



Nanoencapsulation of hydrophobic phytochemicals using poly (DL-lactide-co-glycolide) (PLGA) for antioxidant and antimicrobial delivery applications: Guabiroba fruit (*Campomanesia xanthocarpa* O. Berg) study



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ABSTRACT

Previous studies have reported antioxidant and antimicrobial activity of guabiroba extract (GE), which is associated to its polyphenolic and carotenoid contents. Encapsulation using polymeric materials could improve GE application, bioavailability, and stability in foods and pharmaceuticals. Poly (DL-lactide-co-glycolide) (PLGA) nanoparticles with entrapped GE were synthesized using the emulsion-evaporation method with different lactide to glycolide (50:50 and 65:35) ratios to determine the dependency of polymer composition on nanoparticles antioxidant and antimicrobial activities. Controlled release experiments showed an initial burst followed by a slower release rate of carotenoids inside PLGA matrix. Both nanoparticles showed *Listeria innocua* growth inhibition within the concentration range tested (<1200 µg/mL), that was not observed by the free extract. Lower GE concentrations were required to reduce reactive oxygen species once it was nanoencapsulated; however, equivalent or higher concentrations for free radical scavenging were required. GE-loaded PLGA 50:50 presented the best results for antimicrobial and antioxidant delivery applications. These nanoparticles could be used with other extracts containing carotenoids and other functional lipids as delivery systems for enhanced biological activity.

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1. Introduction

Functional lipids obtained by hydrophobic extraction consist of phytosterols, carotenoids, omega-3 fatty acids, natural antioxidants, and various other compounds have shown to have health benefits that are beyond those associated with their established role in growth, development, and other normal body functions (McClements, Decker, & Park, 2009; Tan & Nakajima, 2005; Wildman & Kelley, 2007). Specifically, the potential for

carotenoids for promoting health through many physiological mechanisms has been shown, including preventing oxidative damage, quenching singlet oxygen, altering transcriptional activity, and vitamin A precursors (Abdel-Aal & Akhtar, 2006). Despite of their benefits, many factors have limited their application and their potential health benefits in functional food or pharmaceutical products including: poor water solubility, high melting point, and chemical instability during production and storage of food and pharmaceutical products (McClements et al., 2009). Decreasing their size and modifying the interfacial layer surrounding them can greatly affect their physicochemical properties and functionalities (McClements et al., 2009).

Encapsulating carotenoids and other functional lipids using nanotechnology approaches could address some of these concerns, prevent their degradation, and improve their water solubility and

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antimicrobial and antioxidant activities. Synthetic polymers and natural macromolecules have been extensively studied as colloidal materials for nanoparticles designed for drug delivery (Benita, 2006). Synthetic polymers have an advantage of higher purity and reproducibility over natural polymers. The polyester family, such as poly (lactide-co-glycolide) (PLGA), has been approved by the US Food and Drug Administration, and is of great interest to the biomedical field because of its biocompatibility, biodegradability and controlled delivery properties (Stevanović & Uskoković, 2009). Furthermore, PLGA can be used to encapsulate hydrophobic compounds through a relatively simple process. Previous studies have shown that entrapped compounds release from nanoparticle and nanoparticle physicochemical properties can be altered by changing synthesis parameters, which include polymer composition and weight, synthesis method, particle size, emulsifier, surface charge, and hydrophobicity (Astete & Sabliov, 2006). These properties will ultimately influence phytochemical compound's functionality and will affect their use in food and pharmaceutical products. PLGA nanoparticle-based delivery system has been widely studied as a drug carrier for controlled delivery applications; however, studies on nanoencapsulation of hydrophobic phytochemical compounds using PLGA and their antimicrobial and antioxidant activities are scarce in the literature (Ganea et al., 2010; Silva, Hill, Figueiredo, & Gomes, 2014).

Guabiroba (*Campomanesia xanthocarpa* O. Berg.), a native fruit from Brazil, has been shown to be a rich source of carotenoids (Pereira et al., 2012). Furthermore, it presented the highest antioxidant activity among six native fruits from Rio Grande do Sul, Brazil, mainly due to its high content of phenolic compounds, vitamin C, and carotenoids (Pereira et al., 2012). Hence, guabiroba demonstrates great potential for further economic exploitation as a value-added antioxidant and preservative by the pharmaceutical and food industry. Therefore, it was selected in this study as it also remains an underutilized fruit. The objectives of this study were to synthesize PLGA nanoparticles with different lactide to glycolide ratios containing hydrophobic extract of guabiroba fruit, to characterize their physico-chemical properties, and to evaluate their potential antioxidant and antimicrobial activities.

2. Materials and methods

2.1. Materials

Guabiroba fruits were harvested in December 2012 from a farm located in the state of Rio Grande do Sul, Brazil. Fully ripe fruits were preselected for absence of visible injury and infection, and uniform size and color. The seeds were removed and the fruits frozen ($-80\text{ }^{\circ}\text{C}$), and freeze-dried ($-50\text{ }^{\circ}\text{C}$) under 1.09 Pa at Federal University of Pelotas, Pelotas, RS, Brazil. Samples were shipped to Texas A&M University, College Station, TX, USA where they were held at $-20\text{ }^{\circ}\text{C}$ for experimental trials.

PLGA, with a copolymer ratio of lactide to glycolide of 65:35 and 50:50 (MW 40,000–75,000 g/mol), poly(vinyl alcohol) (PVA, MW 30,000 to 50,000 g/mol), and β -carotene were purchased from Sigma–Aldrich (St. Louis, MO, USA). D(+) trehalose dihydrate, 98% was obtained from EMD Chemicals (Philadelphia, PA, USA). Dichloromethane was obtained from CTL Scientific Supply Co. (Deer Park, NY, USA). Nanopure water was obtained from Macron Chemicals (Charlotte, NC, USA). Tryptic soy agar (TSA), tryptose phosphate broth (TPB), yeast extract and peptone were purchased from Becton, Dickinson and Co. (Franklin Lakes, NJ, USA). 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from AMRESCO (Solon, OH, USA). Trolox (6-hydroxy-2, 5, 7, 8-tetramethyl-

chroman-2-carboxylic acid) was obtained from Tokyo Chemical (Tokyo, Japan).

2.2. Hydrophobic extract of guabiroba fruit

The hydrophobic extract of guabiroba fruit (GE) was prepared according to method described by Rodríguez-Amaya (2001) for carotenoids extraction. Briefly, freeze dried fruit (200 mg) was mixed with acetone until complete sample discoloration. Then, the extract was concentrated under vacuum (at 6.7 kPa) at room temperature in a rotary evaporator (Buchi R-210 Rotavapor, BuchiCo., New Castle, DE, USA) until final volume of 5 mL. Extract was prepared immediately before use. Total carotenoids concentration was 3.51 ± 0.18 mg of β -carotene per g GPE dry basis.

2.3. Nanoparticle synthesis with entrapped hydrophobic extract of guabiroba fruit

Nanoparticles were synthesized by an emulsion-evaporation method similar to Zigoneanu, Astete, and Sabliov (2008). A solution containing 50 mg of PLGA (50:50 or 65:35) in 2 mL of dichloromethane with 5 mL of GE in acetone was prepared, which constituted the organic phase. An aqueous phase (20 mL) was prepared with 0.3 g/100 mL PVA in nanopure water. The organic phase was added dropwise to the aqueous phase, and this mixture was homogenized for 2 min at 13,000 rpm (Ultra-Turrax T25 basic Ika, Works, Wilmington, NC, USA) forming an oil-in-water emulsion. Next, this emulsion was sonicated in an ice bath ($2\text{ }^{\circ}\text{C}$) at 70 W (Cole Parmer 8890 ultrasonic cleaner, Vernon Hills, IL, USA) for 30 min. The organic phase (dichloromethane and acetone) was evaporated for 20 min using a rotary evaporator ($25\text{ }^{\circ}\text{C}$). Unloaded nanoparticles were prepared using the same procedure described above, without addition of GE to the organic phase. Following synthesis, the nanoparticles were purified by ultrafiltration using a Millipore-Labscale™ TFF system equipped with a 10 kDa cutoff Pellicon XL-Millipore (Millipore Co., Kankakee, IL, USA). The nanoparticles were ultrafiltered with water (200 mL) and 50 mL was collected (retentate). D(+) trehalose (cryoprotectant) was added at a 1:1 (w/w) ratio relative to nanoparticles. Samples were frozen ($-80\text{ }^{\circ}\text{C}$), then freeze-dried ($-50\text{ }^{\circ}\text{C}$) under 1.09 Pa for 48 h. Lyophilized samples were stored at $-20\text{ }^{\circ}\text{C}$ and utilized within one week.

2.4. Particle size and size distribution

Nanoparticle size and distribution (polydispersity) were determined using a Delsa TM Nano C Particle Analyzer (Beckman Coulter, Brea, CA, USA). Nanoparticles were dispersed in nanopure water (10 mg/mL) at $25\text{ }^{\circ}\text{C}$ and sonicated for 15 min before analysis using 1 cm path length plastic cuvettes at 165° scattering angle, with a pinhole set to 20 μm , and a refractive index of 1.3328 for 120 continuous accumulation times (Hill, Taylor, & Gomes, 2013).

2.5. Nanoparticle morphology characterization

Nanoparticles morphology was examined by Transmission Electron Microscopy using FEI Morgagni TEM (FEI Co., Hillsboro, OR, USA). Particles were suspended in water (1 mg/mL) and placed on 0.037 mm copper grids and stained with a 2 g/100 mL uranyl acetate aqueous stain (Electron Microscopy Sciences, Hatfield, PA, USA) to provide contrast under magnification. Excess liquid on the mesh was removed with filter paper and the grid was allowed to air dry before viewing under 50,000 to 100,000 times magnification. Observations were performed at 80 kV.

2.6. Entrapment efficiency (EE)

The EE of GE in the PLGA nanoparticles was measured by total carotenoids analysis (Rodríguez-Amaya, 2001). Nanoparticles samples (10 mg) were suspended in 95 mL/100 mL acetone in water (5 mL), mixed well, flushed with nitrogen gas, protected from light, and left for 72 h at room temperature. Each sample was passed through a syringe filter (0.2- μ m, Acrodisc, Pall Life Sciences, Port Washington, NY, USA) and solvent extraction was performed with hexane. The extract was analyzed spectrophotometrically at 450 nm (Shimadzu UV-1601 spectrophotometer, Columbia, MA, USA). A standard curve for total carotenoids concentration (expressed in β -carotene) was prepared (concentration range 5–250 μ g/mL, $R^2 = 0.99$). EE was calculated according to Equation (1) (Teixeira, Ozdemir, Hill, & Gomes, 2013):

$$EE = \frac{\text{amount of active compound entrapped}}{\text{initial active compound amount}} \times 100 \quad (1)$$

2.7. Controlled release study

Lyophilized re-suspended nanoparticles were dissolved in a release medium, containing 0.1 mol/L sodium dodecyl sulfate and 0.1 mol/L NaCl (pH 6.8 \pm 0.2) that was sufficient to establish sink conditions at a concentration of 3.3 mg/mL using 2-mL Eppendorf tubes as described by Zigoneanu et al. (2008). The controlled release study was performed at 37 °C and 100 rpm (Orbit Shaker, Lab Line Instruments, Melrose Park, IL, USA) with samples withdrawn at predetermined time intervals, filtered with a 0.2- μ m Acrodisc filter, and 1 mL was collected to carry out the total carotenoids analysis as described in Section 2.6.

2.8. Antimicrobial activity

Bacterial Culture: *Listeria innocua* (NRRL B-33076) was used as a non-pathogenic surrogate for *Listeria monocytogenes* due to its importance to the food industry (Gravani, 1999). An aliquot (0.1 mL) of *L. innocua* frozen culture was transferred, by two identical consecutive transfers, onto TPB and incubated for 24 h at 35 °C. The bacterial culture was maintained on TSA containing 0.6 g/100 mL yeast extract (TSAYE) slants at 4 °C. Working cultures were produced daily by transferring a loop of culture from TSAYE slants to 10-mL TPB and incubating for 24 h at 35 °C.

Minimum inhibitory and bactericidal concentrations (MICs and MBCs): a broth dilution assay described by Brandt et al. (2010) was used to determine MIC and MBC values for GE and its corresponding PLGA nanoparticles. *L. innocua* inoculum (3.0 log₁₀ CFU/mL) was prepared in double strength TPB to allow the correct nutrients content in the final sample cuvette upon treatment addition. The MIC experiments used free GE suspended in 0.1 g/100 mL tween 20 solution and GE-loaded nanoparticles suspended in water by two-fold diluted concentrations ranging from 650 to 5000 μ g/mL and 950 to 6500 μ g/mL, respectively. Controls were also prepared containing inoculum and tween 20 solution or control nanoparticles at test concentrations to ensure nanoparticle encapsulant materials and additives had no inhibitory effect on bacterial growth. Samples were incubated for 24 h at 35 °C and OD at 630 nm readings were taken to observe bacterial growth and inhibition. Treatment solutions that showed ≤ 0.05 change in OD at 630 nm were considered inhibited by the treatment solution (after appropriate baseline adjustments). The MIC was the lowest treatment concentration that inhibited growth for all tests replicates.

Treatments that showed microorganism inhibition were then tested for bactericidal activity by spreading 100- μ L aliquot onto

TSAYE plates and incubating for 24 h at 35 °C. The lowest treatment concentration demonstrating bactericidal activity (no colonies on plate surfaces following incubation) across all replicates was considered the MBC.

2.9. Antioxidant activity

Unloaded and GE-loaded nanoparticles (200 mg) were suspended in water (2 mL). Free GE was suspended in acetone (40 mg/mL) as previously described (Section 2.2). These solutions were thoroughly mixed and 6 different dilutions (ranging from 1000 to 8000 μ g/mL of GE for each treatment with corresponding dilutions for unloaded nanoparticles) were prepared.

DPPH radical scavenging: an aliquot of each sample dilution (0.1 mL) was added to 3.9 mL of DPPH radical solution (0.039 g/L in methanol), vigorously mixed and incubated in the dark at room temperature for 30 min, then absorbance was measured at 517 nm (Shimadzu UV-1601 spectrophotometer) (Brand-Williams, Cuvelier, & Berset, 1995). The results were expressed as antioxidant concentration required to reduce the original amount of free radicals by 50% (IC₅₀), and the values were expressed as grams of GE/gram of DPPH.

Trolox Equivalent Antioxidant Capacity (TEAC): blue-green ABTS radical cation chromophore (ABTS^{•+}) was prepared according to Re et al., 1999. The ABTS^{•+} solution was diluted in 50 mL/100 mL ethanol in water to 0.70 \pm 0.2 absorbance at 734 nm. Diluted ABTS^{•+} solution (3 mL) was added to 30 μ L of each treatment or Trolox standard (final concentration 100–2000 μ mol/L in ethanol) suspensions; then absorbance reading was taken 6 min after initial mixing at 734 nm. Samples absorbance measurements were plotted as a function of their concentrations and used to calculate the TEAC, where the absorbance reading was used to find the sample concentration equivalent to 1000 μ mol/L of Trolox. Results were expressed in μ mol/L Trolox Equivalent/g of GE.

2.10. Reactive oxygen species (ROS) generation assay

Cell culture: Non-cancer CCD-18Co colon fibroblast and human colon adenocarcinoma HT-29 cancer cells were obtained from ATCC (Manassas, VA, USA). HT-29 cells were cultured using McCoy's-5a modified medium with 1 g/100 mL penicillin/streptomycin and 10 g/100 mL fetal bovine serum (FBS); CCD-18Co cells were cultured using high glucose Dulbecco's Modified Eagle Medium with 1 g/100 mL penicillin/streptomycin, 1 g/100 mL non-essential amino acids (10 mmol/L), 1 g/100 mL sodium pyruvate (100 mmol/L) and 20 g/100 mL FBS (Invitrogen, Carlsbad, CA, USA). Cells were maintained at 37 °C in 5 mL/100 mL CO₂ atmosphere and seeded in 96-well plates (10,000 cells/well); then grown for 24 h to allow cell attachment before exposure to varying concentrations of each treatment (275 μ g/mL, 8.5 μ g/mL and 10 μ g/mL GE in free, and loaded-PLGA 50:50 and PLGA 65:35, respectively). Values for GE content in loaded nanoparticles were calculated based on EE values. Both cells were also treated with 0.3 g/100 mL acetone and 5 g/100 mL unloaded PLGA nanoparticles in water to ensure nanoparticle encapsulate materials and solvent no inhibitory effect or cell toxicity.

ROS production was analyzed according to Meng, Velalar, and Ruan (2008). Briefly, cells were treated with free GE, unloaded and GE-loaded nanoparticles. ROS generation was induced in CCD-18Co cells with 100 μ mol/L of H₂O₂ with each treatment for 2 h. HT-29 cells were not stimulated with H₂O₂ because they are constitutively under higher than normal oxidative stress (Schumacker, 2006). Cells were washed with PBS and ROS generation was detected after incubation with 100 μ mol/L of 2',7'-

dichlorofluorescein diacetate (H₂DCF-DA) (Sigma–Aldrich) at 37 °C for 30 min. The relative fluorescence units were measured (520 nm emission and 480 nm excitation) with a FLUOstar Omega plate reader (BMG Labtech Inc, Durhan, NC, USA). Fluorescence intensity was used as an indicator for ROS generation.

2.11. Statistical analysis

For all analyses, determinations were made at least in triplicate as independent experiments based on a completely randomized design with equal replications. Data analysis was performed using JMP v.9 software (SAS Institute, Cary, NC, USA) and differences between variables were tested for significance by analysis of variance (ANOVA). Significantly different means ($P < 0.05$) were separated by the Tukey test.

3. Results and discussion

3.1. Particle size and size distribution

Particle size ranged from 145 to 162 nm (Table 1), which were similar to previously reported PLGA nanoparticles with loaded hydrophobic compounds such as *trans*-cinnamaldehyde and eugenol, with sizes of approximately 180 nm for PLGA 65:35 (Gomes, Moreira, & Castell-Perez, 2011), and cinnamon bark extract, between 145 and 167 nm for PLGA 65:35 and 50:50 (Hill et al., 2013). On the other hand, Ribeiro, Chu, Ichikawa, and Nakajima (2008) reported particles ranging from 74 to 77 nm; for β -carotene-loaded PLGA nanoparticles; however, they used a different synthesis method, PLGA lactide to glycolide ratio (75:25), and surfactants (gelatin and tween 20). Although the unloaded PLGA 50:50 showed a significantly larger size than the unloaded PLGA 65:35, both PLGA nanoparticles when loaded with GE were not significantly different in size. The higher amount of lactide present in the PLGA 65:35, which is more hydrophobic than glycolide, could explain the smaller size of the unloaded particles since they are less likely to aggregate (Astete & Sabliov, 2006).

The polydispersity values ranged from 0.21 to 0.28 (Table 1) indicating that it was high (above 0.10) probably because significant amounts of PVA were removed during the ultrafiltration process and this could cause some nanoparticle agglomeration, since surfactant amount plays an important role in the emulsification process and in protecting the particles from coalescence (Astete & Sabliov, 2006). Similar results were reported previously for studies utilizing similar synthesis method, emulsifier, and polymer composition for hydrophobic compounds entrapment, with polydispersity values oscillating from 0.2 to 0.3 (Gomes et al., 2011; Hill et al., 2013; Silva et al., 2014).

Table 1

Average particle diameter, polydispersity index, and entrapment efficiency (EE) of poly-D,L-lactide-co-glycolide (PLGA) nanoparticles unloaded and loaded with hydrophobic guabiroba fruit extract (GE).

Nanoparticles	Size (nm)	Polydispersity Index	EE (%) ^a
GE PLGA 50:50	153.2 ± 1.8a,b	0.28 ± 0.01c	83.7 ± 3.8a
Unloaded PLGA 50:50	162.1 ± 0.6b	0.24 ± 0.01b	–
GE PLGA 65:35	154.8 ± 7.8a,b	0.21 ± 0.01a	98.5 ± 5.2b
Unloaded PLGA 65:35	144.8 ± 1.3a	0.24 ± 0.01b	–

Two types of PLGA were used with lactide: glycolide ratios of 50:50 (PLGA 50:50) and 65:35 (PLGA 65:35). Values are shown as the mean ± standard deviation of three independent repetitions.

^{a,b} Means within a column which are not followed by a common online letter are significantly different ($P < 0.05$).

^a Values were obtained using the Equation of Teixeira et al. (2013).

3.2. Nanoparticle morphology characterization

All particles showed a spherical shape and smooth surface with a darker perimeter on the spheres edge (Fig. 1) similar to PLGA nanoparticles seen in other studies (Hill et al., 2013; Zigoneanu et al., 2008). This dark perimeter is attributed to PVA, which forms connections between its hydrophobic regions and the PLGA chains to form a matrix, while its hydrophilic regions are exposed to the water phase (Zigoneanu et al., 2008). No distinct difference in morphology was noticed among the unloaded and loaded particles and different PLGA proportion. Additionally, the particles presented a broad size distribution and a strong tendency to form clusters, and their sizes (in the range of 200 nm from TEM images) were consistent with the particle sizes measured by the particle analyzer.

3.3. Entrapment efficiency (EE)

Entrapment efficiency of PLGA 65:35 was significantly higher than PLGA 50:50 (Table 1), which could be explained by an increase in interaction or affinity of hydrophobic extract with PLGA with an increase of lactide to glycolide ratio (Budhian, Siegel, & Winey, 2005). Both EE values were within range of previously reported values for PLGA nanoparticles containing hydrophobic components such as eugenol (98%), *trans*-cinnamaldehyde (92%) (Gomes et al., 2011) and α -tocopherol (89–95%) (Zigoneanu et al., 2008). High EE for hydrophobic compounds (80–98%) with PLGA as encapsulant are usually observed using the emulsion-evaporation method (Astete & Sabliov, 2006). There are very few reports on EE for carotenoids-loaded PLGA nanoparticles. Recently, Gharib and Faezizadeh (2014) used a nanoprecipitation method for the formation of lycopene-loaded PLGA nanospheres and obtained 79% EE. Ribeiro et al. (2008) described the solvent displacement method for the formation of β -carotene-loaded nanodispersions containing PLA and PLGA with high EE (up to 95%).

3.4. Controlled release study

The carotenoids release profiles from both PLGA nanoparticles followed similar trend with an initial burst effect in the first hours followed by a gradual decline in cumulative release over time (Fig. 2). PLGA 50:50 presented a more pronounced initial burst effect, reaching approximately 92% of carotenoids release in 1 h, while PLGA 65:35 demonstrated a more gradual initial release, for instance, within 0.5 h only 37% carotenoids have been released and after 6 h only 77% was released. Both nanoparticles showed similar release rate after 12 h which gradually declined until 72 h. This could be explained by carotenoids present in the GE being labile under the *in vitro* release conditions; consequently, this decrease of release after a certain time point would indicate their degradation. Recently, Chiu et al. (2007) carried out a stability test at 35 °C for 120 h for gelatin and poly (γ -glutamic acid) microparticles containing lycopene extract and observed 22% lycopene degradation. Once the GE was released and it was no longer protected by the polymer, presence of oxygen and the temperature of 37 °C influenced carotenoids degradation in our study, as previously reported (Maiani et al., 2009). This temperature was chosen to resemble physiological conditions and also because it is ideal for microbial growth.

The smaller initial burst effect and slower more sustained release from PLGA 65:35 is attributed to the affinity of carotenoids for the polymer, which has a higher lactide content, and its greater hydrophobicity may have made it more difficult for the lipophilic carotenoids to diffuse initially through the polymer matrix (Wischke & Schwendeman, 2008). Moreover, carotenoids could be more easily incorporated deeper within the PLGA 65:35 due to their

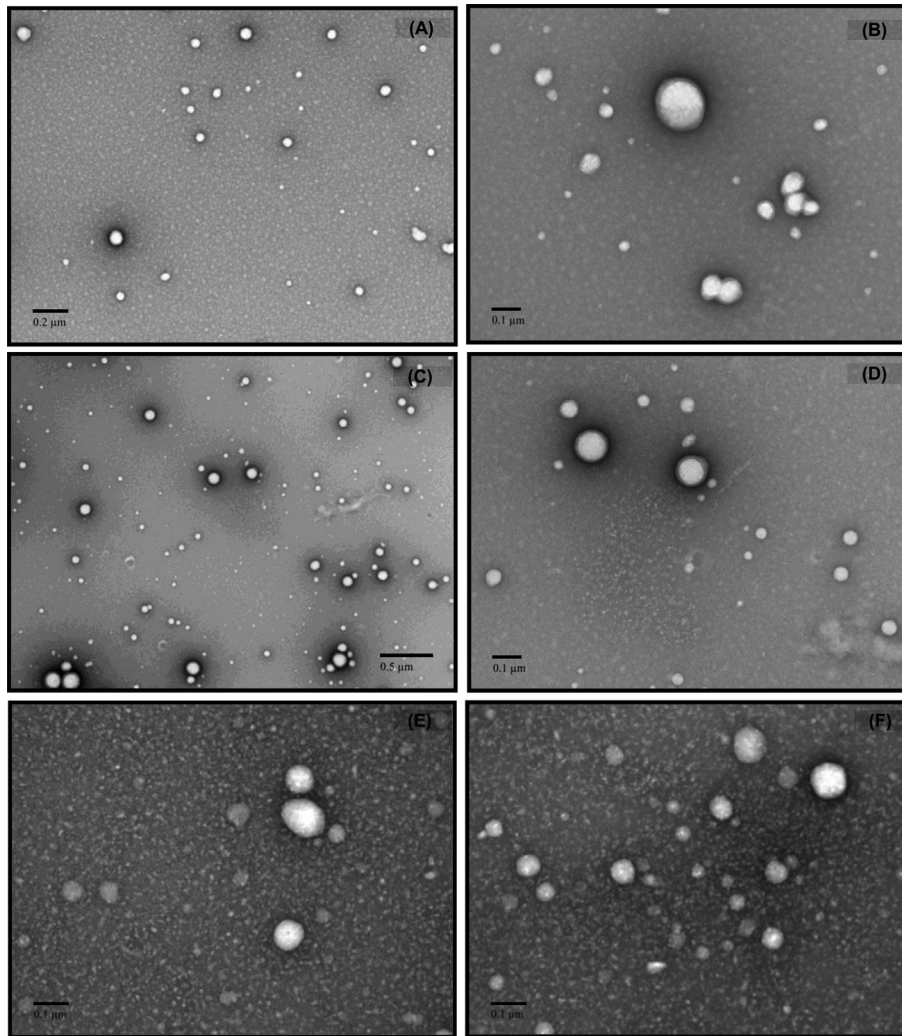


Fig. 1. Transmission electron microscope (TEM) images of PLGA nanoparticles loaded and unloaded with hydrophobic guabiroba extract (GE). Images are representative of samples and depict (A) and (B) GE-loaded PLGA 50:50; (C) and (D) GE-loaded PLGA 65:35; (E) unloaded PLGA 50:50 (F) unloaded PLGA 65:35. Images were taken at 80 kV with (a) 36,000, (b) 56,000, (c) 22,000 (d) 56,000, (e) 71,000 and (f) 71,000 magnification times. Scale bars are shown by horizontal lines.

higher affinity for its more hydrophobic nature (Zigoneanu et al., 2008). Additionally, PLGA 50:50, with its lower lactide content and molecular weight, is more hydrophilic and consequently it exhibits a more rapid water uptake and begins to degrade more quickly through hydrolysis (Stevanović & Uskoković, 2009). The faster degradation of the polymer matrix will lead to a more rapid release of the entrapped material through pores created in the nanoparticle surface. Kaihara, Matsumura, Mikos, and Fisher (2007) have shown that PLGA 50:50 is the fastest degrading composition, with the degradation rate being decreased when either lactide or glycolide content of the copolymer was increased. Furthermore, this study showed that as PLGA molecular weight increases the initial burst during release decreases in magnitude (Kaihara et al., 2007).

3.5. Antimicrobial activity

The free GE did not show antimicrobial activity (Table 2) for the concentrations tested (up to 5000 $\mu\text{g}/\text{mL}$) against *L. innocua*, which is probably related to the extract active compounds having low water solubility, since it is difficult for these compounds to interact with microorganisms in aqueous suspensions. Nevertheless, once

the GE was entrapped in the PLGA, water solubility was increased due to the encapsulant material, enhancing GE interaction with the microorganism, and consequently antimicrobial effects were observed which clearly indicate the benefits of encapsulation with PLGA (Table 2). Controls showed no inhibitory action against *L. innocua* (data not shown). The antimicrobial activity of GE was likely due to other hydrophobic compounds present in the extract other than carotenoids such as essential oils and hydrophobic polyphenolics which are known to present strong antimicrobial activity (Raybaudi-Massilia, Mosqueda-Melgar, Soliva-Fortuny, & Martin-Belloso, 2009).

The MIC of GE-loaded PLGA 65:35 showed to be slightly higher ($P < 0.05$) than PLGA 50:50 loaded nanoparticles, which could be attributed to the more gradual initial burst effect in the GE release exhibited by the PLGA 65:35. Similar to the carotenoids release, it is possible that more antimicrobial compounds in GE were released initially by PLGA 50:50 on the first few hours, consequently these compounds would be able to interact with more pathogens and due to a faster release it would allow less recovery time for pathogens damaged during the initial burst. The release of GE compounds was effective in preventing *L. innocua* growth with antimicrobial compounds being released at a concentration that allowed an inhibitory

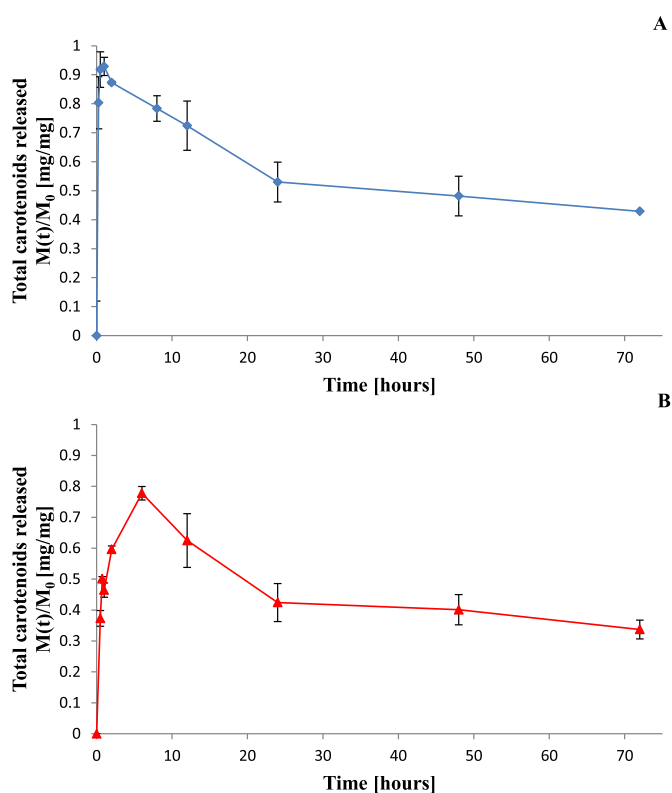


Fig. 2. Hydrophobic guabiroba extract (expressed as total carotenoids) release kinetics from (A) PLGA 50:50 and (B) PLGA 65:35 nanoparticles in 0.1 mol/L SDS and 0.1 mol/L NaCl (pH 6.8 ± 0.2) at 35°C . Symbols are means of 3 replicate measurements and error bars represent standard deviations. $M(t)$ is the carotenoids content in the nanoparticles (in mg) at time t (hours), and M_0 is the initial carotenoids content (in mg). $M(t)/M_0$ is the carotenoid release.

activity over time; however, not high enough antimicrobial compounds were released initially to cause bactericidal effect. *L. innocua* is a Gram-positive microorganism and it is usually considered more resistant to natural antimicrobials than Gram-negative microorganisms because its cell membrane is more difficult to access (Walsh et al., 2003).

The MIC values for GE-loaded nanoparticles can be compared to other natural antimicrobials. For instance, MIC values of 1600, 2000, and 1000 $\mu\text{g/mL}$ for *trans*-cinnamaldehyde, eugenol, and

Table 2

Minimum inhibitory and bactericidal concentration (MIC and MBC) values for *Listeria innocua* for free hydrophobic guabiroba fruit extract (GE) and its loaded poly-D,L-lactide-co-glycolide (PLGA) nanoparticles.

Treatment	MIC ^a ($\mu\text{g/mL}$)	MBC ^b ($\mu\text{g/mL}$)
GE PLGA 50:50	954a	>5000
GE PLGA 65:35	1124b	>5000
Free GE	>5000	>5000

Two types of PLGA were used with lactide: glycolide ratios of 50:50 (PLGA 50:50) and 65:35 (PLGA 65:35). Values are shown as the mean of three independent repetitions. Controls consisting of inoculum exposed to unloaded nanoparticles and tween 20 at the tested concentrations showed no inhibitory action against *Listeria innocua*.

^{a,b} Means within a column which are not followed by a common online letter are significantly different ($P < 0.05$).

^a Values are the lowest concentration of hydrophobic guabiroba fruit extract for which a ≤ 0.05 OD at 630 nm change was observed after 24 h incubation at 35°C in tryptose phosphate broth.

^b Values are the lowest concentration for which a $3.0 \log_{10}$ CFU/mL inactivation in cells was determined by plating. Values preceded by a higher than (>) means that tested concentrations were not sufficient to determine the MBC values.

passion-fruit byproduct extract loaded PLGA nanoparticles; respectively, against *Listeria* spp. have been reported (Gomes et al., 2011; Silva et al., 2014). However, other natural antimicrobials have shown lower MICs values against *Listeria* spp. when encapsulated with PLGA such as cinnamon bark extract (500 $\mu\text{g/mL}$), acerola (250 and 500 $\mu\text{g/mL}$), and guava fruit extract (200–500 $\mu\text{g/mL}$) (Hill et al., 2013; Silva et al., 2014). It is important to note that these fruit extracts were prepared using a different extraction method (ethanolic extraction), which tends to extract more phenolic compounds.

3.6. Antioxidant activity

Antioxidant activity results for free GE and GE-loaded nanoparticles based on DPPH and ABTS radicals' sequestration are shown on Table 3. Unloaded PLGA nanoparticles showed no antioxidant effect (data not shown) for both methods. Generally, high scavenging activity is indicated by a small IC₅₀ value (Brand-Williams et al., 1995). For DPPH method, the free GE extract presented the highest antioxidant activity, respectively 6 to 10 times higher ($P < 0.05$) than GE-loaded PLGA 50:50 and PLGA 65:35. While, for the ABTS method, the free GE extract and GE-loaded PLGA 50:50 showed equivalent ($P > 0.05$) antioxidant activity and both treatments were significantly higher than GE-loaded PLGA 65:35. A high Trolox equivalent number indicates a high antioxidant activity as compared to a standard antioxidant (Trolox) (Re et al., 1999). The observed results could be an indication that encapsulation makes GE less available to react with the free radicals during the reaction time. Moreover, the rate of radical reaction is considerably high compared to the release rate of carotenoids from PLGA, specifically for PLGA 65:35 which showed a slower and gradual release compared to PLGA 50:50. Ganea et al. (2010) synthesized thymoquinone-loaded PLGA nanoparticles using different emulsifiers and contrary to this study, reported higher ($P < 0.05$) or equivalent ($P > 0.05$) antioxidant activity for PLGA-loaded nanoparticles than the free compound, which could be explained by the thymoquinone release profile from PLGA nanoparticles that were either similar to or faster than the free compound release.

These results indicate that GE compounds have different antioxidant mechanisms of action. Previous studies have shown that there is no reaction between the DPPH radical and carotenoids (Müller, Fröhlich, & Böhm, 2011), therefore; the observed results were likely due to other compounds present in the extract. Nonetheless, ABTS radical reacts with carotenoids with increasing reactivity based on their chemical structure (i.e.; number of conjugated double bonds, ionization potentials, etc.) (Müller et al., 2011). GE antioxidant activity in this study was higher ($P < 0.05$) than araticu-do-mato (16,000 g/g DPPH, 3.85 $\mu\text{mol/L TE/g}$, *Rollinia sylvatica* A. St.-Hil.), pindo palm fruit (3848 g/g DPPH, 25.96 $\mu\text{mol/L TE/g}$).

Table 3

Antioxidant activity of free hydrophobic guabiroba fruit extract (GE) and its loaded poly-D,L-lactide-co-glycolide (PLGA) nanoparticles by the DPPH and ABTS free radical methods.

Treatment	^a DPPH (g/g DPPH)	^a ABTS ($\mu\text{mol/L TE/g}$)
GE PLGA 50:50	876.5 \pm 54.6b	484.8 \pm 34.2a
GE PLGA 65:35	1353.7 \pm 26.2c	415.1 \pm 2.7b
Free GE	129.7 \pm 10.8a	505.9 \pm 49.0a

Two types of PLGA were used with lactide: glycolide ratios of 50:50 (PLGA 50:50) and 65:35 (PLGA 65:35). Values are shown as the mean \pm standard deviation of three independent repetitions.

^{a,b} Means within a column which are not followed by a common online letter are significantly different ($P < 0.05$).

^a Results are expressed as IC₅₀ (concentration required to reduce the original amount of free radical by 50%) in g of dry extract per g of DPPH and $\mu\text{mol/L}$ Trolox Equivalent (TE) per g of dry extract for DPPH and ABTS method, respectively.

TE/g, *Butia capitata* (Mart. Becc.) and mandacaru-de-três-quinas (3250 g/g DPPH, 19.61 $\mu\text{mol/L}$ TE/g, *Cereus hildmannianus* K. Schum.) (Pereira et al., 2013), acai (598 g/g DPPH, 64.5 $\mu\text{mol/L}$ TE/g, *Euterpe oleracea*) and cashew apple (906 g/g DPPH, 79.4 $\mu\text{mol/L}$ TE/g, *Anacardium occidentale*) (Rufino et al., 2010).

3.7. Reactive oxygen species (ROS) generation assay

For non-cancer cells (CCD-18Co), results indicated that all cells treated with free GE extract and GE-loaded nanoparticles showed effective concentrations to decrease significantly ROS generation (Fig. 3). Concentrations around 30 times lower ($P < 0.05$) than free GE extract (275 μg extract/mL) were needed when the extract was entrapped in PLGA 50:50 (8.5 μg extract/mL) and PLGA 65:35 (10 μg extract/mL) to obtain the same ROS generation inhibition effect (approximately 7% inhibition compared to positive control cells). Thus, encapsulation using PLGA enhanced GE effect in inhibiting ROS generation by reducing the extract amount needed to obtain the same effect. The negative control cells, which were not H_2O_2 induced oxidative damage, showed 3.5% less ($P < 0.05$) ROS generation than positive control, which were H_2O_2 induced oxidative damage.

For cancer cells (HT-29), the concentration of free GE extract (275 μg extract/mL) used increased significantly the ROS generation

by 2% compared to negative control (not treated); however, when they were treated with a small concentration of GE-loaded PLGA 50:50 (8.5 μg extract/mL) and PLGA 65:35 (10 μg extract/mL) nanoparticles, the ROS production showed a considerable decrease ($P < 0.05$) of 13.4% and 14.9%, respectively; without significant difference between nanoparticles. These results show that the same free GE extract concentration (275 μg extract/mL) that induces oxidative damage in cancer cells also protects the non-cancer cells against ROS. Moreover, encapsulation with PLGA was able to effectively inhibit ROS generation at lower GE concentrations in both non-cancer and cancer cells. The observed ROS increase in cancer cells caused by free GE extract may have been caused by an increased basal oxidative stress which may have driven them beyond a tumor-sustaining threshold, making them more sensitive to oxidative stress that eventually leads to cell death (Schumacker, 2006).

4. Conclusions

GE-loaded PLGA nanoparticles with different lactide to glycolide ratios were successfully synthesized using the emulsion-evaporation method. GE release profiles were different between PLGA types and showed to have a significant effect on nanoparticles biological properties (antioxidant and antimicrobial activities and ROS generation). Overall, GE-loaded PLGA 50:50 presented the best results for nanoencapsulation in terms of release profile, antimicrobial and antioxidant activity and ROS inhibition and it should be the preferred polymer ratio used for GE delivery applications. Results show that nanoencapsulation with PLGA could be useful to other extracts containing carotenoids and other functional lipids as delivery systems for enhanced biological activity.

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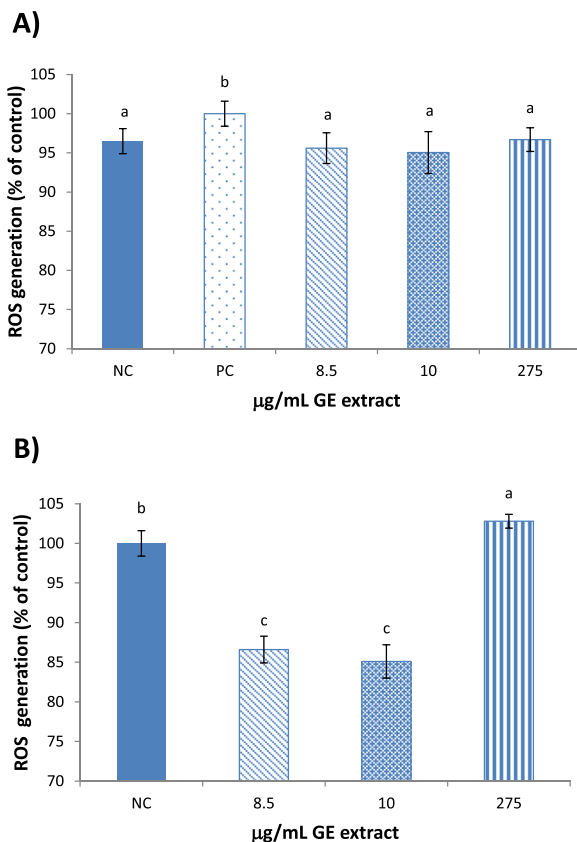


Fig. 3. Generation of reactive oxygen species (ROS) in (A) noncancer colonic myofibroblasts CCD-18Co cells and (B) in human colon adenocarcinoma HT-29 cells treated with free hydrophobic guabiroba fruit extract (□) GE and its loaded poly-D,L-lactide-co-glycolide (▨) GE PLGA 50:50 and (■) GE PLGA 65:35 nanoparticles. Two types of PLGA were used with lactide: glycolide ratios of 50:50 (PLGA 50:50) and 65:35 (PLGA 65:35). HT-29 cells (B) were not stimulated with H_2O_2 because they are constitutively under higher than normal oxidative stress. CCD-18Co cells (A) were stimulated with H_2O_2 . NC (■) = negative control cells were not H_2O_2 induced and PC (▨) = positive control cells were H_2O_2 induced. Values are means of 5 replicate measurements and error bars represent standard deviations; different letters indicate significance at $P < 0.05$.

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