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Cytotoxicity evaluation in cancer cells and bactericidal activity of nanoencapsulated extracts from *Myracrodruon urundeuva*

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Abstract

Plant-derived products have been present in human pharmacopeia for thousands of years, several botanical-based products are unstable and low aqueous solubility compromising their efficiency. The strategy of applying nanotechnology to botanical extracts may potentiate their biological action, promote the sustained release, reduction of required dose and side effects. This study aimed to prepare and evaluate biopolymeric nanoparticles loaded with *Myracrodruon urundeuva* extracts. We performed bioassays using different concentrations to nanoencapsulated extracts against pathogenic bacterial strains and cytotoxicity studies *in vitro*. Nanoparticles were prepared by interfacial biopolymer deposition/solvent displacement. Colloidal suspensions were dried by Spray-Drying. Nanoparticle-based materials were stable and homogeneous. Biological activities ranged according to the kind of prepared nanoparticles, bacterial strains, and dose. The evaluated bacterial strains were: *E. coli*, *S. aureus*, and *S. mutans*. The cytotoxicity assays showed better results in concentration of 300 µg. mL⁻¹. All nanoparticles were three more times toxic to cancer cells than non-encapsulated extract.

Keywords: *Myracrodruon urundeuva*, nanoparticles, bactericidal activities, cytotoxicity activity

Introduction

Nanoparticles are inorganic, organic or polymeric particles that have size dimension in nanoscale (1-1000 nm) (Yuan *et al.*, 2019) [1]. Polymeric nanoparticles are prepared using biocompatible, biodegradable polymers such as poly (lactic-co-glycolic acid) (PLGA), polylactic acid (PLA), useful to drug delivery system (Nascimento *et al.*, 2016) [2]. Drug (botanical extract) nanoencapsulated can't reduce its toxicity, but improving the drug performance through the vectorization to target cells reducing its dose and side effects (Simonetti *et al.*, 2019) [3] besides increase the stability, and dispersion in aqueous medium of active compounds, before reaching the goals (Bonifácio *et al.*, 2014) [4]. A botanical extract is a complex mixture of different organic compounds unstable (Hajialyani *et al.*, 2018) [5]. These features highlight the efficiency of liposomal encapsulated coconut extract enhanced antibacterial and antioxidant properties (Olatunde *et al.*, 2019) [6] besides soy lecithin-derived applications liposome on cancer treatment (Le *et al.*, 2019) [7]. *E. coli* is considered a foodborne pathogenic, as toxin causing gastrointestinal infections such as bloody diarrhea, hemorrhagic colitis, renal failure (Nadzirah *et al.*, 2015) [8]. In its turn, *S. aureus* is a pathogen of a variety of human infections ranging from skin infections to tissues (Kong *et al.*, 2016) [9]. Moreover, *S. mutans*, an etiological factor of dental caries, a microorganism that acquire new properties for expression of pathogenicity (Krzyściak *et al.*, 2014) [10]. In addition to nanomedicine development of bacterial control, its progress is evident in researches for cancer therapies. The literature highlights benefit of nanoparticle application during new pharmaceutical products. Pinto (2011) [11] demonstrates the efficacy of liposomal imatinib-mitoxantrone combination against cancer and emphasizes its potential as treatment. PLGA are attractive vehicles for protein antigen delivery, enhancement in antigen processing, immunogenicity, improved targeted delivery and slow release of antigens (Iranpour *et al.*, 2016) [12]. However, evidence supports the idea of anticancer activity of natural polyphenols, their antioxidant properties,

which help normal cells and increase the surveillance of cancerous cells against oxidative stress caused by anticancer agents (Davatgaran-Taghipour *et al.*, 2017) [13]. Then, the nanoparticles loaded with natural polyphenols may overlap the surveillance system of cancerous cells.

Myracrodruon urundeuva (Anacardiaceae) is popularly known as Aroeira, a medicinal plant widely used in Brazil and application includes activity against tumors, inflammations, infections, allergy, itching, bleeding, gastritis, and wound healing (Gomes *et al.*, 2013) [14]. Studies highlight the antimicrobial activity of *M. urundeuva* and evaluated the extracts action bioassays as bactericide and/or bacteriostatic to *S. mutans*, *S. mitis*, *S. sobrinus*, *S. sanguis* and *L. casei*, and antifungal against *C. albicans*, *C. tropicalis* and *C. krusei* (Alves *et al.*, 2009) [15]. The evaluation of the antimicrobial activity of *M. urundeuva*, and its effectiveness to *S. aureus* were related the presence of phenolic compounds with antimicrobial activity, alkaloids flavonoids and tannins in antifungal activity, antioxidant, anti-inflammatory, and anticancer properties (Gomes *et al.*, 2013) [14]. Bonifácio *et al.* (2015) [16] concluded *M. urundeuva* extract has increased antifungal activity in incorporation into a nanostructured system enhance. Ferreira (2011) [17] emphasize the antiproliferative power of *M. urundeuva* against HL-60 lines (leukemia) cancer, SF-295 (glioblastoma), HCT-8 (colon) and MDA/MB-435 (melanoma) cells assign the antitumor effects. Thus, the main aims of this work were to certify the bacterial activities from nonencapsulated *M. urundeuva* extract against *E. coli*, *S. aureus*, and *S. mutans* evaluating if nanoencapsulated extract are able to keep their activities, as well as to investigate the *in vitro* cytotoxicity activity against pancreatic ductal adenocarcinoma (S2-013).

Material and methods

Method of extraction to *M. urundeuva*

M. urundeuva leaves were collected in 2015, at Agudos, Sao Paulo, Brazil. The exsiccate was deposited in herbarium Rioclarense (HRCB) at Department of Botanic-IB-UNESP-Rio Claro (identification n° exsiccata-HRCB59831). The leaves were ground using an industrial blender and analytical knife mill (IKA A11 basic, Wilmington). The powder material was macerate in ethanol: water (7:3, v/v) for 12 days. In each 72 h, we replaced the solvent mixture, stirred for 3 min before each maceration using a T25 disperse Ultra-turrax (IKA, Wilmington). After extraction, the solvent was filtered, removed using rotaevaporator (Büchi R-215) (Germany, Labortechnik GmbH) and stored in amber glass (Machado *et al.*, 2016) [18].

Nano encapsulation of *M. urundeuva* extract

We obtained the nanocapsules (NC), and nanosphere (NS) in colloidal suspension by interfacial deposition/solvent displacement using preform polymer, according to Fessi *et al.* (1989) [19], and Forim *et al.* (2013) [20], with modifications. In organic phase the nanocapsules containing biopolymer poly(ϵ -caprolactone) PLA (1,000 mg), a surfactant Span 60 (770 mg), isodecyl oleate (3100 mg), *M. urundeuva* extract (250/500 mg), acetone (267 mL). The aqueous phase was prepared with Tween 80 (770 mg) and ultrapure water (533 mL) under magnetic stirring (RT10P/IKA, Wilmington) at 40°C. For nanospheres, the process was carried out under similar conditions, except by presence of isodecyl oleate in organic phase. After synthesizing nanoparticles, the solvent was eliminated using rotaevaporator preparing the colloidal suspension, then was dried by a mini-Spray-Dryer B-290

(Büchi, Brasil), added one drying support (Aerosil 200, Labsynth) into a colloidal suspension (1,5 g/1 L), submitting it by nebulization in hot air obtaining microparticles in powder (Forim *et al.*, 2013) [20]. The yield was available to each formulation. The colloidal suspension total volume was 500 ml, whereas 100 ml to characterization analysis and 400 ml by Spray-Dryer. For each proportion 800 mL in each nanoparticle NC1, NC2, NS1 and NS2 yield powder total volume of 5890, 6140, 2790 and 3040 mg, respectively. Then, the compositions of organic and aqueous phases were previously investigated, the best stability, pH values, colloidal nanoparticle size, and nanoencapsulation efficiency are conditions to prepare the nanoparticles. The best-reached designs antibacterial and cytotoxicity activity were formulations prepared, changing the composition of nanocapsules and nanospheres respectively, previously prepared and achieved best results to nanoformulations will be select to future studies.

Characterization of colloidal suspensions and powder nanoparticles

Physicochemical analyses of nanoparticles were carried out immediately after their preparations, pH values to colloidal suspensions were used by potentiometer (B474; Micronal, Sao Paulo, Brazil). The particle size analysis of nanoparticles was performed by Photon Spectroscopy by measurements (nm). This analysis yields the mean particle hydrodynamic diameter and polydispersity index (Pd_i), which is a dimension less measure of the broadness of the particle size distribution. The zeta potential values were obtained by dynamic light scattering technique on Zetatrak (Microtrac Inc, Montgomeryville, PA) which was controlled by Flex version 10.5.0 software (Microtrac Inc, Montgomeryville, PA). For particle size and zeta potential (mV) measurements, 0.1 mL of each colloidal suspension was diluted into 10 mL of ultrapure water and 10 mM sodium chloride. Like drying support in 1% m/V amounts to powder nanoparticle obtention. For statistical analysis in triplicate, average values and standard deviation of measurements were calculated (Kuma *et al.*, 2017) [21]. Morphological analyses of nanoparticles were performed by Scanning Electron Microscopy (SEM) (Field Emission Gun) (Nanolab Technologies, CA, U.S.A.). The samples were photomicrographed by Magellan XHR 400L FE-SEM-FEG (Field Emission Gun) (Nanolab Technologies, CA, U.S.A.) using different magnifications (Santos *et al.*, 2012) [22].

Microbial bioassays

The following microbial strains were evaluated: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 13565, and *Staphylococcus mutans* ATCC 25175 since pathogens monitored by Brazilian Public Health System (Wang & Dacheng, 2017) [23]. The strains were provided by Research Intensive Center, Bauru Dentistry Faculty. The bacterial suspensions were standardized during 24 h in Mueller Hinton broth. The growth media were centrifuged at 600 ×g, and supernatants were discarded. The bacterial pellets were resuspended in tubes using PBS at pH 7.2, until reach a turbidity similar to one tube suspension in 0.5 scale McFarland (1.5×10⁸ UFCXmL⁻¹), homogenized and washing was repeated. The microbial dilution was detected in spectrophotometric readings at 590 nm (Spectrophotometer; Beckman Coulter; Du 730; Life Science UV-VIS, CA, U.S.A.). The suspensions were diluted to 10 µg·mL⁻¹ in BHI, resuspended 1.0×10⁶ UFC·mL⁻¹. The bioassays were carried out in 96 wells plate, incubated 37°C, for 24 h, and the sample

microdilution until 10^6 UFC·mL⁻¹, and transferred to other 96 wells plate. Afterward, added NC1, NC2, NS1, NS2 nanoparticles and extract in 1000 to 0.1×10^{-1} µg·mL⁻¹ dilutions, and negative control and well dilutions were placed in 20 µL dilution in 4 quadrants in each Petri dish (3 Petri dish/dilution). The assay was triplicate ($n = 3$), the scattering for seeding offered the growth and bacterial colonies score. Both *E. coli*, and *S. aureus* strains were cultivated in Sabouraud Dextrose Agar (BHI) (Brain Heart Infusion Agar, Acumedia, Neogen Corporation, Michigan, USA). But *S. mutans* was cultivated in *Mitis salivarius* Agar (Difco, Becton Dickinson and Company, USA), incubated for 24 h. We reported all results as mean \pm standard error of three independent biological replicates and employed ANOVA followed by Turkeys HSD test ($p \leq 0.05$) to analyze the antibacterial activity data.

In vitro cytotoxicity assay

The Sulforhodamine RB Method (Skehan *et al.*, 1990) [24] evaluated nanoencapsulated extract mechanism on Human pancreatic ductal adenocarcinoma (S2-013) cell line growth, supplied from Laboratory of the Government Chemist (LGC Standards) (Barcelona, Spain). Briefly, the S2-013 cells were seeded in 96-well dishes at cell density of 1000 cells/well, under normal conditions of 5% CO₂, at 37°C for 24 h and treated for 48 h with the samples NC1, NC2, NS1 and NS2 in 0.3 to 300 µg·mL⁻¹ dilutions, fixed using trichloroacetic acid 10% (TCA) (w/v) for 60 min in ice bath, washed with ultrapure water and stained with 50 µl of SRB solution. The unbound dye was removed by washing with acetic acid solution 1% (v/v). The dried cells and protein-bound stain solubilized with 10 mM Tris solution. The SRB absorbance was measured at 560 nm in Synergy HT Multi-Mode Microplate Reader (Biotech Instruments Inc., Winooski, VT, USA). The IC₅₀ (concentration for 50% of cell survival) values determined. The wells absorbance containing nanospheres or nanocapsules and untreated cells following 48 h incubation compared with wells containing cells fixed at time zero.

Results and discussion

Polymeric nano/microparticles and their characterization

Nanoparticles have created opportunities for development of new therapeutic applications (Khan & Gurav, 2018) [25], especially phytotherapies. Polymeric nanoencapsulated with natural compound bioactivities proven successful increasing the bioavailability, permeability, bioavailability, prolonged half-life, tissue targeting, and minimal side effects (Aljuffali *et al.*, 2016) [26]. The nanocapsules and nanospheres was chosen due to simple operation without applied targeted delivery, efficiency reduces stability and dispersion of natural products (Deng *et al.*, 2020) [27]. After syntheses of nanoparticle, suspensions presented a milky and blue feature resulted from the Brownian movement (Tyndall effect), which

occurs due to scattering of light in individual nanoparticles in Random motion in dispersion medium (Uma *et al.*, 2011) [28]. This characteristic confirms the nanoparticle synthesis. The Spray-Dryer process are observed control with minimal increase in temperature (Oliveira & Petrovick, 2009) [29]. The calculated yields of powder for NC1, NC2, NS1, and NS2 were 69.7, 55.7, 77.1, and 77.0%, respectively. For spray dryers, typical process yields are usually in 50-70% range. Gallo *et al.*, (2015) [30] did a comparative study by spray dryer medicinal plant extract of *R. pushiana*, *H. virginiana*, *V. officinalis*, *H. perforatum* and *C. scolymus* and the yield was 65 to 95%, being similar to our study.

The preparation of nanoparticles in powders can't only increase the stability but also simplified their final as capsules or pills for oral administration, besides biological effects, as complete oral drug absorption from powders dispersed in water, reducing gastrointestinal irritant (Guterres *et al.*, 2009) [31]. Nanoparticle characteristics (size, shape and surface properties) for biological interactions at molecular level created development of nanoparticles for therapeutic (Khan & Gurav, 2018) [25]. Nanoparticles have been used as drug delivery vehicles due to better encapsulation, control release and less toxic (Kumari *et al.*, 2010) [32]. To available the nanoparticle stability in colloidal suspension, did not observe precipitate formation, only a variation in the pH according to time of formulation preparation. In Figure 1 the pH suffered a slight variation in time function kept constant after 30 days, and variations were linked to hydrolysis of polymer chain, changing the pH of colloidal suspension (DiDonato *et al.*, 2017) [33].

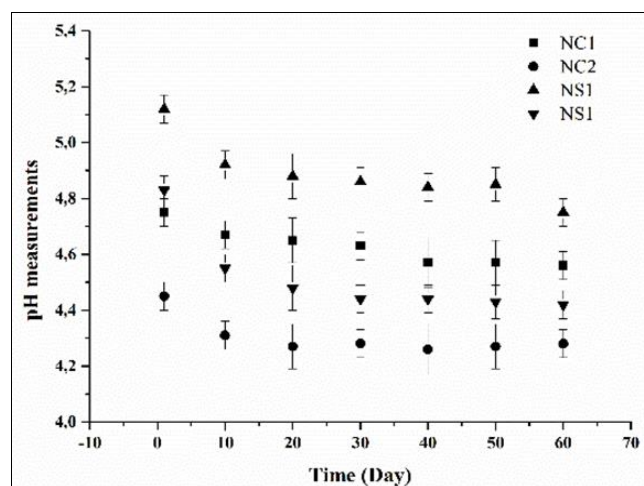


Fig 1: pH variation as in time function in each nanoparticle

The table 2 described the physicochemical properties of NC1, NC2, NS1 and NS2 nanoparticles characterized according zeta potential (ZP), particle diameter (PD) and polydispersity index (PdI) values.

Table 2: Physicochemical properties of nanoparticles applied in biological assays.

Nano formulation	Particle size (nm)	Polydispersity Index (PdI)	Zeta Potential (mV)
NC1	169.0 \pm 5.72	0.185 \pm 0.940	-28.0 \pm 5.07
NC2	258.3 \pm 4.31	1.000 \pm 0.383	-40.6 \pm 7.31
NS1	255.9 \pm 6.53	0.512 \pm 0.512	-26.1 \pm 7.20
NS2	432.1 \pm 6.94	0.912 \pm 0.912	-38.6 \pm 5.34

Values express the average result \pm standard deviation ($n = 3$)

The nanoparticles showed a particle size ranging from 169.0 to 432.1 nm. Usually, the nanoparticles prepared by preform

polymer deposition present average diameter between 100 to 300 nm. Particle sizes are mainly influenced by the oil core

and rate between the quantity of active compounds and polymers. The nanoparticles prepared with twice, the extract presented a larger particle size in nanocapsules and nanospheres. While the PDI was identified 0.6 in nanoparticles prepared extract in the lower level (250 mg) and 1.0 to formulas with an extract in the higher (500 mg) (Table 2) indicating narrow and broad particle size distributions, respectively. The nanoparticles (NC and NS) showed a homogenous population when prepared a composition PCL and extract of 1001,2 g /250 mg range. A lower dosage compared our studies was realized by Busari *et al.* (2017) [34] in cytotoxicity assay with curcumin (5 mg drug/50 mg PLGA).

The zeta potential of colloidal suspensions showed negative values to prepared nanoformulations using polymer/extract rate of 1001,2 g/500 mg. The values -28.0 and -26.1 mV obtained to NC1 and NS1, respectively and nanoparticles prepared with polymer/extract rate of 1001,2 g/500 mg with values of -40.6 and -38.6 mV to NC2 and NS2, respectively. This result is compatible with a system where compounds in extract may be adsorbed in nanoparticle surfaces. Independently of zeta potential, the precipitate in colloidal suspension after 180 days by Spray-dryer was uniform materials. Nanocapsules and nanospheres (Fig 2.) showed compact and spherical structures homogeneous in colloidal suspension and powder.

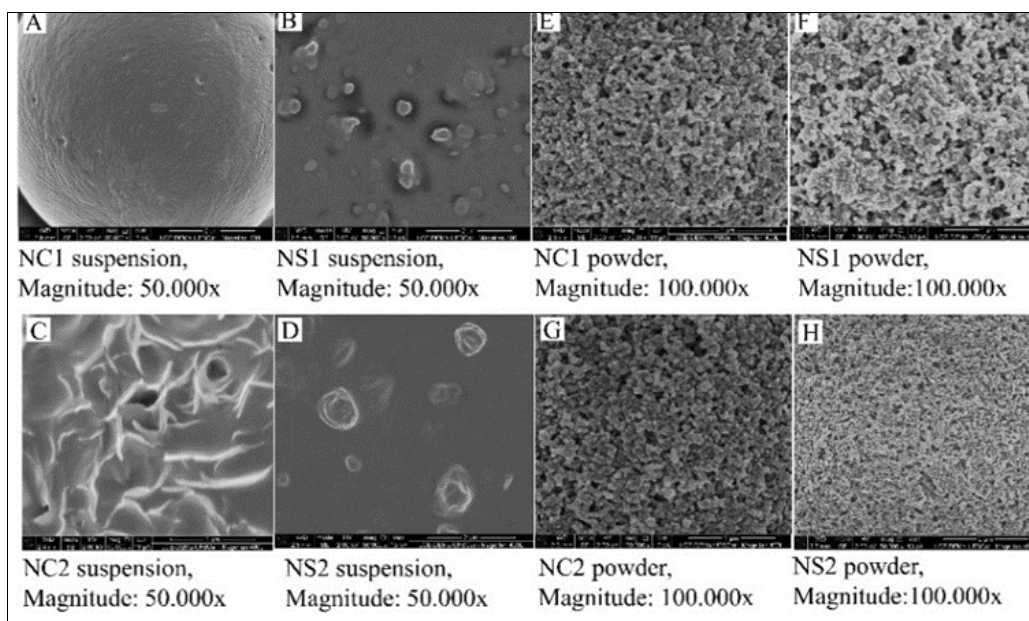
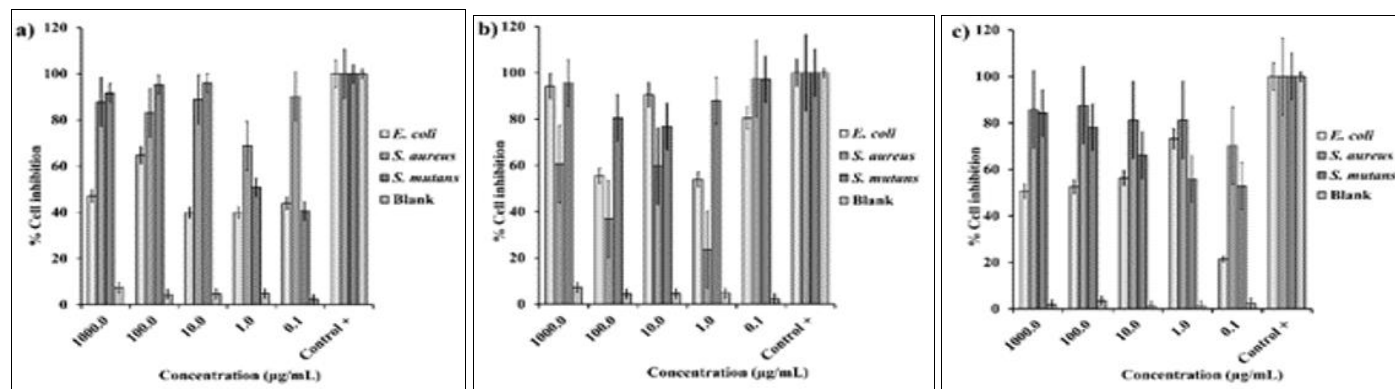


Fig 2: Morphological analyses of nanoencapsulated *M. urundeuva* extract by Scanning Electron Microscopy (SEM); A-D: NC1(A), NC2 (C), NS1 (B), NS2 (D) in colloidal suspension, magnification 50,000 \times ; E-H: NC1 (E), NC2 (G), NS1(F), NS2 (H) in powder, magnification 100,000 \times .

Bacterial bioassays with nanoencapsulated *M. urundeuva* extract

The antibacterial effects of nanoparticles were evaluated in powder loaded with extract and control against pathogenic bacterial strains like *S. aureus*, *S. mutans* and *E. coli*. At first NC1 presented smaller cells inhibition against *E. coli* in all evaluated dilutions, while NC2 inhibited 50 to 100%, but NC1 presented a high inhibition in all dilution against *S. aureus*. Then, NC2 was able to inhibit against *S. aureus* around 30 % to 100 and 1.0 $\mu\text{g}\cdot\text{mL}^{-1}$ dilution, and 60% to

1,000 and 10 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively and the control was similar inhibition to 0.1 $\mu\text{g}\cdot\text{mL}^{-1}$ dilution. NC1 and NC2 inhibited the most dilutions against *S. mutans*. The NS2 have slightly lower inhibition compared NS1 against *E. coli* in the most dilution. While NS2 presented better inhibition than NS1 against *S. aureus* and the NS2 was slightly better than NS1 against *S. mutans*. The extract was efficiently active to *E. coli* but to *S. aureus* showed 70% inhibition only to 1,000 and 10 $\mu\text{g}\cdot\text{mL}^{-1}$ dilution. *S. mutans* presented lower inhibition in all evaluated dilution.



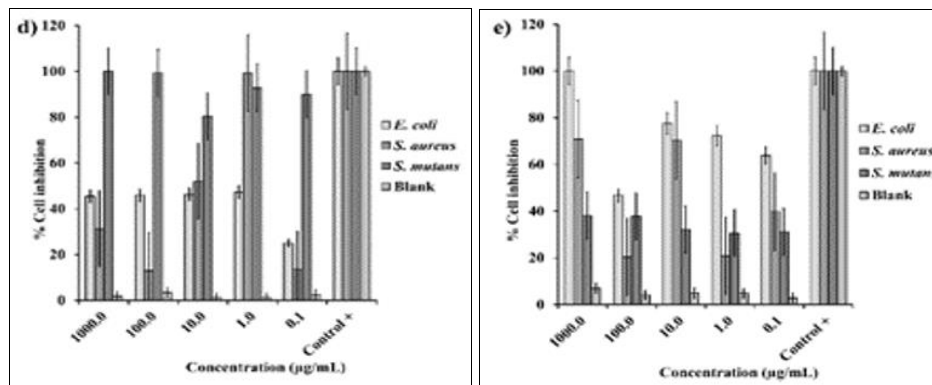


Fig 3: Evaluation antibacterial activities of *M. urundeuva* extract and its nanoparticles in powder against *S. aureus*, *S. mutans* and *E. coli*. A) NC1; B) NC2; C) NS1; D) NS2; E) Non-encapsulated *M. urundeuva* extract. Positive control: 100%.

Therefore, the extract gained efficiency when nanoencapsulated, especially NC1, NC2 and NS1, because optimized the bacterial activities against the investigated strains. Previously, Bonifácio *et al.* (2015) [16] described the fungicide activities from *M. urundeuva* extract in a nanostructured lipid system. Similar to our results, they observed gains to eliminate the infectious agent to encapsulated extract.

In vitro cytotoxicity activity of *M. urundeuva* nanoparticles on pancreatic ductal adenocarcinoma cells

The NS1, NS2, NC1, NC2 nanoparticles and extract was evaluated cytotoxicity activity using in Fig. 4. the pancreatic adenocarcinoma cells exposed to the samples between 0.3 to 300.0 $\mu\text{g}\cdot\text{mL}^{-1}$ dilutions. According Machado *et al.* (2016) [18], dilution range is compatible with cytotoxicity bioassay. The cell growth was monitored by SRB method, applied in studies chemotherapeutic drugs or small molecules in adherent cells (Orellana & Kasinski, 2016) [35].

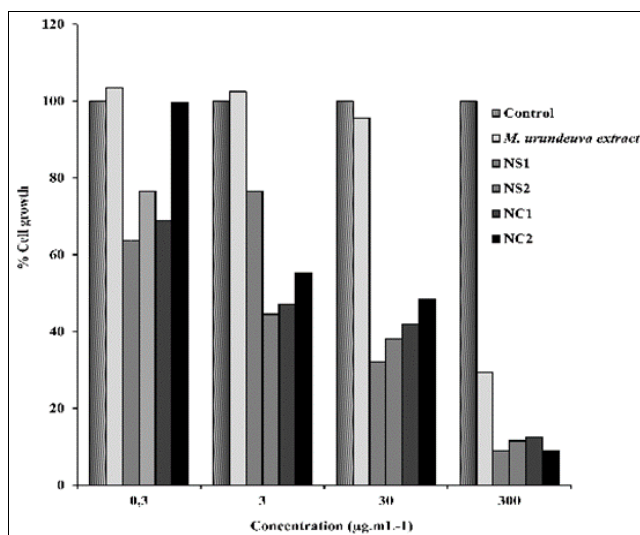


Fig 4: Cytotoxicity activity effects of *M. urundeuva* extract and its NS1, NS2, NC1, and NC2 nanoparticles after 48 h to human pancreatic ductal adenocarcinoma cells.

In 0.3 $\mu\text{g}\cdot\text{mL}^{-1}$ dilution, the NS1, NS2 and NC1 nanoparticles present 70% cell growth inhibition and 100% in NC2. The same is observed for inhibition of cell growth in response to the NC1 compared with NC2 only for extract in 0.3 $\mu\text{g}\cdot\text{mL}^{-1}$ dilution. At 3 $\mu\text{g}\cdot\text{mL}^{-1}$ dilution, the toxicity NS2, NC1 and NC2 reduced the cell growth around 45% to 50%, but the NS1 present around 70%. At 30 $\mu\text{g}\cdot\text{mL}^{-1}$ dilution, the toxicity on pancreatic cancer cells S2-013 were with a growth of 32%,

38% and around 42% for NS1, NS2 and NC1, respectively. At 300 $\mu\text{g}\cdot\text{mL}^{-1}$ dilution all nanoparticle presents only 10% cell growth. The results were similar except for NC2 and NS1 at 0.3 and 3 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively. According to ANOVA statistical analysis, there was general best results the nanoparticles 300 $\mu\text{g}\cdot\text{mL}^{-1}$ dilutions. The similar results to nanoformulations may be explained by natural products with therapeutic, bioavailability limited and nanoparticle-delivered, naturally synthesized for treatment and prevention of cancers (Bharali, *et al.*, 2011) [36]. In both assays we observed gains and efficiency in biological activity of extract nanoencapsulated than extract alone. *M. urundeuva* is a medicinal plant very well-known for its biological properties rich in polyphenols and nanoparticles vectored the Aroeira bioactive compounds (Zhang *et al.*, 2010) [37].

We conclude using PCL is possible to prepare nanocapsules and nanospheres loaded with *M. urundeuva* extract by interfacial deposition of a preformed polymer and the nanoencapsulated extract was obtained in powder by *Spray-drying*. We found different physicochemical features according to rate between polymer/*M. urundeuva* extract mainly to particle size and zeta potential. All nanoparticles showed similar morphology. The nanoencapsulated extract showed better biological activities against pathogenic bacteria and pancreatic adenocarcinoma cells than the one non-formulated. The biological efficiency of nanoparticles changed according to their particle size.

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