Mechanism of formation, characterization and cytotoxicity of green synthesized zinc oxide nanoparticles obtained from *Ilex paraguariensis* leaves extract

Marina Bandeira[a,c], *André L. Possan*[a], *Sandra S. Pavin*[b], *Camila S. Raota*[a], *Mario C. Vebber*[a], *Marcelo Giovanela*[a], *Mariana Roesch-Ely*[b], *Declan M. Devine*[c] *and Janaina da S. Crespo*[a,*]

[a]Universidade de Caxias do Sul, Rua Francisco Getúlio Vargas, 1130, Caxias do Sul, 95070-560, RS, Brazil

[b]Instituto de Biotecnologia, Universidade de Caxias do Sul, Rua Francisco Getúlio Vargas, 1130, Caxias do Sul, 95070-560, RS, Brazil

[c]Materials Research Institute, Athlone Institute of Technology, Athlone, Co. Westmeath, Ireland

Phone/Fax: + 55 54 32182159 / *Corresponding author. E-mail : jscrespo@ucs.br

ABSTRACT

Zinc oxide nanoparticles (ZnONPs) was produced using *Ilex paraguariensis* (*mate*) leaves extract using a green synthesis process. The influence of ethanolic and aqueous plant extract and zinc source on the green synthesis of ZnONPs was studied. Cyclic voltammetry and Fourier transform infrared spectroscopy (FTIR) were used for the assessment of the mechanism route of ZnONPs while the formation of this nanomaterial was confirmed by X-ray diffraction (XRD) analysis. The morphology and size of the ZnONPs synthesized were evaluated using field emission scanning microscopy (FESEM) and transmission electron microscopy (TEM) analysis. In general, all different synthesized ZnONPs exhibited a hexagonal crystalline structure whereas the size and shape varied depending on the extract and zinc salt used. Nonetheless, the most uniform and smallest ZnONPs were obtained using ethanolic extract and zinc nitrate, showing spherical morphology and a diameter of about 18 nm. With the use of cyclic voltammetry and FTIR analysis, it was concluded that the formation of ZnONPs through green synthesis occurred due to complexation of Zn(II) ions by antioxidants compounds present in the *Ilex paraguariensis* extract and further thermal degradation of the complexes. Concerning the cytotoxicity assays, the L929 cell viability decreases in a dose-dependent manner for all samples tested. In general, nanoparticles with reduced size and uniform shape exhibited no cytotoxic effects up to a concentration of 10 $\mu g\ mL^{-1}$. However, higher ZnONPs concentrations caused a decrease in cell viability. This was possibly due an autophagic induction process triggered by the internalization of the nanomaterial. Finally, this work provides a better understanding of the mechanism route to obtain ZnONPs *via* green method and their potential to be used as a biomedical material.

Keywords: *Ilex paraguariensis*; green synthesis; zinc oxide nanoparticles; mechanism route; cytotoxicity

1 INTRODUCTION

Nanotechnology is a growing field of science and researchers believe that the use of nanomaterials will increase considerably in a variety of subjects [1–4]. Within this context, zinc oxide nanoparticles (ZnONPs) have been widely investigated due to their unique physical, chemical and optical properties that enables them to be utilized in a variety of technological applications, including novel biomedical devices and therapies [5–8]. More specifically, ZnONPs enhanced the efficacy of bone regeneration when incorporated to polymer scaffolds by inducing early mineralization and preventing infections due its antibacterial activity [9]. This nanomaterial has also been investigated for its wound healing performance. The incorporation of ZnONPs on polymeric beads, for example, led to faster wound closure and avoided the formation of microbial biofilms on the skin [10]. This characteristics corroborates with the findings of related studies [11–15].

The toxicity of ZnONPs for use in human health applications is still a subject of discussion, as nanomaterials have different toxicity mechanism routes, such as reactive oxygen species (ROS) production, cell internalization and metal ions release, among others, which can affect human cells in different manners compared to the bulk material form [16]. Recent developments on this topic have been made, and the cytotoxicity of oxide nanoparticles are now known to be not only dose-dependent, but also associated with properties like size, morphology and surface characteristics [17–19].

Several methods are applied to obtain these nanomaterials through chemical, physical or biological synthesis [20,21]. However, the biological approach has gained much attention as it is more environmentally friendly than conventional methods, considering that it substitutes hazardous solvents by plant extracts, and can improve the

properties of the nanomaterials. Commonly known as green synthesis, many plant extracts have been used to synthesize metal and metal oxides nanoparticles with enhanced properties [22–30]. Although the mechanism of formation of nanoparticles through green synthesis is not completely understood, it is believed that antioxidant compounds present in the plant, such as flavonoids and polyphenols, reduce or form coordinated complexes with the targeted metal [31–33].

Many plant extracts have been applied to the green synthesis of ZnONPs [26,32,34–37]. Gunalan et al. [38], for example, used *Aloe vera* extract and obtained ZnONPs with enhanced antibacterial effect in comparison to ZnONPs synthesized by chemical method. Shahriyari Rad et al. [39] also confirmed the antimicrobial activity of ZnONPs obtained using *Menta pulegium L* leaves. Nava et al. [31] obtained ZnONPs using fruit peel extract for photocatalytic degradation of dyes with enhanced degradation rate than ZnONPs commercially available.

In this work, we report the green synthesis of ZnONPs and its optimization using *Ilex paraguariensis* leaves extract. This plant, also called *mate*, is commonly found in the south of Brazil, Uruguay and Argentina, where it is used to prepare a traditional tea [40]. Anxiolytic, neuroprotective and anti-inflammatory properties have been described in m*ate* tea and have been associated to a variety of antioxidant compounds found in the plant, which includes chlorogenic acid, caffeic acid and caffeine [41–43].

Although many tea extracts have been used for synthesizing ZnONPs [44–46], to the best of our knowledge, the use of *Ilex paraguariensis* leaves to obtain ZnONPs has not been reported in the literature. In addition, even though much research has designed a probable mechanism route for the green synthesis, none have used cyclic voltammetry to evaluate and confirm it. In this sense, this work contributes to a better understanding of the green synthesis of ZnONPs in a molecular level, which is essential to develop and

implement a sustainable large-scale production of nanoparticles. Moreover, a carefully study on the cytotoxicity and cell internalization of ZnONPs was performed to evaluate their application in the biomedical field.

2 MATERIALS

The materials used to perform the green synthesis include zinc nitrate hexahydrate (Sigma-Aldrich, São Paulo, Brazil), zinc acetate dihydrate (Sigma-Aldrich, São Paulo, Brazil) and ethanol 99% (Didática Artigos para Laboratório Ltda, São Paulo, Brazil). The *Ilex paraguariensis* leaves were collected in the municipality of Caxias do Sul (Rio Grande do Sul, Brazil) and identified at the Natural Science Museum of University of Caxias do Sul (registration number 46.334).

For the characterization of the leaves extract Folin Ciaocalteau 2N (Didática Artigos para Laboratório Ltda, São Paulo, Brazil), calcium carbonate P.A. (Didática Artigos para Laboratório Ltda, São Paulo, Brazil), trishydrochloride (Sigma-Aldrich, São Paulo, Brazil) and 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma-Aldrich, São Paulo, Brazil) were used.

Regarding the cellular tests the following materials were used: mouse fibroblast L929 cells, cell medium Dulbecco´s Modified Eagle's Medium (DMEM, Sigma-Aldrich, São Paulo, Brazil), fetal bovine serum (FBS, Sigma-Aldrich, São Paulo, Brazil), penicillin-streptomycin (P/S, Gibco, Thermo Fisher Scientific, Waltham, MA, United States of America), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, São Paulo, Brazil), dimethyl sulfoxide (DMSO, Sigma-Aldrich, São Paulo, Brazil), Giemsa staining (Sigma-Aldrich, São Paulo, Brazil), rapamycin (Sigma-Aldrich, São Paulo, Brazil), methanol (Sigma-Aldrich, São Paulo, Brazil), monoclonal anti-LC3A/B antibody (autophagy kit, Cell Signaling, Danvers, MA, United

States of America), anti-mouse fluorescein isothiocyanate (FITC, Sigma-Aldrich, San Luis, MO, United States of America) and bovine serum albumin (BSA, Sigma-Aldrich, São Paulo, Brazil).

3 METHODS

3.1 Ilex paraguariensis leaves extract preparation

The *mate* leaves were cleaned carefully with the following sequence of rinses to remove impurity particles: tap water, 50% (v/v) ethanol/water solution and distilled water, respectively. Subsequently, the leaves were submitted to 60 °C for 1.5 h and were ground in a mill. The ground leaves were stored in an amber flask under refrigeration (4 °C) for further use.

To obtain the plant extract, 100 g L^{-1} of the ground *mate* leaves were heated and stirred with two different solvents: distilled water and a 50% (v/v) ethanol/water solution. Three ranges of temperature (25 °C, 50 °C and 100 °C) and time of extraction (10 min, 20 min and 30 min) were evaluated, respectively. The extract was then filtered and centrifuged for 20 min at 5,000 rpm to remove particles of the medium.

3.2 Green synthesis of zinc oxide nanoparticles

Zinc salt (nitrate and acetate) in a Zn(II) concentration of 21.8 g L^{-1} was added to 50 mL of the *mate* extract previously prepared. The solution was stirred for 1 h at room temperature and then heated for 4 h at 70 °C. After that, the mixture was submitted to a hot air oven at 140 °C for 1 h, being finally calcinated at 400 °C for 1 h. The resulting powder was collected for further characterization.

To evaluate the effect of the *mate* extract on the synthesis of ZnONPs, two samples containing only zinc nitrate hexahydrate (named Nitrate) and zinc acetate dihydrate

(named Acetate) were calcinated under the same conditions used in the green synthesis (at 400 °C for 1 h). **Table 1** describes the synthesized ZnONPs samples and their conditions. Only the sample prepared with zinc nitrate and ethanolic extract (Nit_EtOHa) produced ZnONPs without calcination.

Table 1 Description of the synthesized ZnONPs samples

Sample	Zinc source	Plant extract solvent	Heat treatment
Nitrate	Zinc nitrate	–	C
Nit_H_2O		H_2O	O + C
Nit_EtOH		50% (v/v) EtOH	O + C
NIt_EtOHa		50% (v/v) EtOH	O
Acetate	Zinc acetate	–	C
Act_H_2O		H_2O	O + C
Act_EtOH		50% (v/v) EtOH	O + C

C = calcination; O = hot air oven

3.3 Characterization of the mate leaves extract

The antioxidant activity (AA) of the *mate* leaves extracts was evaluated through an oxidation-reduction reaction of the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), following the method described by Yamaguchi et al. with slight modifications [47]. To perform the analysis, 100 µL of the plant extract was added to 400 µL of trishydrochloride 0.1 mol L^{-1} (pH = 7.0) and 500 µL of a DPPH 0.5 mmol L^{-1} solution. The control sample was prepared with the substitution of the plant extract by its solvent (water or 50 % (v/v) ethanol/water solution). After 20 min of incubation in the absence of light, the absorbance was measured in a UV-Vis spectrophotometer (Thermo Scientific, model Evolution 60, Waltham, MA, United States of America) at 517 nm.

The total polyphenolic content was determined following the procedure of Singleton and Rossi with adaptations [48]. For this, 150 µL of the plant extract was added

171 to 750 µL of Folin-Ciocalteau 10% (v/v) reagent and 600 µL of calcium carbonate 7.5%
172 (w/v). The mixture was slightly hand-shaken and incubated at 55 °C for 5 min. Then, at
173 room temperature, the absorbance was measured in a UV-Vis spectrophotometer
174 (Thermo Scientific, model Evolution 60, Waltham, MA, United States of America) at
175 760 nm. Total phenolic content was determined by comparison with a standard curve of
176 gallic acid (5, 25, 50, 75 and 100 µg mL^{-1}) and expressed in terms of micrograms of gallic
177 acid per milliliter of plant extract (µg EAG mL^{-1}).

178 The content of six main *mate* constituents was evaluated by high performance
179 liquid chromatography (HPLC, HP model 1100, Waldbronn, Germany), equipped with a
180 UV detector operating at 272 nm, a column Lichrospher RP18 5 µm and a quaternary
181 pumping system. Prior to the analysis, *mate* extracts were filtered through a Millipore
182 membrane (0.45 µm). The analysis in reverse phase mode was constituted of solvent A
183 (Milli-Q water and 1% (v/v) acetic acid) and solvent B (methanol). The mobile phase was
184 pumped with 75% of solvent A at 0.4 mL min^{-1} flux and the column was kept at 40 °C
185 [49]. For the quantitative evaluation, a standard calibration curve was obtained using
186 standard solutions with a known concentration of the pure compounds (chlorogenic acid,
187 caffeic acid, theophylline, theobromine, rutin and caffeine).

188

189 *3.4 Evaluation of the green synthesis mechanism*

190 *3.4.1 Cyclic voltammetry*

191 ZnONPs synthesis mechanism was investigated through cyclic voltammetry using
192 a conventional three electrode cell at room temperature and a potentiostat (Ivium
193 Technologies, model Compactstat.h, Eindhoven, The Netherlands), operating with a
194 potential range from –1.7 V to 0.0 V, scanning speed of 50 mV s^{-1} and 1.0 mV step. Cyclic
195 voltammograms were obtained with a 3.0 mm glassy carbon working electrode, a

platinum wire as a counter electrode, an Ag/AgCl reference electrode and KCl 0.1 mol L^{-1} as the electrolyte solution. The working electrode was polished with alumina powder and cleaned with acetone in a 5 min ultrasonic bath prior to each analysis. The peak current of the reduction or oxidation reaction is given according to the Rendles-Sevcik equation (**Equation 1**):

$$i_p = 2{,}69\text{x}10^5 n^{3/2} A D_0^{1/2} C_o v^{1/2} \quad (1)$$

where,

i_p = peak current (A);

n = number of electrons involved in the redox reaction;

A = working electrode area (cm^2);

D_o = diffusional coefficient ($cm^2\ s^{-1}$);

C_o = analyte concentration (mol cm^{-3});

v = scanning speed ($V\ s^{-1}$).

Pure solutions of caffeine were reacted with the zinc precursors for comparison with the green synthesis. It was used a concentration of 0.05 mol L^{-1} of zinc salt and concentration of the antioxidants were determined considering a 1:4 molar ratio of Zn(II) to caffeine molecules.

3.4.2 Fourier transform infrared spectroscopy (FTIR)

The functional groups present in the samples were determined by FTIR spectroscopy (Thermo Scientific, model Nicolet iS10, Waltham, MA, United States of America) using KBr pellets. The spectra were registered in a range of 4,000 to 400 cm^{-1},

with a resolution of 1 cm^{-1}, using 32 scans per sample. Samples were vacuum dried at 60 °C for 24 h, prior to the analysis to remove any possible adsorbed water.

3.5 Characterization of ZnONPs

3.5.1 X-Ray diffraction (XRD)

XRD patterns of the ZnONPs synthesized were obtained using a powder diffractometer (Shimadzu, model XRD-6000, Tokyo, Japan) equipped with a Cu anode ($K\alpha_1$ =1.54056 Å) in a range of 2θ from 20° to 80°, with an acquisition time of 5 s. The crystallite size was estimated by using the Scherrer equation [50] and the software *Match!*.

3.5.2 Field emission scanning electron microscopy (FESEM) coupled with energy dispersive spectroscopy (EDS)

ZnONPs morphology was examined with a field emission scanning electron microscope (TESCAN, model MIRA 3, Brno, Czech Republic) using an energy beam of 15 kV and a SE detector. All the samples were placed in an aluminum stub and were covered with a thin layer of gold by sputtering method (Denton Vacuum, Desk V, Moorestown, NJ, United States of America) for 30 s at 0.13 mbar vacuum to perform the analysis. EDS analysis was performed coupled with FESEM using a silicon drift detector (SDD).

3.5.3 Transmission electron microscopy (TEM)

The particle size of synthesized ZnONPs was investigated by the analysis of images obtained in a transmission electron microscope (Jeol, model JEM 1200 EX II,

Peabody, MA, United States of America) using a working voltage of 80 kV. The mean size of the particles was determined with Image J software [51].

3.5.4 Ultraviolet and visible spectroscopy (UV-Vis)

The surface plasma resonance (SPR) and the energy band gap (E_{bg}) of the green synthesized ZnONPs were evaluated by UV-Vis spectroscopy in a wavelength range of 250-600 nm. ZnONPs were suspended in distilled water (200 mg L^{-1}) and exposed to an ultrasonic bath for 30 min. The band gap energy was obtained using the **Equation (2):**

$$E_{bg} = hc / \lambda_{max} \quad (1)$$

where,

h = Plank constant (6.63×10^{-34} J s);

c = velocity of light (2.99×10^{8} m s^{-1});

λ_{max} = maximum absorption wavelength.

3.5.5 Cell viability assay

Cellular cytotoxicity was evaluated by means of the MTT method. The L929 strain of mouse fibroblasts were used according to the standard method described by ISO 10993-5[52]. Cells were cultured in DMEM, supplemented with 10% (v/v) of FBS and 1% (v/v) of P/S. The cultures were maintained in a humid atmosphere at 37 °C with 5% (v/v) CO_2. The study was performed as cells reached 70–80% confluence. Briefly, cells were seeded into the 96-well plates at a density of 5.0×10^{4} cells mL^{-1}. After 24 h, cells were treated with different concentrations (1–80 μg mL^{-1}) of the compounds and incubated for 24 h. The compounds were solubilized in DMSO. Negative controls were treated with the same amounts of 0.5% (v/v) DMSO solution.

The medium was removed and 1.0 mg mL^{-1} MTT dye in serum-free medium was added to the wells. Plates were incubated at 37 °C for 2 h in a humidified controlled atmosphere with 5% (v/v) CO_2. Subsequently, the MTT solution was removed and the obtained formazan violet product was dissolved in 100 µL DMSO for 30 min. Absorbance was measured using a microplate reader (Molecular Devices, model Spectra Max 190, San Jose, CA, United States of America) at 570 nm. All readings were compared with the negative control, which represented 100% viability. The IC50 (concentration in µg mL^{-1} that inhibits cell growth by 50%) was also calculated. Each experiment was performed in triplicate and independently repeated at least four times.

3.5.6 Cellular morphological analysis

Mouse fibroblast cells (L929) were seeded into 24-well plates in similar conditions to the cell viability analysis. After 24 h of treatment with different concentrations (1–25 µg mL^{-1}) of ZnONPs, culture medium was removed and cells were prepared to FESEM and optical microscopy analyses. To perform the optical microscopy, culture medium was removed after treatment and cells were dyed with Giemsa staining 1% (v/v). To perform FESEM analysis, after removing culture medium, cells were fixed with glutaraldehyde 2% (v/v) and dehydrated with ethanol.

3.5.7 Indirect immunofluorescence analysis

L929 cells were seeded into 24-well plates containing coverslips. After 24 h incubation, cells were treated with a range of different concentrations (5–15 µg mL^{-1}) of ZnONPs and incubated for 24 h. Negative controls were treated with the same amount of growth medium. Positive control received rapamycin (100 nmol L^{-1}). Cells were fixed with methanol at –20 °C, blocked with 2% BSA for 1 h, and incubated with the

monoclonal anti-LC3A/B antibody (1:100 v/v) for 1 h, followed by incubation with secondary anti-mouse fluorescein isothiocyanate (FITC) (1:150 v/v) for 1 h. The slides were mounted with coverslip and analyzed with a fluorescence light microscope (Olympus, model BX43, Miami, FL, United States of America).

4 RESULTS AND DISCUSSION

4.1 Characterization of the mate leaves extract

The extraction of active compounds of the *mate* leaves was optimized in terms of time and temperature, considering the antioxidant activity and total phenolic content of the plant extract. The results are presented in the Supplementary Material and the extraction time of 20 min and temperature of 50 °C was chosen to be optimal to prepare the *mate* extract for the green synthesis of ZnONPs.

Thus, the concentration of six main compounds present in *mate* leaves extract obtained with the optimized conditions was quantified by HPLC in both aqueous and ethanolic extracts (**Table 2**).

Table 2 Concentration of the main *mate* compounds determined by HPLC

Substance	**Concentration (μg mL^{-1})**	
	H_2O	**50% (v/v) EtOH**
Chlorogenic acid	42.86 ± 0.68	47.96 ± 1.27
Caffeic acid	7.18 ± 0.02	7.53 ± 0.09
Caffeine	541.39 ± 0.72	654.22 ± 0.99
Theophylline	13.22 ± 0.35	28.26 ± 0.64
Theobromine	111.00 ± 0.92	125.44 ± 0.72
Rutin	n.d.	n.d.

n.d. = not detected

In comparison to the aqueous extract, the 50% (v/v) ethanol extract exhibited a higher concentration of the substances evaluated, mainly for caffeine, theophylline and theobromine. This result agrees with the work of Vieira et al. [53], where ethanol increased the extraction of antioxidants substances compared to the extraction performed exclusively with water. The substance rutin, commonly found in *mate* leaves was not detected in either of the extracts with the applied methodology. Conversely to our findings, Berté et al. (2011) reported that chlorogenic acid was the main antioxidant present in the *mate* leaves, followed by caffeine and theobromine. Anesini et al.[54] have also found a major state for chlorogenic acid in commercial *mate* leaves; however, the rutin content showed to be higher than caffeine. Nonetheless, the concentration of antioxidants is significantly affected by climate, leaves age, soil characteristics, extract conditions, among others [55,56].

4.2 Evaluation of the green synthesis mechanism

4.2.1 Cyclic voltammetry

Cyclic voltammetry was performed in the samples Nit_EtOH, Nit_H_2O, Act_EtOH and Act_H_2O (before heat treatment) to evaluate the mechanism of formation of ZnONPs. This analysis observes change in the current when reduction or oxidation reactions occur in a system due to the transference of electrons. The cyclic voltammetry test was performed from the lowest to the highest potential, i.e., from the reduction to oxidation potential. **Equation (3)** illustrates the redox reactions for zinc:

$$Zn^0 \rightarrow Zn^{2+} + 2e^- \text{ (oxidation)} \quad (3)$$

$$Zn^{2+} - 2e^- \rightarrow Zn^0 \text{ (reduction)}$$

Several studies support the idea that antioxidant compounds present in plants have the capacity of forming complexes with or reducing metals ions [22,31,57]. Thus, if the Zn(II) ions were reduced by the *mate* extract, only an oxidation peak will be visualized. Conversely, if the Zn(II) ions are free in solution, both reduction and oxidation peaks will be observed, and if the Zn(II) ions were complexed by the plant extract, no peak will be observed as no redox reaction will take place.

Figure 1 shows the voltammograms of the 50% (v/v) ethanol and aqueous extracts with a concentration of 50 mg L^{-1} in KCl 0.05 mol L^{-1}. Oxidation and reduction peaks were not observed for the *mate* extract solutions in comparison to the electrolyte voltammogram, which indicates that no electrochemical reaction occurred.

Figure 2a shows a typical zinc nitrate voltammogram where the peaks between –0.7 V and –1.0 V are related to the oxidation-reduction reaction of Zn(II), and the peaks between –1.0 and –1.25 V are related to the redox reaction of the nitrate ion, corroborating with the literature [58,59]. The cyclic voltammograms of Nit_EtOH and Nit_H_2O (**Figure 2b)** suggest that Zn(II) were complexed by both *mate* extracts as no peak was observed in comparison to the electrolyte (KCl 0.1 mol L^{-1}).

Different from the samples with zinc nitrate, the samples Act_EtOH and Act_H_2O (**Figures 3a** and **3b**) displayed peaks of reduction and oxidation, but with a lower current than the pure zinc acetate solution. The peak current is linearly proportional to the concentration of the targeted substance (Zn(II) in this case) [60]. **Figure 3c** indicates the linearity relation between the peak current and zinc acetate concentration of the pure solution.

From this relation, the concentration of zinc acetate that was reduced in the Act_EtOH and Act_H_2O samples was determined as 0.041 mol L^{-1} and 0.008 mol L^{-1}, respectively, being the rest complexed by the antioxidants of the *mate* extract. Thus,

364 considering the concentration of zinc that did not undergo the redox reaction, only 71%
365 of zinc available in the sample Act_EtOH and 95% in sample Act_H_2O was possibly
366 complexed by the *mate* extract.

367 In general, Zn(II) can form coordinated complexes with nitrogen, sulfur, halogens
368 and oxygen ligands, resulting in complexes with tetrahedral (sp^3 hybridization) or
369 octahedral (sp^3d^2 hybridization) geometry, being the octahedral the less
370 thermodynamically stable form [61]. Antioxidant compounds available in plant extracts
371 usually have hydroxyl groups (from phenolic compounds) in their composition or atoms
372 with lone electrons pairs, such as nitrogen and oxygen (methylxanthines) that will form
373 metal complexes by originating an anion or by the element with a lone electron pair.

374 Methylxanthines are found in high concentrations in *mate* extracts [49,53,62].
375 Thus, the mechanism of formation of the green synthesis of ZnONPs here developed is
376 based on the molecular structure of this group and is shown in more detail in **Figure 4**.
377 According to the HPLC results, caffeine is the methylxanthines with the highest
378 concentration in the plant extract used in this study. This substance has one dissociation
379 site, where a lone pair of electrons in the nitrogen reacts to form the respective conjugated
380 acid of caffeine as indicated in **Figure 4** [63]. Besides, several studies report the formation
381 of complexes of Zn(II) and caffeine and Zn(II) and theophylline, supporting the
382 mechanism proposed here [63–67].

383 The formation of this complexes was further evaluated using pure solutions of
384 caffeine. **Figure 5** shows that the cyclic voltammograms of caffeine with zinc nitrate and
385 zinc acetate have the same pattern of the solutions prepared with the *mate* extracts,
386 confirming the involvement of this methylxanthine compound on the green synthesis of
387 ZnONPs. In addition, the analysis with zinc acetate (**Figure 5b**) also indicates that zinc

388 salt did not react completely with the antioxidant, corroborating with the results observed
389 in **Figure 3b**.

390 According to the calibration curve of zinc acetate (**Figure 3c**), approximately 60%
391 of the Zn(II) in the Act_EtOH_caffeine and 70% in the Act_H_2O_caffeine were
392 complexed by caffeine, which is lower than the concentration obtained in the analysis of
393 the green synthesis. However, the *mate* extract has different types of active compounds,
394 which might have improved the formation of complexes when using the plant extract
395 instead of the pure caffeine solution.

396

397 *4.2.2 Fourier transform infrared spectroscopy*

398 **Figure 6** shows the FTIR spectra of Act_EtOH_caffeine in comparison to pure
399 caffeine. Caffeine illustrates a broad and weak peak at 3460 cm^{-1} related to the OH
400 stretching and two weak peaks at 3115 cm^{-1} and 2950 cm^{-1} associated with the vibration
401 of CH and CH_3 bonds. The peaks at 1700 cm^{-1} and 1655 cm^{-1} represent the symmetric
402 and asymmetric vibration of C=O of ketones, respectively, and a strong peak at
403 1550 cm^{-1} indicates the presence of C=C and C=N groups. At 1240 cm^{-1}, a strong and
404 narrow peak indicates the vibration of CH bend and CN stretching. The peaks at
405 973 cm^{-1} and 645 cm^{-1} are associated with ring deformation while the weak peak at
406 860 cm^{-1} is related to the wagging of CH bonds [67–69].

407 Regarding to the Act_EtOH_caffeine, a broad and strong peak at 3440 cm^{-1} and a
408 weak peak at 2358 cm^{-1} related to the vibration of OH indicate the presence of water in
409 the complex structure. Also, the appearance of both peaks associated with the vibration
410 of –C=O groups (1700 cm^{-1} and 1650 cm^{-1}) indicates that complexation of Zn(II) did not
411 occur via the oxygen atom.

412

An analysis of specific peak areas was developed to evaluate the difference associated with the transmittance and presence of functional groups between the two substances (**Table 3).** The vibration of CH_3 (1405 cm^{-1}) was taken as a reference as the methyl groups should not be affected by the Zn(II) complexation, once they are radicals attached to the main structure of the caffeine molecule. Peak areas were taken considering the same wavenumber interval.

A higher Act_EtOH_caffeine ratio for the OH stretching and H_2O vibration peaks in relation to the caffeine confirms the presence of water bonded to the complex. A decrease in the peaks related to the CN group (1240 cm^{-1} and 1023 cm^{-1}) was observed for the Act_EtOH_caffeine, indicating the complexation of the nitrogen with the Zn(II) due to a lower vibration of CN bonds. A lower ratio of ring deformation (973 cm^{-1} and 645 cm^{-1}) for the Act_EtOH_caffeine sample indicates a reduced vibration of the ring due to the formation of complexes [64]. All these findings corroborate with the complexation of the Zn (II) ions and the mechanism route for the green synthesis here proposed.

Table 3 Ratio of peak areas of FTIR spectra of caffeine and Act_EtOH_caffeine samples

Wavenumber	**Peak area**		**Ratio**	
(cm^{-1})	**Caffeine**	**Act_EtOH_caffeine**	**Caffeine**	**Act_EtOH_caffeine**
1405 (-CH_3)	2551.54	2819.11	–	–
3460 (-OH)	2666.18	13493.57	1.04	4.79
2358 (H_2O)	90.62	277.94	0.04	0.10
1240 (-CH/-CN)	1434.47	689.36	0.56	0.24
1023 (-CN)	434.98	283.82	0.17	0.10
973 (rd)	379.94	125.44	0.15	0.04
645 (rd)	448.36	263.46	0.18	0.09

rd = ring deformation

The mechanism of formation was designed specifically for ZnONPs synthesized with *Ilex paraguariensis* extract. However, the green synthesis of other metal oxides obtained from plant extract reported in the literature probably follows the same mechanistic route [46,70–72]. Likewise, the *Ilex paraguariensis* extract used in this study has great potential to be applied in the production of metal and metal oxides nanoparticles other the zinc oxide.

4.3 Characterization of zinc oxide nanoparticles

4.3.1 X-Ray diffraction (XRD)

XRD patterns of all samples synthesized is shown in **Figure 7**. All the peaks observed correspond to *zincite* (JCPDS nº 36-1451), a hexagonal form of ZnO, confirming the formation of this oxide [73]. Patterns of other crystalline structures were not observed, suggesting that the nanomaterial synthesized have high purity. The average crystallite size was estimated to be 19 nm for Nit_EtOH, 37 nm for Nit_H_2O, 24 nm for Nit_EtOHa, 61 nm for Act_H_2O, 59 nm for Act_EtOH and 31 nm and 65 nm for Control A and Control N, respectively. The lattice parameters were determined according to the Bragg equation and considering all the identified planes of the hexagonal structure. The parameters were found to be a = 3.2518 Å and c = 5.2162 Å for samples obtained from zinc nitrate, and a = 3.2564 Å and c = 5.2166 Å for samples obtained from zinc acetate, corroborating with the literature [74,75].

4.3.2 Field emission scanning electron microscopy (FESEM) coupled with energy dispersive spectroscopy (EDS)

The surface morphology of all samples was studied by FESEM micrographs (**Figures 8** and **9**). The samples produced from calcination of zinc nitrate and zinc acetate

(Nitrate and Acetate) showed triangular and rod particle morphology, respectively (**Figures 8a–b** and **Figures 9a–b)**. Samples synthesized with the green route and zinc nitrate showed spherical shape (**Figures 8c–h)**, while those synthesized with the green route and zinc acetate have irregular shapes and sizes (**Figures 9c–f**).

Samples obtained with *mate* extracts and zinc nitrate have the smallest particle size (**Figures 8c–h**). In addition, the synthesis with 50% (v/v) ethanol extract (**Figures 8e–h)** resulted in more uniform shape and size particles than the sample obtained with an aqueous extract (**Figures 8c–d**). According to the HPLC analysis, the ethanolic extract has a higher concentration of antioxidants compounds, favoring the complexation of Zn(II), which results in more uniform particles. The influence of calcination can be evaluated from **Figures 8e–h**, where the sample Nit_EtOH (calcinated) presented more homogeneous particles than the sample Nit_EtOHa (without calcination).

Different morphologies were observed for ZnONPs obtained by the biological method using a variety of plant extracts. For instance, Nava et al. [31] obtained ZnONPs from fruit peel extract and zinc nitrate with polyhedral shape. Bala et al. [35] used *Hibiscus subdariffa* and zinc acetate to produce spherical ZnONPs that formed agglomerates with a cauliflower shape. On the contrary, Anbuvannan et al. [5] produced ZnONPs from *Phyllantus niruri* leaves extract and zinc nitrate with irregular morphology. However, the methodologies applied in each synthesis were different and a comparison among the morphologies obtained is not plausible.

EDS results of all green synthesized ZnONPs are shown in **Figure 10.** In theory, ZnO has 80.3% of Zn content. Thus, the ZnONPs synthesized from *Ilex paraguariensis* extract have shown high purity with approximately 70-80% of Zn content, with exception of the sample Act_H_2O that presented a lower Zn content (67%). The presence of C, O

and a small peak related to K can be originated from the plant compounds. Similar results were observed for ZnONPs obtained from other plant extracts [32,35].

4.3.3 Transmission electron microscopy (TEM)

Particle size was determined by TEM micrographs (**Figure 11**), except for the sample Nitrate because its size is greater than the size limit of analysis of a transmission electronic microscope (~ 1 μm) and was determined by FESEM. **Table 4** shows the mean particle size for each sample. Samples green synthesized with zinc nitrate are formed by a single crystal, as the particle sizes are similar to the crystallite size determined by XRD, while the other samples are formed by several crystallites.

Table 4 Mean size of the ZnONPs samples

Sample	Mean size (nm)
Nitrate	53 ± 8 μm*
Nit_H_2O	33 ± 9
Nit_EtOH	18 ± 5
Nit_EtOHa	29 ± 10
Acetate	389 ± 249
Act_H_2O	176 ± 50
Act_EtOH	116 ± 103

*Result is given in micrometers (μm)

Samples synthesized by green synthesis showed reduced particle size in comparison to the samples obtained from the calcination of zinc salts (Nitrate and Acetate), confirming the influence of *mate* extract to obtain ZnONPs in nanometric size. It was observed that using zinc nitrate (**Figures 11a–c**) in the green synthesis route resulted in particle sizes in the approximate range of 18 to 33 nm, which exhibited a reduction of particle size in comparison to using zinc acetate (**Figures 11d–e**). This

difference in the particle size possibly occurred due to the number of nucleation sites that were formed during the synthesis.

The nucleation process is the first stage of a crystal formation in which the atoms organize in a crystal structure thermodynamically stable forming the crystal nucleus from where the particle grows [76]. In the case of Nitrate and Acetate, Zn(II) ions were very close, resulting in fewer nucleation sites and consequently larger particle sizes. Conversely, the samples Nit_EtOH, Nit_EtOHa and Nit_H_2O were complexed by the antioxidants present in the *mate* extracts that kept the Zn(II) separated, originating more nucleation sites and smaller particle sizes.

The samples Act_EtOH and Act_H_2O showed increased particle size and more irregular shape than the samples Nit_EtOH, Nit_EtOHa and Nit_H_2O. This may have occurred because the Zn(II) of the samples containing zinc acetate were not completely complexed by the *mate* compounds as shown in the cyclic voltammetry analysis, resulting in fewer nucleation sites.

ZnONPs obtained by plant extracts and zinc nitrate have exhibited particle sizes of approximately 10 to 50 nm [5,31,35]. Nonetheless, when using zinc acetate as the metal precursor, research studies have presented a contrast on the ZnONPs size that varied from *quantum dots* of only 3–9 nm to clusters of 500 nm [57,77]. However, the extract composition and synthesis method differ among these works and cannot be directly compared.

4.3.4 Ultraviolet and visible spectroscopy (UV-Vis)

Figure 12 shows the UV-Vis spectra of all green synthesized ZnONPs and their respective E_{bg}. All samples showed absorption peaks between 360 and 380 nm and E_{bg} around 3.3 eV characteristic of ZnONPs [78–80]. The difference between the samples

526 corroborates with the findings of Goh et al., where a decrease in particle size led to a shift
527 of the maximum absorbance to lower wavelengths and E_{bg} [81].
528
529 *4.3.5 Cell viability assay*
530 The cytotoxicity of ZnONPs were evaluated with L929 cells for the samples
531 Nit_ETOH, Nit_EtOHa e Nit_H_2O, as they showed reduced size and regular shape, and
532 for the sample Act_EtOH to compare the cytotoxicity of both zinc sources. Cell viability
533 for ZnONPs concentrations varying from 1–25 μg mL^{-1} is shown in **Figure 13**.
534 Cell viability decreased with the increase of ZnONPs concentration in all samples.
535 However, the samples Nit_EtOH exhibited low cytotoxicity for concentrations varying
536 from 1 to 10 μg mL^{-1} as cell viability was not affected in this range of concentration.
537 Syama et al. [82] also observed no cytotoxicity up to a concentration of 10 μg mL^{-1}, when
538 treating L929 cells with spherical ZnONPs ranging from 20 nm to 40 nm. Different from
539 our findings, the authors found that cell viability decreased only around 20% for samples
540 treated with 20 μg mL^{-1} while cells treated with Nit_EtOH resulted in a decrease of around
541 50%. Paino et al. [83], by other hand, reported low levels of necrosis and apoptosis, when
542 treating the same type of cells with ZnONPs in concentrations lower than 10 μg mL^{-1}.
543 The LC50 (concentration to reduce cell viability by 50%) was determined as
544 18 μg mL^{-1} for Nit_EtOH and Act_EtOH, 9 μg mL^{-1} for Nit_H_2O and 7 μg mL^{-1} for
545 Nit_EtOHa. These results suggest that the samples Nit_EtOH and Act_EtOH have lower
546 cytotoxicity in comparison to the other samples. Hence, a higher concentration of
547 nanoparticle was required to reduce cell viability by 50%. On the contrary, the sample
548 Nit_EtOHa was the most cytotoxic. This sample was not calcinated which may have
549 resulted in the presence of Zn(II) species, $–NO_2^–$ and $–COO^–$ that may affect cell viability.

Therefore, as the sample Nit_EtOH exhibited the least cytotoxic, further analysis were performed to evaluate the difference of cell viability through the different concentrations.

4.3.6 Morphological analysis of L929 cells

Morphological tests in L929 cells were developed with the samples (Nit_EtOH, Act_EtOH, Nit_EtOHa and Nit_H_2O) to evaluate the morphological parameters with Giemsa staining through optical microscopy (**Figures 14a**, **d**, **g**, **j** and **m**) and FESEM (**Figures 14b**, **e**, **h**, **k** and **n**). Micrographs of both methods show similarities with elongated cytoplasm in control samples (**Figures 14a**, **b** and **c**) and cytoplasmic retraction and chromatin condensation points for the sample treated with Nit_EtOH (**Figures 14d**, **e** and **f**). Other treatment conditions with Act_EtOH, Nit_EtOHa and Nit_H_2O showed cellular morphology with intermediary nuclear and cytoplasmatic condensation when compared to the control and Nit_EtOH samples. These results may have been related to the reduced size and regular morphology of Nit_EtOH nanoparticles which can facilitate the internalization of this nanomaterial into the cell, altering its biochemical and morphological structure [84]. It is relevant to observe that samples exposed to Nit_EtOH exhibited a cell survival *plateau* up to a maximum concentration of about 10 μg mL^{-1}, displaying cytotoxicity in higher concentrations.

These findings corroborate with similar reports related to the cytotoxicity of ZnONPs nanostructures. Syama et al. [82] observed a change from elongated to round shape after treating L929 cells with ZnONPs at 50 μg mL^{-1}, whereas Satimano et al. [85] report morphological changes in human A549 cells from polygonal to granules appearance, after treatment with different concentrations of ZnONPs.

575 *4.3.7 Indirect immunofluorescence analysis*

576 Indirect immunofluorescence tests were developed in L929 cells to evaluate if the
577 morphological alterations in treated cells observed using Giemsa staining and FESEM
578 were related to autophagosome effects due to internalization and deposition of ZnONPs
579 on this vesicle. Cells were exposed to the ZnONPs (Nit_EtOH, Act_EtOH, Nit_EtOHa
580 e Nit_H_2O) for 24h with concentrations related to the LC50. An increase of LC3A/B
581 expression in cells exposed to Nit_EtOH (**Figure 14f**) was verified in comparison to
582 negative control and to cells exposed to other samples (**Figures 14c**, **i**, **l** and **o**).
583 Expression parameters of LC3A/B are evaluated with topographic qualitative reading in
584 the cell cytoplasm, more specifically in phagocytic regions, evidencing more intense
585 green staining for the sample treated with Nit_EtOH (LC50 concentration). All conditions
586 exhibited blue staining on the nuclear region to locate cellular structures.

587 To evaluate the LC3A/B protein expression in L929 cells exclusively to the
588 sample Nit_EtOH, a second investigation was developed with a crescent concentration
589 gradient treatment (5–15 μg mL^{-1}) with an exposure period of 24 h. This analysis showed
590 an evident increase on the LC3A/B expression after exposure to Nit_EtOH in
591 concentrations higher than 10 μg mL^{-1} (**Figures 15e** and **f**). For comparison, cells were
592 exposed to a positive control (rapamycin 100 nmol L^{-1}) that exhibited LC3A/B expression
593 compatible with cells treated with high concentrations of Nit_EtOH (**Figures 15e**, **f** and
594 **g**). Topographical distribution using indirect immunofluorescence analysis revealed that
595 cells treated with different ZnONPs progressively increased LC3A/B expression,
596 confirming the affinity of this protein in the autophagosome of cellular structures.

597

598

599

5 CONCLUSIONS

ZnONPs were synthesized using *Ilex paraguariensis* leaves extract. A mechanism route for the green synthesis of this nanomaterial was designed in which the antioxidants of the plant extract form coordinated complexes with Zn(II) and nanoscale particles of ZnO were formed via thermal degradation of these complexes. Samples obtained with zinc nitrate exhibited reduced particle size and a more regular shape than those synthesized with zinc acetate. These findings might be related to the fact that Zn(II) present in the synthesis with zinc acetate did not react with the plant compounds in total, as observed in the cyclic voltammograms.

Ethanol increased the extraction of active compounds from the plant resulting in more uniform particles. Therefore, the sample obtained with zinc nitrate and 50% (v/v) ethanol extract showed the smallest and more uniform size, which can be effective for photocatalytic and biological applications.

Cell viability decreased with increasing of ZnONPs concentration to all samples. However, sample Nit_EtOH showed no cytotoxic effects for concentrations up to a limit of 10 µg mL^{-1}. Morphological analysis showed a degree of nucleus and cytoplasmic condensation for cells treated with all ZnONPs samples. Small and uniform ZnONPs were able to internalize cells and form deposits in phagosomes, which can initiate metabolic processes of cell death in elevated concentrations. Finally, the ZnONPs synthesized here can be applied in biocompatible materials in low concentrations and in the development of novel therapies within the cells.

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FIGURE CAPTIONS

Fig. 1 Voltammograms of *mate* extracts

Fig. 2 Cyclic voltammograms: **(a)** zinc nitrate 0.1 mol L^{-1}; **(b)** Nit_EtOH and Nit_H_2O containing 0.1 mol L^{-1} of zinc nitrate in 100 g L^{-1} mate extract

Fig. 3 Cyclic voltammogram: **(a)** zinc acetate 0.1 mol L^{-1}; **(b)** Act_EtOH and Act_H_2O containing 0.1 mol L^{-1} of zinc acetate in 100 g L^{-1} *mate* extract; **(c)** linear relation between peak current and zinc acetate concentration

Fig. 4 ZnONPs mechanism of formation *via* green synthesis

Fig. 5 Cyclic voltammetry of caffeine solutions reacted with **(a)** zinc nitrate and **(b)** zinc acetate

Fig. 6 FTIR spectra: **(a)** Act_EtOH_caffeine; **(b)** caffeine

Fig. 7 XRD pattern of ZnONPs synthesized with **(a)** zinc nitrate and **(b)** zinc acetate

Fig. 8 FESEM micrographs of ZnO synthesized from zinc nitrate: **(a-b)** Nitrate; **(c-d)** Nit_H_2O; **(e-f)** Nit_EtOH; **(g-h)** Nit_EtOHa

976 **Fig. 9** FESEM micrographs of ZnO synthesized from zinc acetate: **(a-b)** Acetate;
977 **(c-d)** Act_H_2O; **(e-f)** Act_EtOH
978 **Fig. 10** EDS spectra of green synthesized ZnONPs
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980 **Fig. 11** TEM micrographs of **(a)** Nit_H_2O; **(b)** Nit_EtOH; **(c)** Nit_EtOHa; **(d)** Act_H_2O;
981 **(e)** Act_EtOH; **(f)** Acetate
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983 **Fig.12** UV- vis spectra of the green synthesized ZnONPs
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985 **Fig. 13** L929 cell viability after treatment with ZnONPs. Samples with different letters
986 represent significant difference ($p < 0.05$)
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988 **Fig. 14** LC3A/B expression in cells L929 after 24 h of exposure to ZnONPs (Nit_EtOH,
989 Act_EtOH, Nit_EtOHa and Nit_H_2O) with concentrations related to each IC50.
990 **a**, **b**, **c** – control samples; **d**, **e**, **f** – samples exposed to Nit_EtOH; **g**, **h**, **i** – samples exposed
991 to Act_EtOH; **j**, **k**, **l** – samples exposed to Nit_EtOHa; **m**, **n**, **o** – samples exposed to
992 Nit_H_2O.
993
994 **Fig. 15** LC3A/B expression in L929 cells after 24 h of exposure to Nit_EtOH in a crescent
995 gradient of concentration (**b** – 5 μg mL^{-1}; **c** – 7,5 μg mL^{-1}; **d** – 10 μg mL^{-1};
996 **e** – 12,5 μg mL^{-1}; **f** – 15 μg mL^{-1}). **a** – negative control, without treatment; **g** – positive
997 control, treated with autophagy inducer rapamycin (100 nmol L^{-1}), with a cellular marker
998 pattern of LC3A/B similar to elevated concentrations of Nit_EtOH (**e**, **f**)
999
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TABLE CAPTIONS

Table 1 Description of the synthesized ZnONPs samples

Table 2 Concentration of the main *mate* compounds determined by HPLC

Table 3 Ratio of peak areas of FTIR spectra of caffeine and Act_EtOH_caffeine samples

Table 4 Mean size of the ZnONPs samples

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