Green synthesis of silver nanoparticles using *Ilex paraguariensis* extracts: antimicrobial activity and acetylcholinesterase modulation in rat brain tissue

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**Abstract**

The silver nanoparticles (AgNPs) green synthesis has been investigated by selecting naturally occurred reduction and stabilizing/capping agents. In this work, *Ilex paraguariensis* aqueous extract in the one-step biosynthesis of AgNPs is reported. The synthesis was carried out at room temperature under different pH (5.0, 6.8 and 8.5) and extract concentrations (2.5, 5.0 and 7.5% v v⁻¹) resulting in average diameters ranging from 34 to 144 nm and polydispersity index (PDI) up to 0.51. The morphological characterization was performed by Transmission Electron Microscopy, associated with the UV–Vis Spectroscopy, showing that at higher pH the shape of AgNPs was more spherical rather than ellipsoidal. X-ray diffraction (XRD) pattern of AgNPs exhibited 2θ values corresponding to silver nanocrystals. Colloidal AgNPs solution synthesized with pH 8.5 and extract concentration of 2.5% v v⁻¹ remained stable for 10 months of storage at ambient temperature. Fourier Transform Infrared Spectroscopy suggested that the polyphenols were responsible for the AgNPs formation. AgNPs presented bacteriostatic and bactericidal activity. AgNPs presented in vitro rat brain acetylcholinesterase (AChE) activity, with higher inhibition potential at 200 µL. So, it is concluded that AgNPs were synthesized with success, the pH and extract concentration influences in AgNPs size and morphology, consequently in its antimicrobial and AChE activity.

**Introduction**

Nanostructured materials have been highlighted for their wide applicability (1). Metal nanoparticles can be mainly obtained by two methods: top-down and bottom-up (2), but both methods generate toxic residues and are potentially harmful to the environment and human health. Therefore, researchers have been investigated alternatives with less impact on the environment and health, with green synthesis being a promising alternative (3).
Among various metal nanoparticles, silver nanoparticles (AgNPs) have attracted attention because of their proven antimicrobial activity (4) with several applications as pharmacology, human and veterinary medicine, food industry, water purification and other uses (5).

Literature reports several research works employing green synthesis to obtain silver AgNPs. Green synthesis of AgNPs has been investigated since it does not involve any hazardous material (6–8). AgNPs have been synthesized by green methods, for many researchers with different plant extracts (6,9–15), that uses an aqueous vegetable extract that acts as a reducing agent for silver ions in metallic silver, and as a stabilizing/capping agent, providing stability to suspended nanoparticles.

The main advantage of using plant extracts for the AgNPs synthesis is because they have a broad variety of metabolites (e.g. enzymes, proteins, amino acids, vitamins, polysaccharides and organic acids) that can be used in the reduction of silver ions and capping the nanoparticles synthesized, so in this way, green process proves to be environmentally eco-friendly, low cost and biocompatible (16).

Erva mate or yerba mate (Ilex paraguariensis), a typical plant from the south of South America, is rich in antioxidants, such as purine alkaloids, flavonoids, tannins, chlorogenic acid and its derivatives, and numerous triterpenic saponins (17), which are capable of acting as reducing agents for AgNPs synthesis (18,19). Due to the rich composition of the Ilex paraguariensis, it becomes important to understand how the extract may act in the synthesis of AgNPs.

AgNPs presented a wide array of biological activity such as antimicrobial and antioxidant (20). However, there is a concern about their safety (21), about the mechanisms involved in vivo distribution (22,23) and their clearance from the body (24). Also, the action of AgNPs in brain biochemistry is worth investigating, particularly the cholinesterase equilibrium. Whereas the possible action of the AgNPs in the cholinergic system is not fully understood and literature on this topic is still scarce.

The present study describes the use of an aqueous extract of Ilex paraguariensis as reducing/capping agent for the AgNPs synthesis. Different conditions of pH and aqueous extract concentrations were evaluated and the influence on nanoparticles size and morphology were reported. Also, antibacterial activity was tested against three bacteria responsible for foodborne diseases as well as the in vitro activity of the nanoparticles in the activity of rat brain acetylcholinesterase (AChE) enzyme.

### Experimental

#### Materials

The commercial erva mate (Ilex paraguariensis) was purchased at the local market. Silver nitrate (Proquímios, Brazil), hydrochloric acid and sodium hydroxide (Vetec, Brazil) were used in the AgNPs synthesis. Potassium bromide (KBr, spectroscopic standard, Sigma Aldrich) was used to obtain the infrared spectra. Muller-Hinton broth (Biomark, Brazil) and nutrient agar (Biomark, Brazil) was used for microbial analysis. Acetylthiocholine, 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB), sodium chloride, potassium phosphate monobasic and dibasic were obtained from Sigma Aldrich and used in the AChE assays, and rat brain (donated from Federal University of Santa Maria, Brazil).

#### Microorganisms

All standard microorganisms were provided by the Adolfo Lutz Institute, Sao Paulo, Brazil that included Gram-positive bacteria (Bacillus cereus (ATCC 14579) and Staphylococcus aureus (ATCC 6538)) and Gram-negative bacteria (Pseudomonas aeruginosa (ATCC 9027)). Bacteria were grown in Mueller–Hinton broth at 37°C for 24 h and maintained on slopes of nutrient agar.

#### Preparation of the Ilex paraguariensis extract

The erva mate aqueous extract was prepared mixing 10 g of Ilex paraguariensis in 100 mL of boiling ultrapure water and maintained for 20 min, then the extract was cooled, and vacuum filtered.

#### Silver nanoparticles synthesis

The AgNPs were synthesized according to Mariselvam et al. (6) methodology with modifications. Three pH levels (5, 6.8 and 7.5) and three extract concentration levels (2.5, 5 and 7.5% v v⁻¹) were included in the experimental conditions as presented in Table 1.

#### Table 1. Experimental conditions to AgNPs green synthesis using erva mate extract and average particles size (Dz) and polydispersity index (PDI) result of AgNPs synthesized with Ilex paraguariensis aqueous extract.

| Experiment | pH  | Extract concentration (%v v⁻¹) | Dz (nm) | PDI (-) |
|------------|-----|--------------------------------|---------|---------|
| E1         | 5.0 | 2.5                            | 70      | 0.36    |
| E2         | 8.5 | 2.5                            | 34      | 0.51    |
| E3         | 5.0 | 7.5                            | 144     | 0.34    |
| E4         | 8.5 | 7.5                            | 69      | 0.29    |
| E5         | 6.8 | 5.0                            | 56      | 0.39    |
Briefly, the erva mate extract was added to ultrapure water according to the experimental condition described in Table 1 yielding 50 mL of total volume. Then, the pH was adjusted, using HCl 0.1 M or NaOH 0.1 M. After that, the silver nitrate solution (50 mL, 0.02 M) was dripped under gentle stirring during 2 min at room temperature (25°C). The AgNPs colloidal dispersions were stored protected from light.

**AgNPs characterization**

The reduction of silver ions and the formation of the nanoparticles was verified by UV–Visible spectroscopy (UV–Vis, Ocean Optics, USB 650UV) from 200 to 800 nm, in the next day after the green synthesis for all samples. The final colloidal dispersions obtained at the AgNPs synthesis were diluted with ultrapure water and the measurements were done with a quartz cuvette (1 cm optical path length).

Nanoparticles morphology and chemical composition were accessed by transmission electron microscopy (TEM, JEOL JEM-2100 at 80 kV) and by energy dispersive X-ray spectrometry (EDS, Carl Zeiss, EVO MA 15), respectively. XRD patterns were obtained with an X-ray diffractometer (Shimadzu XRD-7000) equipped with a Cu Ka radiation source (λ = 0.154 nm) to evaluate the crystalline characteristics of AgNPs from 5° to 70° (2θ). The average crystallite size of the silver particles was calculated using Scherrer equation (Equation (1)), where \( d \) is average crystallite size, \( \beta \) is the width of the peak at half maximum intensity of a specific phase in radians, \( \lambda \) is the wavelength of incident rays and \( \theta \) is the center angle of the peak in radian.

\[
d = \frac{0.9 \lambda}{\beta \cos \theta}
\]

Average diameter (Dz) and PDI of the AgNPs were measured by dynamic light scattering (DLS, Malvern – Zetasizer Nano S). Nanoparticles stability during storage was also evaluated after 10 months of the synthesis (stored at ambient temperature) by DLS. The characterization of functional groups on the surface of AgNPs was carried out using infrared spectroscopy Fourier transform spectroscopy (FTIR, Affinity-1, Shimadzu), from 4000 to 400 cm\(^{-1}\) using 32 accumulations.

**AgNPs antibacterial activity**

According to Andrews (25), minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial substance that inhibits the visible growth of a given microorganism after overnight incubation, while minimum bactericidal concentration (MBC) is the lowest concentration of an antimicrobial substance that prevents the growth of a given microorganism after subculture on to antibiotic-free media. Therefore, MIC and MBC were determined against the main bacteria responsible for foodborne diseases, namely Bacillus cereus (ATCC 14579), Staphylococcus aureus (ATCC 6538) and Pseudomonas aeruginosa (ATCC 9027). MIC and MBC were performed according to Clinical and Laboratory Standards Institute (CLSI) methodology, document M07-A9 (26).

**Acetylcholinesterase activity assay**

Rat brain was homogenized (1:10 w/v\(^{-1}\)) in 50 mM potassium phosphate buffer pH 7.2 and centrifuged (S1) by 10000 rpm for 10 min at 4°C. AChE activity was determined by the method of Ellman et al. (27), modified as described by Pereira et al. (28). The final mixture assay contained 1.04 mM dithiobisnitrobenzoate (DTNB), 24 mM potassium phosphate buffer pH 7.2, 75 µL of enzymatic material (S1) and different AgNPs volumes (100, 150 and 200 µL) or 100 µL of AgNO3. The medium was pre-incubated for 2 min at 25°C and the reaction was started with the addition of 0.83 mM acetylthiocholine iodide (AcSCh). The rate of hydrolysis of AcSCh was measured via the release of the thiol compound that reacts with DTNB producing the color-forming compound 5-thio-2-nitrobenzoic acid (TNB). The specific activity was expressed as µmol AcSCh hydrolyzed h\(^{-1}\).

**Statistical analysis**

AChE graphic data were expressed as mean ± SEM. Statistical evaluation of the AChE results was carried out using the analysis of variance followed by post-hoc Tukey test whereas control groups. \( p \) values lower than 0.05 (\( p < 0.05 \)) were considered statistically significant. Data were analyzed using GraphPad Prism 5.0 software (San Diego, CA, USA).

**Results and discussion**

**Nanoparticles characterization**

The AgNPs formation by the AgNO\(_3\) reduction using Ilex paraguariensis aqueous extract was evidenced by color change of the reactional mixture from transparent to brown as can be observed in Figure 1.
Particles formation was also evidenced by UV–Vis spectroscopy as presented in Figure 2. According to Zhang and Noguez (29), metals such as silver exhibit strong absorption in the visible region of the spectrum in nanoscale. The origin of this absorption is attributed to collective conduction band electron oscillation in response to the electrical field of the electromagnetic radiation of light. This optical absorption is termed surface plasmon resonance, partly because net charges are displaced transiently on the particle surface during electron oscillation. While the color and surface plasmon absorption band are somewhat dependent on the size of spherical nanostructures, they strongly depend on the shape of the nanostructures. For a metal, such as silver, almost any color or absorption in any part of the visible spectrum can be produced by controlling the shape or structure of the nanomaterial.

The color change indicates the AgNPs formation, which can be confirmed by UV–Vis absorption spectra in the 300–500 nm range (30). The aqueous extract of *Ilex paraguariensis* had translucent, yellow color and changed to light brown or dark brown after 5 min of reaction in all experiments in this work (Figure 1).

UV–Vis peaks (Figure 2) ranged from 433 to 448 nm for all samples which is in accordance with previous studies. Loo et al. (31) showed that using *Camellia sinensis* extract as a reducing agent the resulting absorption peak was obtained at 436 nm, while Vilchis-Nestor et al. (32) also using *C. sinensis* observed the peak at 430 nm. When *Ocimum sanctum* aqueous extract was used as a reducing agent the authors observed the peak at 450 nm (33). And when aqueous extract of *C. forskohlii* was used as a reducing agent it was noticed peaks at 440 nm indicating the nanoparticles formation (10).

TEM images presented in Figure 3 showed that spherical nanoparticles were only obtained under the experimental condition E2 (pH 8.5 and extract concentration 2.5% v v⁻¹). Sathishkumar et al. (34) also observed that higher pH values led spherical nanoparticles rather than ellipsoidal. Shankar et al. (35) produced AgNPs with geranium (*Pelargonium graveolens*) leaf extracts and observed at the UV–Vis spectra a shoulder on absorption at 370 nm and associated this behavior to a transverse plasmon vibration in the AgNPs whereas the higher peak, located at 440 nm, to excitation of longitudinal plasmon vibrations. Authors concluded that wavelengths separated by 70 nm indicate the AgNPs in solution are assembled into open, quasilinear superstructures which were also observed here.

Particles size (Figure 4(a)) showed bimodal distribution with large PDI with the minority of AgNPs presenting diameters lower than 40 nm (Table 1). It may also be observed (comparing E1 and E2) that the size distribution shifted to lower diameters and the lowest population peak increased when pH was increased from 5.0 to 8.5. Cumberland and Lead (36) observed that DLS size distribution peaks heights increased slightly at lower pH values for AgNPs synthesized with sodium citrate. On the other hand, Sathishkumar et al. (34) described the same behavior and stated that pH played a major role in size control of the particle synthesized with *Cinnamon zeylanicum* bark extract. They suggested that the aggregation AgNPs forming larger nanoparticles is favored over nucleation to form new nanoparticles at lower pH. At higher pH values, the large number of functional groups from the extract available in the aqueous phase for silver binding facilitates a higher number of silver ions to bind and subsequently form a large number of new nanoparticles.
nanoparticles with smaller diameters. Also, it is known that protonation of the low-molecular compounds present in the aqueous extract significantly changes their hydrophobicity probably resulting in a more ineffective availability for silver ions to bind and form new nanoparticles (37).

Average diameter, shown in Table 1, increased from 70 to 144 nm with increasing extract concentration (comparing E1 and E3) while PDI was not affected. Since the availability of more reducing biomolecules for the reduction of silver ions there is a higher production of AgNPs (34), resulting in further growth of the nanoparticles by Ostwald ripening and shifting the sizes distribution toward higher diameters (38). All AgNPs colloidal dispersions were monitored for 10 months, in order to observe their storage stability. It was detected the presence of precipitates in all samples except of E2 sample which remained stable during this period with Dz and PDI equal to 34 nm and 0.46, respectively. This result can be observed in Figure 4(b).

Analyzing the extract FTIR spectrum in Figure 5, it is possible to verify the presence of characteristics bands in the range of 1240–1452 cm\(^{-1}\) related to caffeine, in 1642 cm\(^{-1}\) related to the flexural vibration of (\(-OH\)) hydroxyl, between 1408 and 1603 cm\(^{-1}\) of deprotonated carboxylic groups (COO\(^{-}\)) representing changes within of chlorogenic acid (12), 1588 cm\(^{-1}\) that can be attributed to the aromatic (C=C) vibrations (e.g. chlorogenic acid), 1259 and 1153 cm\(^{-1}\) that confirm the presence of (C–O) groups of polyols, as well as in 1042 and 1072 cm\(^{-1}\) bands attributed to the vibrational stretch of (C–O–C) and (C–O) of the phenolic compounds. According to Bracesco et al. (17), the main organic components of the Ilex paraguariensis (non-roasted) extract are 42% chlorogenic acid, 21% gallocatechin, 11% gallic acid, 8% caffeine and other minor compounds which are in accordance with the functional groups found in the extract. Since the phenolic compounds are free radical and metal scavengers (39,40), it can be noted that the disappearance or alteration of these bands in AgNPs spectrum (E2) could be due to the action of these groups as reducing agents of Ag\(^+\) ions.

In AgNPs spectrum (Figure 5), the appearance of a band in 1722 cm\(^{-1}\) indicates the presence of carbonyl groups of aldehydes or ketones, which may have been formed due to the oxidation of the (OH) groups during Ag\(^+\) ions reduction. Also, in AgNPs spectrum, the bands located in 1515 and 1358 cm\(^{-1}\) can be attributed to symmetric or asymmetric vibrations of (NO\(_3\))\(^-\), residual from the precursor silver nitrate. And, the appearance of a shoulder in 1652 cm\(^{-1}\) evidences the presence of quinones which are probably only present after the oxidation of phenolic compounds after the reduction of
Figure 4. Size distribution results of AgNPs synthesized with *Ilex paraguariensis* aqueous extract (E1: pH 5.0 and extract concentration (Ec) 2.5% v v\(^{-1}\); E2: pH 8.5 and Ec 2.5% v v\(^{-1}\); E3: pH 5.0 and Ec 7.5% v v\(^{-1}\); E4: pH 8.5 and Ec 7.5% v v\(^{-1}\); E5 pH 6.8 and Ec 5.0% v v\(^{-1}\)). (a) Size distributions after preparation. (b) Size distributions after 10 months of storage (E2: pH 8.5 and Ec 2.5% v v\(^{-1}\)).

Figure 5. Results of FTIR spectra of *Ilex paraguariensis* extract, silver nitrate and AgNPs (E2: pH 8.5 and Ec 2.5% v v\(^{-1}\)).
Ag\(^+\) ions to the formation of AgNPs (41). Furthermore, when comparing the spectra of the extract and AgNPs (E2), it can be observed that there were several band shifts and intensity–shape changes in the fingerprint zone (800–1500 cm\(^{-1}\)), that can be attributed to the reduction of Ag\(^+\) to Ag\(^0\) due to the activity of phenolic compounds (12).

The characteristic peaks found in the X-ray analyses shown in Figure 6, were at 38°, 44°, 64.4° and their corresponding (hkl) values were (111), (200), (220) which stands for a face-centered cubic (fcc) structure and confirms the crystalline nature of the AgNPs (10,33,42–44). The slight shift in the peaks position could be an indicative of the presence of strain in the crystalline structure which is characteristic of nanocrystallites (10).

EDS results presented in Table 2 showed the presence of silver in all samples and other components such as oxygen and carbon mostly from the extracts. Differences from the percentages may be attributed by the fact that EDS scans only a small area of each sample to determine its composition being susceptible to local variations in the concentration of each component.

### Nanoparticles activity

Results of MIC and MBC are presented in Table 3. After analyzing the results, it was observed that MIC was higher in the case of *P. aeruginosa* for all experimental conditions when compared to the other microorganisms. This is probably due to its Gram-negative structure which presents a thicker peptide membrane layer.

### Table 2. Average crystallite size (d) from DRX analysis and EDS results of the AgNPs synthesized with *Ilex paraguariensis* aqueous extract (E1: pH 5.0 and extract concentration (Ec) 2.5% v \(\text{v}^{-1}\); E2: pH 8.5 and Ec 2.5% v \(\text{v}^{-1}\); E3: pH 5.0 and Ec 7.5% v \(\text{v}^{-1}\); E4: pH 8.5 and Ec 7.5% v \(\text{v}^{-1}\); E5 pH 6.8 and Ec 5.0% v \(\text{v}^{-1}\)).

| Experiment | C (%) | O (%) | Na (%) | Mg (%) | Al (%) | Cl (%) | Ca (%) | Ag (%) | Total (%) | \(d\) (nm) |
|------------|-------|-------|--------|--------|--------|--------|--------|--------|-----------|-----------|
| E1         | 7.41  | 48.84 | 10.92  | 2.88   | 0.68   | 2.07   | 6.91   | 20.29  | 100.00    | 30.46     |
| E2         | –     | 19.54 | 1.18   | 0.42   | 0.80   | –      | 0.88   | 77.98  | 100.00    | 25.26     |
| E3         | 22.76 | 45.46 | 8.93   | 2.68   | 0.70   | 1.45   | 5.25   | 12.79  | 100.00    | 12.62     |
| E4         | 12.72 | 10.44 | 1.25   | 0.72   | –      | 0.62   | 4.06   | 69.60  | 100.00    | 41.4      |
| E5         | –     | 35.03 | 8.05   | 2.52   | –      | 4.54   | 8.01   | 41.84  | 100.00    | 12.09     |

\(d\): average crystallite size from DRX analysis and calculated from Equation (1).
only formulation that presented bactericidal properties against all the tested bacteria was experiment E1 which was expected since AgNPs synthesized using natural extracts present low bactericidal activity as reported elsewhere (16). Formulation E4 was the one that presented the poorest results of antimicrobial activity and this result can be associated with AgNPs morphology. Hong et al. (45) evaluated the effect of AgNPs with specific shapes, spheres, cubes and nanowires on antimicrobial activity and observed that morphology may influence the contact between the nanoparticles and bacterial cells. Agnihotri et al. (46) evaluated the MIC and MBC for AgNPs synthesized using a co-reduction approach (NaBH4 and trisodium citrate) against E. coli and S. aureus. Authors obtained similar results with the present work for MIC (20 μg mL⁻¹) of AgNPs with average size of 5 nm. AgNPs (average size of 15 nm) produced with Zingiber officinalis root extract as a reducing and capping agent showed a MIC against Staphylococcus spp of 20 μg mL⁻¹ (47). Ghaedi et al. (48) obtained MIC values of 193.3 μg mL⁻¹ against P. aeruginosa and 386.62 μg mL⁻¹ against E. coli for AgNPs (29 nm) synthesized with Rosmarinus officinalis leaf extract.

Table 3. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of the synthesized AgNPs.

| Experiment | E1 | E2 | E3 | E4 | E5 |
|------------|----|----|----|----|----|
| MIC (μg mL⁻¹) | P. aeruginosa | 17 | 17 | 17 | – | 17 |
| S. aureus | 7.1 | 7.1 | 5.7 | 5.7 | 7.1 |
| B. cereus | 14.2 | 1.6 | 4.3 | – | 4.3 |
| MBC (μg mL⁻¹) | P. aeruginosa | 17 | 17 | + | + | + |
| S. aureus | 17 | + | 17 | + | + |
| B. cereus | 14.2 | 17 | + | + | + |

*No inhibition; (+) Presence of bacterial growth.

Conclusion
The ability of Ilex paraguariensis extract to be used as stabilizing/capping agent in the green synthesis of AgNPs was demonstrated. AgNPs obtained in a more alkaline
medium were smaller and presented greater stability than those synthesized in an acid medium. Therefore, it can ensure that XRD pattern revealed that AgNPs formed by *Ilex paraguariensis* aqueous extract are crystalline in nature. AgNPs presented bacteriostatic and bactericidal activity for *P. aeruginosa*, *B. cereus* and *S. aureus*. However, *P. aeruginosa* required a higher concentration of AgNP for bacterial inhibition due to its Gram-negative characteristic. Differences in bacterial activity were accounted by the different morphology of the obtained nanoparticles. AgNPs were able to modulate the activity of AChE enzyme exhibiting inhibition effect after demonstrated AChE inhibition activity at 150 (E2) and 200 µL (E4) nanoparticles solutions depending on the extract concentration. Therefore, the green synthesis approach using *Ilex paraguariensis* extract is an efficient alternative to conventional physical and chemical methods to obtain AgNPs synthesis that could be scaled up for large-scale production.

Disclosure statement

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