at 37 °C and 5% CO_2 for 24 h. Ethanolic extract from *M. salicifolia* roots at various concentrations (3, 10, 30, 100, and 300 µg/mL) was then added. An exposure period of 48 h was chosen to determine the in vitro cytotoxicity. After incubation, the supernatant was removed, and MTT solution (0.5 mg/mL) was also added to each well 60min prior to the end of the experiment. Water-insoluble dark blue formazan crystals formed in viable cells were solubilized in DMSO, and the absorbance was measured at 550nm using a microplate reader (Biotek µQuant). Cell survival was determined by comparing the absorbance values obtained for treated and untreated cells.

3.3. Synthesis of AgNPs-MS

The ethanolic extract from *M. salicifolia* roots (MS) was diluted from 3.0 mg of the plant extract in 8.0 mL of ethanol and 12 mL of distilled water. A sample containing 1.0 mL of this solution was collected. After that, 9.0 mL aqueous solution of silver nitrate ($AgNO_3$) 1.0 mM was added. In order to carry out the synthesis of the nanoparticles, the mixture was heated at 65 °C under stirring. The change of the solution color was monitored to determine the exact point of formation of the AgNPs-MS, which was indicated by a colloidal yellow color.

3.4. Characterization of the AgNPs-MS

The reduction in silver ions to form nanoparticles was monitored observing the UV-Vis spectra of AgNPs-MS. The spectra were obtained by a UV-VIS NIR Varian Cary 50 spectrophotometer in the range 200-800 nm. The size and morphology of the AgNPs-MS were characterized with a particle size analyzer, Zetasizer Nano ZS90 and a field emission electron gun (FEG) microscope by a MYRA 3 LMH (Tescan).

3.5. Green analysis of the synthesis

The greenness analysis of the procedure adopted was verified according to the adequacy to the 12 principles of the Green Chemistry. A comparison of the methodology used in this work with a procedure used by Antunes et al. (2013) was made, using the Green Star.

4. Conclusions

The ethanolic extract from *M. salicifolia* roots (Mart ex. Reissek) Biral was able to reduce silver ions in AgNPs. The nanomaterials obtained are spherical, stable and exhibit varied shapes. This eco-friendly method has the advantage of practicality in the execution, in addition to minimizing the formation of toxic waste. Further studies will now be carried out to evaluate the biological activities of the particles. The

construction of the Green Star was useful to emphasize the greenness of the phytosynthesis of AgNPs and allowed the verification of possibilities to make the reactions more sustainable.

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Author Contributions

The work described in this paper was done during the scientific initiation work of S. Grzygorczyk, who performed the experiments and took part in the discussion. J. F. P. Paula and C. G. Magalhães are supervisors. Both suggest the subject of the work, revised the methodologies, discussed the results, and check on the final version of the manuscript. J. T. Ezpinoza revised the characterization of the silver nanoparticles. P. M. D. Boscardin supervised the evaluation of cytotoxicity of the extract of *M. salicifolia*; D.S. Nunes contributed in funding acquisition and supervised the prepation of the extract. M. C. M. Sandri analyzed the greenness of the conventional synthesis of AgNPs and the biosynthesis of AgNPs using that extract.

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