

Innovative encapsulation for enhanced drug delivery using stingless bee propolis and honey *Tetragonisca angustula* (Hymenoptera: Meliponini), and their antimicrobial activity

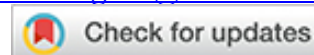
Diego Otuna-Hernández ^{1,2}, Alexandra D. Hernández Hernández ², Leslie Espinoza-Castro ¹,
Lilian M. Spencer ¹, Hortensia Rodríguez ^{1,3,*}

¹ School of Biological Sciences and Engineering, Yachay Tech University, Urcuquí 100119, Ecuador;
diego.otuna@yachaytech.edu.ec; alehernandez2301@gmail.com; leslie.espinosa@yachaytech.edu.ec;
lspencer@yachaytech.edu.ec

² Instituto Nacional de Biodiversidad (INABIO), Quito 170501, Ecuador.

³ Yachay Tech Medicinal Chemistry Research Group (MedChem-YT), School of Chemical Science and Engineering, Yachay Tech University, Urcuquí 100119;

* Correspondence: hmrodriguez@e-mail.com



ABSTRACT

Propolis, renowned for its immunological and therapeutic properties due to its flavonoids and phenolic acids, has been utilized in alternative medicine for many years. In Ecuador, the commercialization of stingless bee honey has been increasing recently, although the physical-chemical and biological characteristics of honey from each species are unknown due to their different behaviors and diets. The objective of this research was to synthesize chitosan nanoparticles by encapsulating commercial honey (NPMC), honey (NPMA), and propolis (NPP) from *Tetragonisca angustula* by ionic gelation and glutaraldehyde cross-linking. The polymeric nanoparticles were structurally characterized (TGA and FT-IR), and their antimicrobial activity against *E. coli* and *S. aureus*, cytotoxicity, and controlled release were evaluated in two phases at different pH levels, one gastric (pH 1.8) and intestinal (pH 7.4). FT-IR analysis of the polymeric nanoparticles revealed the presence of hydroxyl and alkyl groups. NPMA and NPP showed characteristic vibrations of flavonoids and C=O, regardless of the molecular weight of chitosan. In the TGA, the NPMC had decomposition efficiencies of 97.22% (LMW) and 92.10% (HMW). Propolis nanoparticles achieved encapsulation efficiencies of 94.31% and 89.21%. NPPA (HMW-chitosan-based nanoparticles encapsulating propolis from stingless bees) was the most effective against *S. aureus*. NPPB (LMW-chitosan-based nanoparticles encapsulating propolis from stingless bees) at 10 µg/mL showed higher cytotoxicity than NPMMA (HMW-chitosan-based nanoparticles encapsulating honey from stingless bees). In summary, propolis nanoparticles maintained efficiencies above 76%, while NPMC and NPMA exhibited loading capacities greater than 27%. In contrast, formulations with high molecular weight chitosan showed slightly lower values, although these differences were not statistically significant.

Keywords: *Tetragonisca angustula*, stingless bee; polymeric nanoparticles; controlled released; antimicrobial activity

INTRODUCTION

Bees, with more than 20,000 species identified globally, are crucial for pollination, a vital process for sustaining life on land. The family Apidae, particularly the stingless bee tribe Meliponini, plays an essential role in tropical and subtropical ecosystems. The *Apis mellifera* species is known for its efficiency in honey production, but it is essential to highlight the importance of native species, such as *Tetragonisca angustula*, for their ecological functions and highly beneficial products. This species can be found only in Latin America, from Mexico to northern Argentina^{4,5}. In Ecuador, there are many information gaps about the total number and

distribution of stingless bee species. However, several researchers specializing in tropical bees have recorded this species and conducted studies that demonstrate its importance in various ecosystems. Meliponiculture emerges as a sustainable practice that benefits the conservation of stingless bees⁷. Additionally, pollination by these species is vital for 30% to 80% of plants in their respective biomes, underscoring their importance in the conservation of faunal and floral communities⁸. The morphological features of *Tetragonisca angustula* are presented in Figure 1, supporting its identification and distribution in Latin America. Its honey is prized for its unique flavor and health benefits, and is commonly used in respiratory and eye treatments in communities⁹. The propolis of this species stands out for its antimicrobial properties, attributed to flavonoids⁹. These products are of great interest to the population of the Amazon region because they have low humidity and therapeutic potential¹⁰.

Nanotechnology has become an important alternative for stabilizing applications, ranging from proteins to drug delivery.¹¹ Their versatility and nanometer size make them excellent candidates for a variety of applications, including drug discovery, water purification, imaging, and others¹². Methods such as ionic gelation and microemulsion are commonly used for its synthesis, offering a promising approach for drug delivery and other biomedical applications¹³. Conversely, chitosan is a versatile and biodegradable polysaccharide with unique properties^{14,15}. Modulating deacetylation and its molecular weight significantly influence its physical and biological properties^{16,17}. Chitosan nanoparticles offer a safe and effective method of drug delivery, as they enable the controlled loading of drugs due to their amino and hydroxyl groups. Drug delivery systems are crucial for improving therapeutic efficacy and minimizing side effects. The combination of drug delivery systems with natural products offers a promising strategy for treating chronic diseases, such as cancer, by leveraging the biocompatible and non-toxic properties of these materials. Finally, antimicrobial activity requires an essential criterion of antibacterial susceptibility testing; normally, the strains used to perform these tests are *E. coli* and *S. aureus*²¹.

Herein, this study aimed to synthesize, characterize, and test the antimicrobial activity of LMW and HMW-chitosan-based nanoparticles (NPQ) encapsulating commercial honey (NPMC), stingless bee propolis (NPP), and honey (NPMA) *Tetragonisca angustula* (Hymenoptera: Meliponini). Both spectroscopies (Fourier-transformed Infrared FT-IR and Thermogravimetric analysis TGA) were applied for the structural characterization of nanoparticles. Likewise, antimicrobial activity against two strains of *E. coli* and *S. aureus*, cytotoxicity using a standard Roche protocol, and controlled release simulating two phases at different pH, one gastric (pH=1.8) and intestinal (pH=7.4), were examined. The outcomes may lead to a novel design, improved stability, and enhanced biocompatibility of propolis *Tetragonisca angustula* bioactive compounds during processing, with potential biomedical applications.

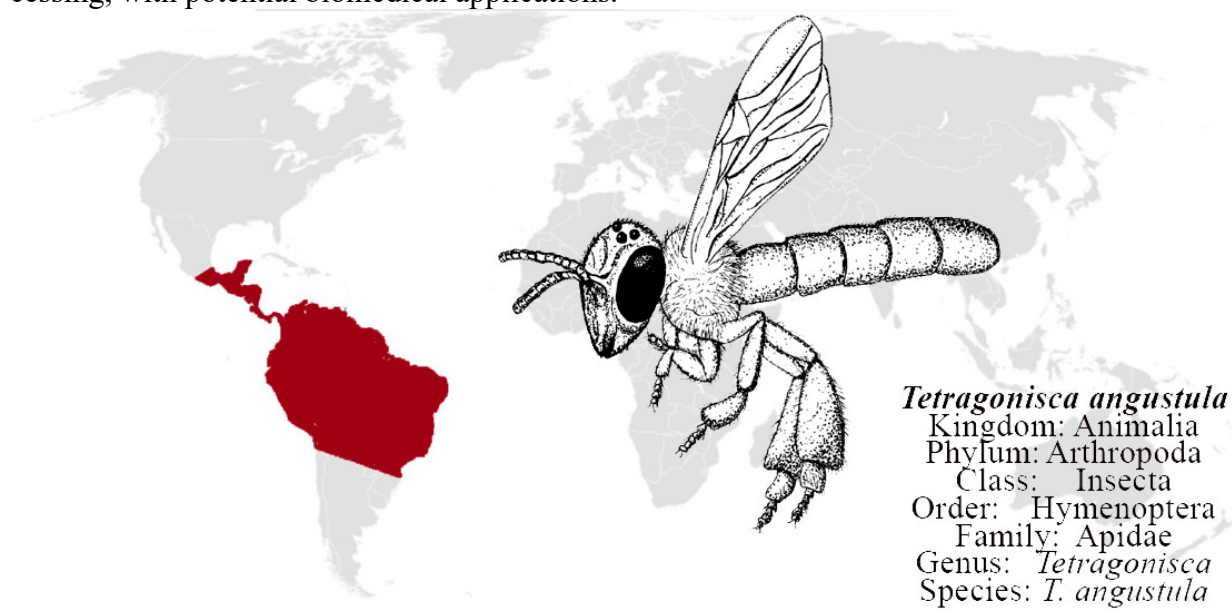


Figure 1. *Tetragonisca angustula*. Scientific illustration of the stingless bee species studied, native to Latin America and valued for its honey and propolis with medicinal properties. (*Tetragonisca angustula* illustration by Alexandra D. Hernández Hernández, 2024)

MATERIAL AND METHODS

Materials

Propolis and stingless bee honey *Tetragonisca angustula* (Hymenoptera: Meliponini) were obtained directly from a meliponary (Puyo, Ecuador). Chitosan (low and high molecular weight), acetic acid (1%), arabic gum (0.3%), glutaraldehyde (1%), and all other chemicals were acquired at the Laboratory of Chemical Sciences and Engineering, and Biological Sciences and Engineering of Yachay Tech University, respectively (Urcuquí, Ecuador).

Equipment

Fourier transform infrared spectroscopy (FT-IR) was performed on a Cary 630 FT-IR spectrometer; the analysis was performed with the method (Attenuated total reflection). Thermogravimetric analysis (TGA) was carried out on a TGA-1000/1500 using a small microfurnace with a small scanning volume. ThermoFisher Scientific™ Company Heratherm IMP-180 refrigerated incubator, ThermoFisher Scientific™ Company Nanodrop Uv-Vis spectrophotometer, and Telstar AV-30/70 vertical laminar flow cabinet were used to perform biological essays. Finally, drug delivery release was performed using a ThermoFisher Scientific™ Company pH meter, a ThermoFisher Scientific™ Company AccuSpin 24C centrifuge, and a Thermo Scientific™ TX-400 4 x 400 ml tiltable rotor (75003180).

Extraction of the propolis

Propolis was extracted using 70% ethanol by the maceration method²². The ratio of solvent and propolis was 10:1 (v/w). First of all, 10 g of propolis was vigorously mixed with 100 mL of 70% ethanol for 10 minutes at room temperature, 25°C. This mixture was left to rest for 24 hours at room temperature in a stirrer with a speed of 120 rpm. It was then vacuum-filtered through filter paper. The filtrate was then concentrated using a rotary steamer at 40°C at 120 rpm (Stirring plate) to remove the solvent ethanol. Finally, 3.6 g of concentrate was obtained and stored at room temperature.

Preparation of chitosan-based nanoparticles encapsulating honey and propolis extract

15mL of chitosan solution (0.7%) was prepared by dissolving the chitosan powder in acetic acid (1%) under continuous stirring for one hour. 7.5 mL arabic gum (0.3%) solution was prepared in ionized type I water at room temperature, to which 1.5 mL of propolis extract *Tetragonisca angustula* (Hymenoptera: Meliponini) (25%) was added gradually, by vigorous agitation, until a homogeneous mixture was achieved. Arabic gum/propolis extract mixture was added to the chitosan solution, and then 7.5 mL of glutaraldehyde (1%) was injected as a cross-linking agent by syringe, under continuous agitation, for two hours, thus allowing the formation of nanoparticles. For FT-IR, TGA, and biological activity measurements, the nanoparticles were super-frozen (-80°C), then lyophilized, and finally stored at room temperature until characterization and biological assays. The same procedure was used to make the different nanoparticles of commercial honey and stingless bee honey *Tetragonisca angustula* (Hymenoptera: Meliponini) of LMW and HMW-chitosan, respectively.

Structural characterization

Polymeric nanoparticles were investigated by FT-IR (Fourier Transformed Infrared Spectroscopy) in the range 4000-900 cm⁻¹, using the Agilent Cary 630 FT-IR spectrometer, which used an ATR system. TGA (Thermogravimetric analysis) was performed with a Q500 instrument. The nanoparticle samples (approximately 5.0 ± 2.0 mg) were placed on a standard platinum palette. The scan was performed at a rate of 10 °C/min under a nitrogen flux, and the change in mass from 25 °C to 700 °C was recorded.

Antimicrobial activity

The strains used to evaluate antimicrobial activity were *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ECBI-UI TEY through the Kirby-Bauer disk diffusion susceptibility test on Mueller-Hinton (MH) agar medium²³. Reserve bacterial cultures were incubated for 12 hours at 37°C and 120 rpm in a sterile Luria Bertani broth base (LB). Lyophilized nanoparticle samples to be tested were prepared in a circular form using a press. All samples were made with a weight of 0.6 mg at a concentration of 0.16 µg/mL. The nanoparticles

were neither diluted nor filtered to avoid loss; they were only hydrated with previously autoclaved Type I water. Once this process was completed, each MH agar plates were seeded with bacterial strains (*E. coli* ATCC 25922 and *S. aureus* ECBI-UI TEY) whose turbidity was adjusted to the 0.5 McFarland standard. The inoculated plates were then incubated at 37 °C for 24 hours. After this period, the inhibition zones were observed. Ampicillin (1 mg/mL) and chitosan-based nanoparticles of HMW and LMW were used as positive control and blank, respectively.

MTT colorimetric assay for cytotoxicity

The standard protocol of Roche Corporation for colorimetric cell proliferation assays²⁴ was employed to assess the cytotoxicity of the nanoparticle samples. These samples were previously treated, meaning that initially a concentration of 1 mg/mL was obtained, and subsequently, they were diluted to achieve concentrations of 10 µg/mL and 100 µg/mL, respectively. On the other hand, the 3T3 fibroblast cell culture was prepared in advance. Cell line was harvested using a cell scraper, and cell viability was assessed by staining with trypan blue in a Neubauer chamber. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% antibiotics and 10% heat-inactivated fetal bovine serum (FBS) was used, and this medium was prepared in two different concentrations, both 1x and 4x. Culture medium was added to the 96-well microplates for cell culture. First, 50 µL of 1x medium was added, followed by 50 µL of 4x medium, then 50 µL of each previously diluted nanoparticle sample (10 µg/mL and 100 µg/mL, respectively), and finally 50 µL of cells. Finally, the microplate was incubated at 37 °C for 72 hours with 5% CO₂. Subsequently, 20 µL of the MTT reagent was added to each well and incubated for 4 hours at 37 °C in a humidified chamber. During this incubation, absorbance was measured at 630 nm with the microplate reader at 0 hours, 4 hours, and 4 hours + buffer.

Encapsulation efficiency, loading capacity, and controlled release

The encapsulation efficiency of the nanoparticles was assessed through a filtration procedure as follows: the nanoparticles were separated from free propolis in the mixture using a membrane filter (PVDF Millex filter unit), and the amount of free propolis in the filtrate was measured spectrophotometrically at 450 nm. Encapsulation efficiency (EE%) and loading capacity (LC%) of the propolis extract in the nanoparticles were determined using the following equations:

$$EE(\%) = \left(\frac{C_t - C_f}{C_t} \right) \times 100 \quad (1)$$

$$LC(\%) = \left(\frac{C_t - C_f}{C_d} \right) \times 100 \quad (2)$$

Where:

C_t corresponds to the total amount of propolis used in the loading stage.

C_f denotes the amount of free propolis in the filtrate.

C_d represents the amount of propolis present in the nanoparticles after the freeze-drying process.

Cumulative release of propolis was investigated in simulated gastrointestinal solutions with pH=1.8 and pH=7.4. Simulated solutions were prepared according to the literature^{25, 26} as follows:

Simulated Gastric Fluid (SGF), an artificial medium designed to simulate stomach acid, was prepared by dissolving 0.66 g of sodium chloride and 1.06 g of purified pepsin (derived from the stomach mucosa of pigs) in 2.32 mL of hydrochloric acid and water, reaching a total volume of 95.96 mL. The final solution was adjusted to a pH of 1.8.

Simulated Intestinal Fluid (SIF), was prepared by dissolving 0.31 g of monobasic potassium phosphate in 25 mL of water. Subsequently, 7.7 mL of 0.2 N sodium hydroxide and 50 mL of water were added. Then, 0.45 g of pancreatin was added, and the resulting solution was adjusted with 0.2 N hydrochloric acid to a pH of 7.4. Finally, the solution was diluted to a total volume of 100 mL.

0.23 g of lyophilized polymeric nanoparticles encapsulating propolis/honey was used, and they were dispersed in the two previously simulated fluids. The release rate was also determined by dividing the gastric phases (0-180 minutes) and small intestinal phases (180-300 minutes), respectively. However, the solution containing released propolis must be centrifuged at 3000 rpm for 10 minutes because propolis is insoluble in water; subsequently, the sedimented propolis was spectrophotometrically measured at 300 nm. Finally, the concentration of released propolis was calculated using a standard absorption curve. Moreover, the percentage of released propolis was calculated using the following equation:

$$R(\%) = \left(\frac{C_{pr}}{C_{pt}} \right) \times 100 \quad (3)$$

Where:

CPr stands for the concentration of released propolis after a specific time interval.

CPt denotes the total amount of propolis encapsulated in the nanoparticles.

Statistical analysis

Data normality was used before further analysis was performed. Shapiro-Wilk and Kruskal-Wallis H-test (for non-parametric variables) were used to test the level of significance in the differences between chitosan-based nanoparticle samples in the cytotoxicity and drug delivery assays. Statistical analysis was performed using IMB SPSS Statistics for Windows version 22.0.

RESULTS

Polymer nanoparticles were synthesized and chemically characterized using FT-IR, HPLC, and TGA. The synthesis was carried out as described in Chapter 2, resulting in eight samples of chitosan-based nanoparticles (with low and high molecular weight) encapsulated with honey and propolis, respectively. All synthesized nanoparticles underwent a freeze-drying process after being frozen in a super-freezer to facilitate chemical testing and biological assays.

Characterization of chitosan-based nanoparticles

The structure of polymeric nanoparticles was corroborated by TGA and FT-IR spectroscopy. Structural characterization of powdered chitosan-based nanoparticles (NPQ) encapsulating commercial honey (NPMC), stingless propolis (NPP), and honey (NPMA) *Tetragonisca angustula* was using low molecular weight (LMW) chitosan-based nanoparticles and secondly using high molecular weight chitosan. For example, Figure 2 shows the chitosan-based nanoparticles with LMW spectrum, with the presence of the distinctive band of the flavonoids and lipids ($\nu C=O$), aromatic rings ($\nu C-H$), and polyols ($\nu C-O$, $\nu C-OH$) groups around 1700 cm^{-1} , 1463 cm^{-1} , and 1032 cm^{-1} respectively, attributed to phosphate groups and proteins, but also the broad characteristics bands of the hydroxyl (νOH), and alkyl ($\nu C-H$) groups at around 3351 cm^{-1} , and 2936 cm^{-1} . On the other hand, Figure 3 shows the chitosan-based nanoparticles with the HMW spectrum. Nevertheless, this spectrum shows one additional group attributed to the presence of chitosan HMW, corresponding to a symmetrical stretch ($\nu C-O-C$) at around 1155 cm^{-1} , which was also observed.

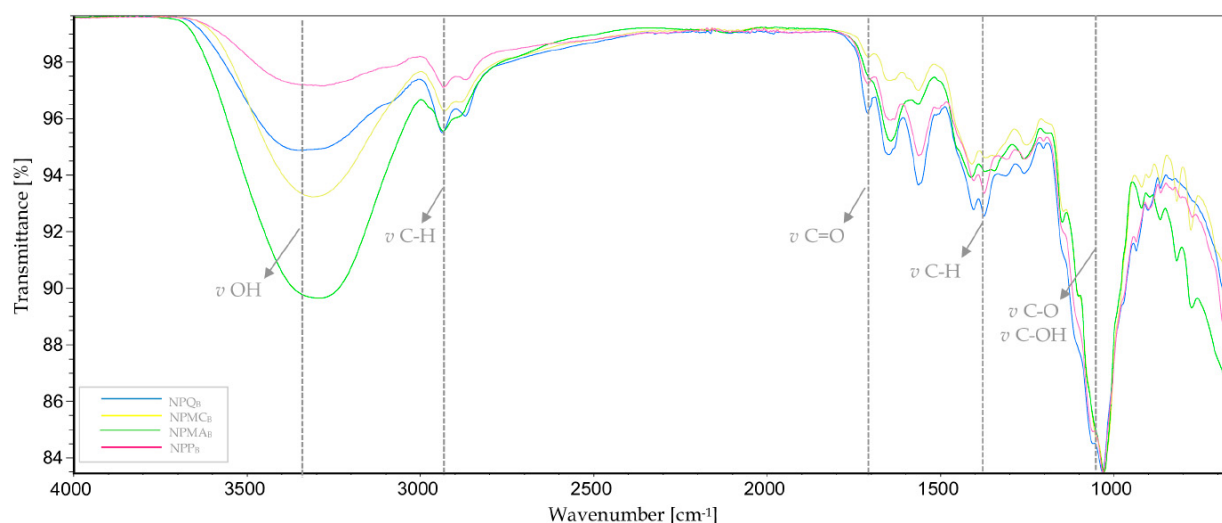


Figure 2. FT-IR spectrum of low molecular weight (LMW) chitosan nanoparticles encapsulating honey and propolis from *T. angustula*. Characteristic bands of flavonoids, lipids, and hydroxyl groups confirm the incorporation of phenolic compounds into the polymeric matrix.

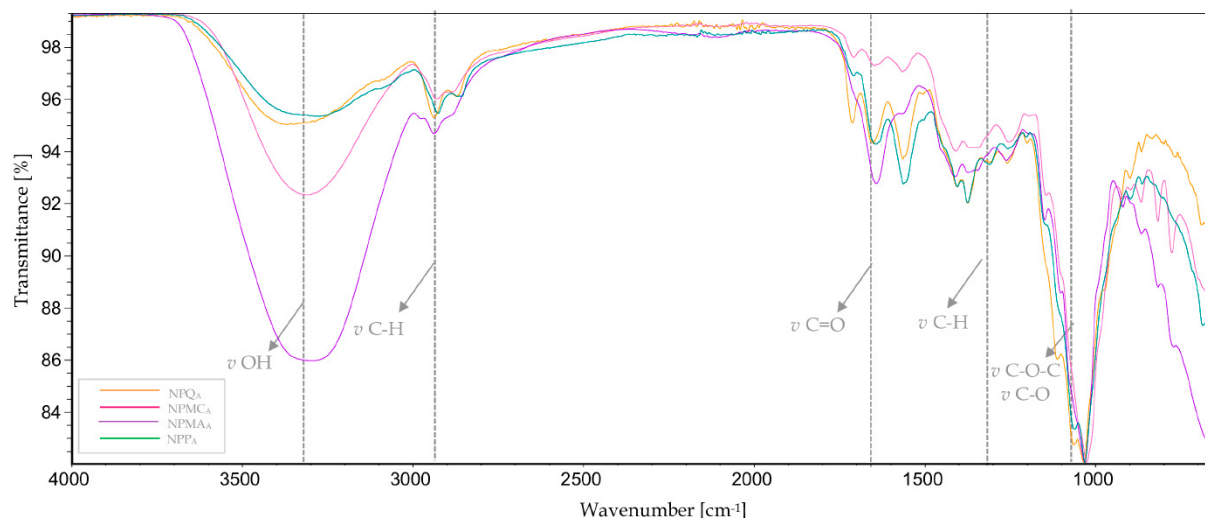


Figure 3. FT-IR spectrum of high molecular weight (HMW) chitosan nanoparticles. An additional signal corresponding to a symmetrical stretch (C–O–C) is observed, attributed to the HMW chitosan structure, highlighting structural differences compared to LMW formulations.

Related to TGA, all analyzed nanoparticle samples showed decomposition similarities. Each experiment was divided into three main groups. Loss of water and chitosan compounds, degradation of chitosan, commercial, stingless bee honey and propolis, and Organic impurities and polymer network decomposition around 50-175°C, 176-300°C, and 500-700°C, respectively (Figures 4 and 5). Based on the data provided, the decomposition of LMW and HMW chitosan-based nanoparticles encapsulating stingless honey (NPMA) exhibits the highest decomposition efficiency, registering 97% for LMW and 92% for HMW. Second, stingless bee propolis (NPP) exhibits high decomposition, reaching 94% for LMW and 89% for HMW. Finally, HMW chitosan-based nanoparticles (NPQ) show relatively lower decomposition at 77%.

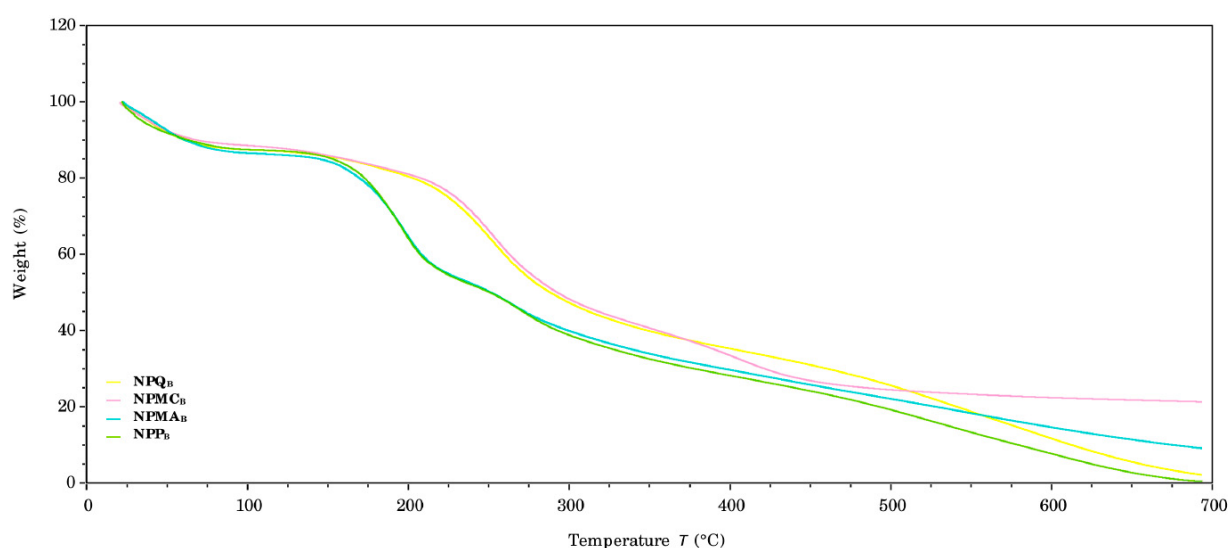


Figure 4. Thermogravimetric analysis (TGA) of low molecular weight chitosan nanoparticles. Three main degradation phases are identified: water loss and volatile compounds (50–175 °C), chitosan and phenolic compounds degradation (176–300 °C), and polymer network decomposition (500–700 °C).

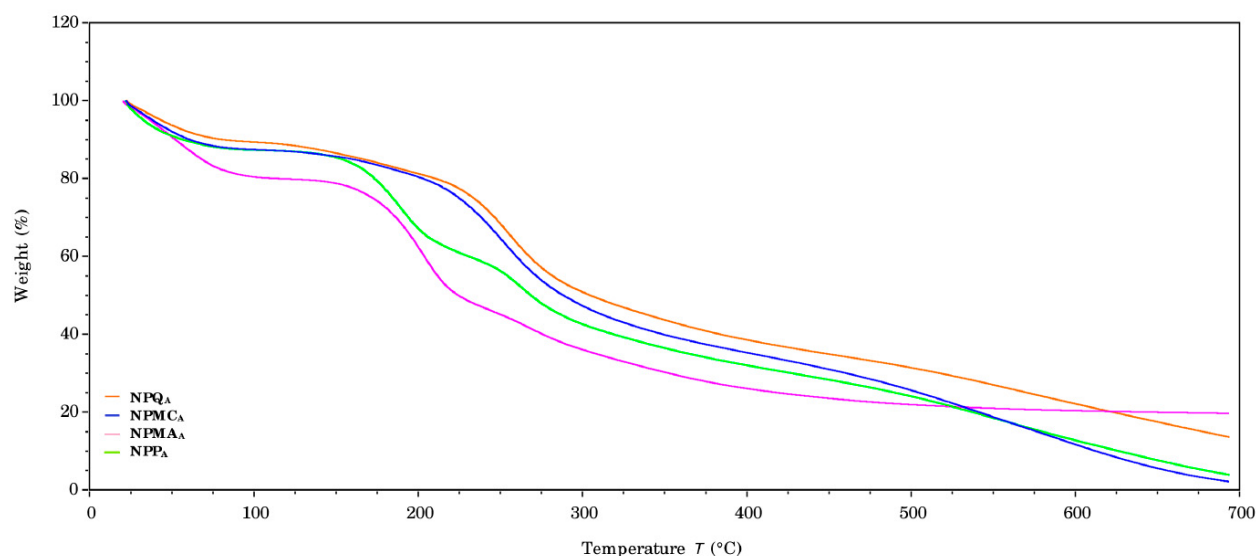


Figure 5. Thermogravimetric analysis (TGA) of high molecular weight chitosan nanoparticles. The profile shows greater thermal stability compared to LMW formulations, reflecting the formation of a denser polymeric network.

Antimicrobial activity analysis

Related to antimicrobial activity, the chitosan-based nanoparticles encapsulating stingless bee propolis (NPP) showed good inhibitory activity against Gram-positive bacteria and low activity against Gram-negative bacteria, as shown in Figure 5 and Table 1. *S. aureus* was more susceptible to all the tested nanoparticle samples, maintaining the same concentration (0.16 µg/mL). Regarding the bactericidal properties of stingless honey, a halo of 0.70 mm was observed. Importantly, this halo occurs specifically in the *S. aureus* ECBI-UI TEY strain and not in *E. coli* ATCC 25922. This difference is explained by the gram-positive nature of the *S. aureus* ECBI-UI TEY strain, which makes it more susceptible to the bactericidal action of stingless honey. In contrast, gram-negative bacteria, such as *E. coli* ATCC 25922, possess an outer cell membrane that makes them more resistant to these effects²⁷.

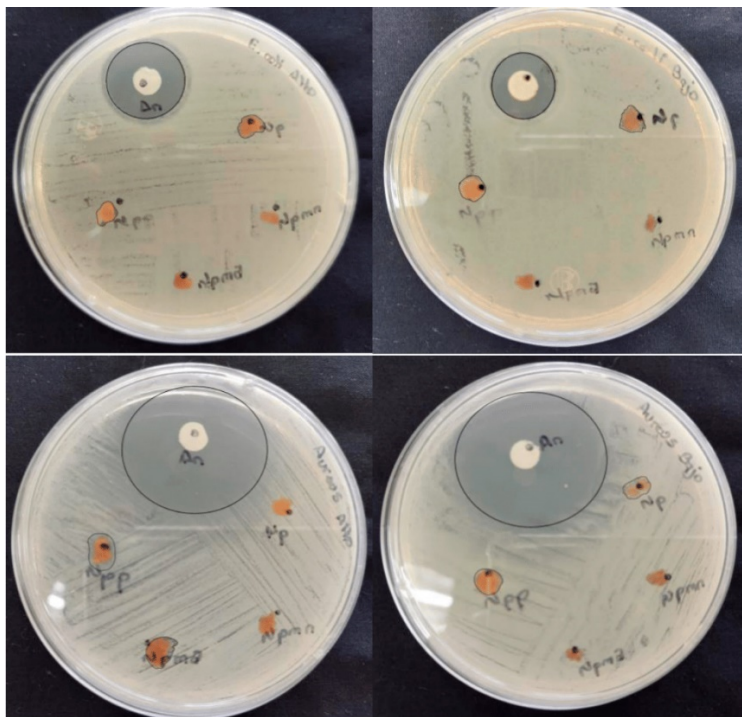


Figure 6. In vitro antimicrobial activity of nano- particles at 0.16 µg/mL against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. In- hibition zones demonstrate greater susceptibility of *S. aureus* to the encapsulated compounds.

Chitosan-based nanoparticles	Concentration (µg/mL)	Diameter of Inhibition zone (mm) ^b	
		<i>S. aureus</i> ECBI-UTEY ^a	<i>E. coli</i> ATCC 25922 ^a
NPQ _B	0.16	0.37 ± 0.11	0.45 ± 0.15
NPQ _A		-	0.40 ± 0.15
NPMC _B		-	-
NPMC _A		-	-
NPMA _B		-	-
NPMA _A		0.70 ± 0.15	-
NPP _B		0.66 ± 0.18	0.71 ± 0.22
NPP _A		1.10 ± 0.10	0.84 ± 0.15

Table 1. Antimicrobial activity of chitosan nanoparticles encapsulating honey and propolis from *T. angustula* against *S. aureus* (Gram-positive) and *E. coli* (Gram-negative). Inhibition zone diameters (mm ± SD) were evaluated at 0.16 µg/mL using the disk diffusion assay.

Cytotoxicity assay analysis

In this study, the cytotoxicity of the various polymeric nanoparticles was evaluated at concentrations of 10 and 100 µg/mL, respectively. The results were recorded at 630 nm using 96-well microplates for a cell culture reader at three time points: at time zero, after four hours, and after four hours with the buffer. The four-hour measurement was previously selected because it is the time needed for the reaction to fully develop, thus ensuring optimal measurement, as illustrated in Figure 7. To assess possible significant differences, a one-way analysis of variance ($P > 0.05$) was applied. The data associated with the 10 µg/mL concentration are parametric in nature, revealing that there are no significant differences between nanoparticles encapsulated with commercial honey, stingless bee propolis, and honey, compared to the control. Similarly, it is observed that nanoparticles at concentrations of 100 µg/mL do not generate harmful effects, since the Kruskal-Wallis statistical analysis did not reveal significant differences between the nanoparticles and the control.

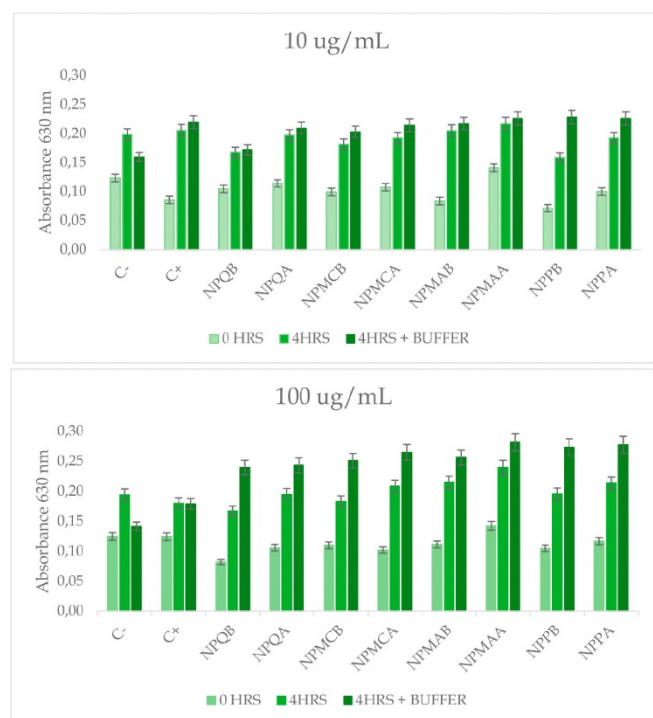


Figure 7. Cytotoxicity (MTT) assay of nanoparticles at 10 and 100 µg/mL on 3T3 fibroblasts. Absorbance values at 630 nm were recorded after four hours of incubation. No significant differences with the control were observed, confirming the biocompatibility of the formulations.

Encapsulation efficiency and loading capacity

Encapsulation efficacy (EE%) and loading capacity (LC%) of propolis extract and stingless honey in a chitosan matrix reinforced with arabic gum and cross-linked with glutaraldehyde were calculated according to ratios (2.1) and (2.2) previously described in the Methodology. Importantly, the trial was conducted in triplicate. The EE% and LC% results for two different pH phases are presented in Table 2. It is observed that chitosan-based nanoparticles encapsulated with propolis show encapsulation efficiencies greater than 76% under both simulations. Additionally, it is intriguing to note that the encapsulation efficiency (EE%) for the LMW-chitosan-based nanoparticles encapsulated in stingless honey is similar in both phases. Regarding loading capacities, nanoparticles encapsulated with stingless bee propolis exhibit higher values, exceeding 29% in both conditions. Notably, LMW-chitosan-based nanoparticles, both encapsulating commercial honey and stingless bee honey, show good loading capacities, exceeding 27%.

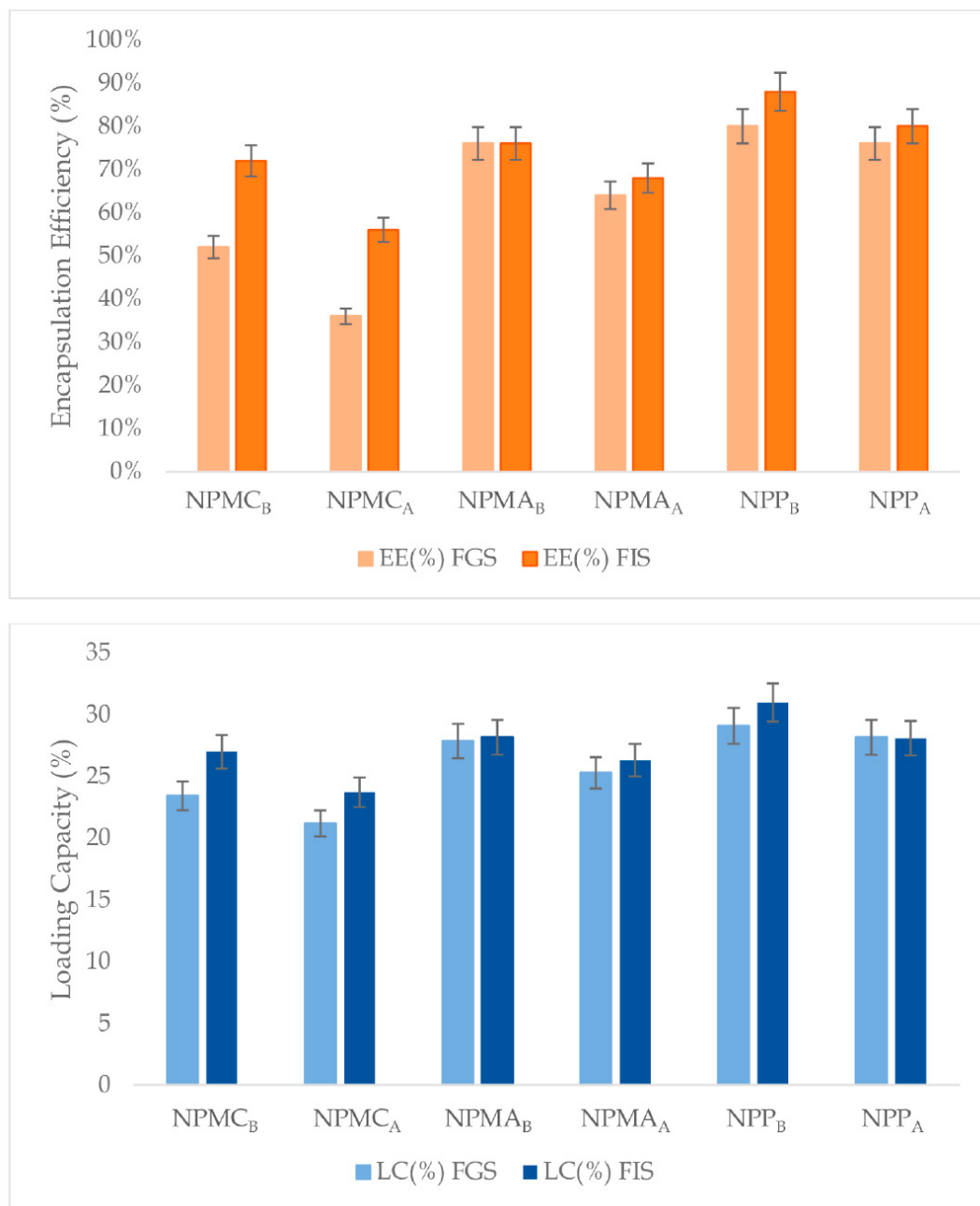


Figure 8. Encapsulation efficiency (EE%) and loading capacity (LC%) of chitosan nanoparticles loaded with honey and propolis from *T. angustula* in simulated gastric (pH 1.8) and intestinal (pH 7.4) fluids. Results indicate higher retention and controlled release for propolis compared to honey.

Chitosan-based nanoparticles	EE% FGS	EE% FIS	LC% FGS	LC% FIS
NPMC _B	52.00 ± 0.28	72.00 ± 0.46	23.44 ± 0.93	27.00 ± 0.80
NPMC _A	36.00 ± 0.14	56.00 ± 0.31	21.20 ± 1.16	23.73 ± 0.82
NPMA _B	76.00 ± 0.50	76.00 ± 0.50	27.88 ± 0.80	28.18 ± 0.75
NPMA _A	64.00 ± 0.39	68.00 ± 0.42	25.31 ± 0.81	26.33 ± 0.74
NPP _B	80.00 ± 0.53	88.00 ± 0.60	29.11 ± 0.75	31.00 ± 0.76
NPP _A	76.00 ± 0.50	80.00 ± 0.53	28.18 ± 0.75	28.11 ± 0.75

Table 2. Encapsulation efficiency (EE%) and loading capacity (LC%) of chitosan nanoparticles loaded with honey and propolis from *T. angustula* in simulated gastric fluid (SGF, pH 1.8) and simulated intestinal fluid (SIF, pH 7.4). Propolis-loaded nanoparticles achieved the highest EE% and LC% values under both conditions.

In vitro drug delivery release

The results of the determination of the encapsulation concentrations of the natural products used in this study are detailed in Figures 9, 10, and 11, respectively. It is noted that the best encapsulations are achieved with LMW-chitosan. Despite the nature of commercial honey, FGS with LMW-chitosan is observed to reach 0.09 mg/mL encapsulation, while FIS with the same chitosan variant reaches 0.15 mg/mL. Nevertheless, there was a loss of 16% in FGS and 12% in FIS, with an improvement of 4% for FIS compared to FGS, both using low molecular weight chitosan. This loss could be associated with commercial residues of honey in synthesis or freeze-drying.

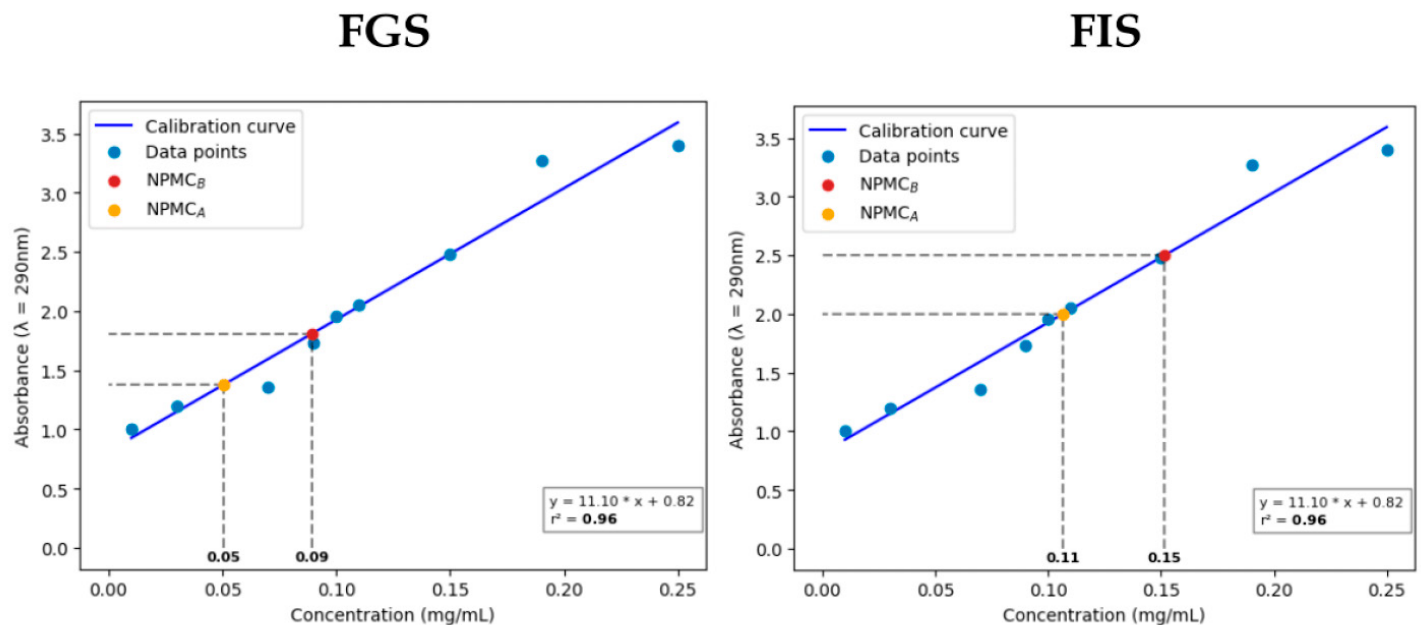


Figure 9. Encapsulated concentrations of commercial honey in simulated gastric (SGF) and intestinal (SIF) fluids, measured at 290 nm. A higher loss is observed in the gastric phase, associated with honey crystallization.

In contrast, Figure 10 shows the results of the concentration of chitosan nanoparticles encapsulated with stingless honey. Compared to commercial honey, significant differences are observed: in FGS, the encapsulation concentration reaches 0.16 mg/mL, while in FIS, 0.17 mg/mL is recorded. Nonetheless, in the concentrations of stingless honey encapsulations, there is evidence of a lower loss of natural product compared to commercial honey, around 12% for FGS, while in FIS there is a loss of 8%. This variation is associated with the crystallization of commercial honey, while stingless honey remains stable due to its acidity.

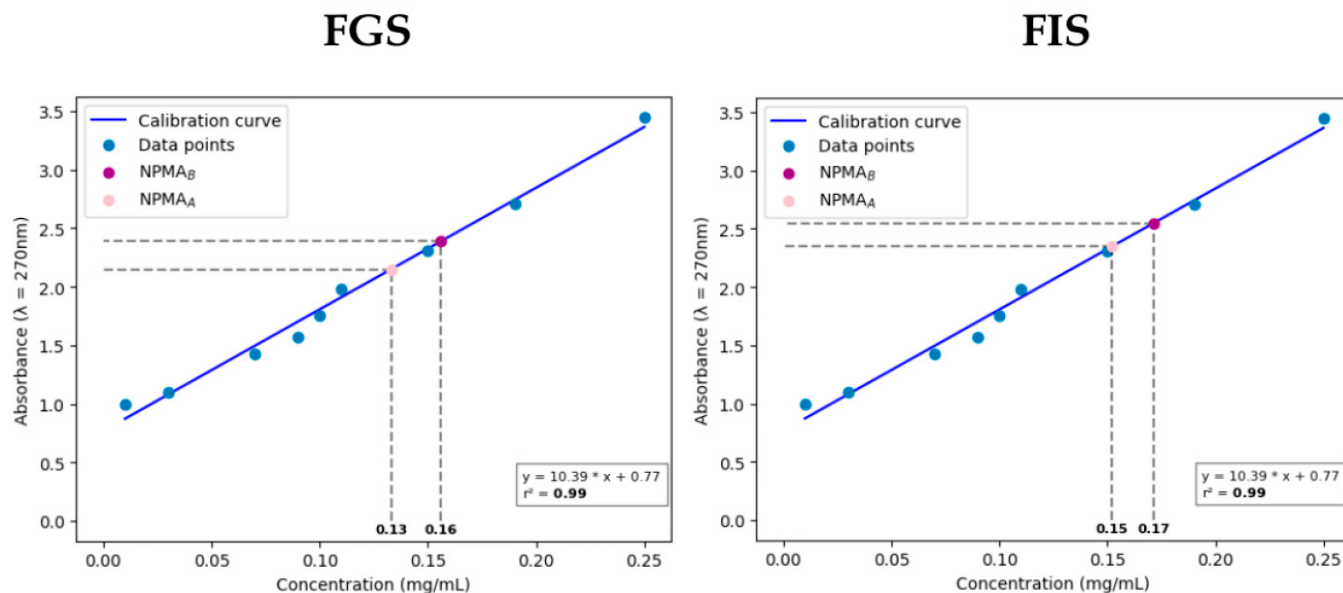


Figure 10. Encapsulated concentrations of *T. angustula* honey in simulated gastric (SGF) and intestinal (SIF) fluids, measured at 270 nm. Lower losses compared to commercial honey are evident due to the acidic stability of stingless bee honey.

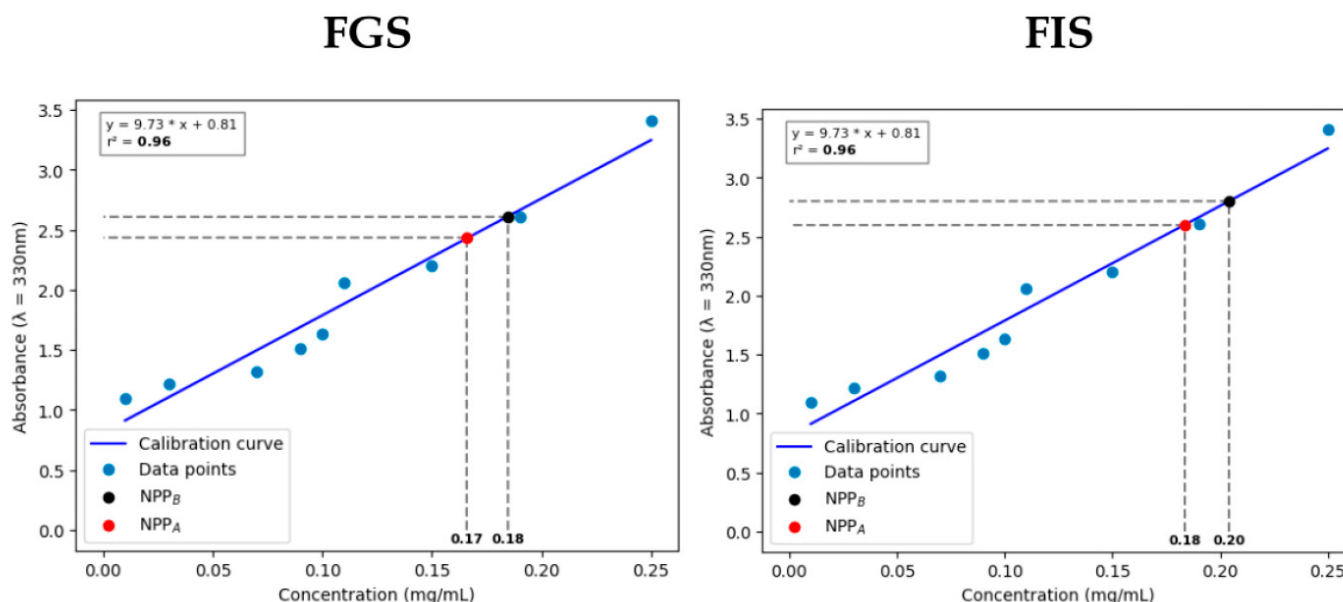


Figure 11. Encapsulated concentrations of *T. angustula* propolis in simulated gastric (SGF) and intestinal (SIF) fluids, measured at 330 nm. Nanoparticles show the highest encapsulation efficiency and the lowest loss of the natural product.

The release profiles of the products encapsulated in the nanoparticles, as a function of incubation time in FGS (pH=1.8), and FIS (pH=7.4), were calculated according to the ratio (2,3) described earlier in Methodology. The results of these calculations are presented in Figure 12. Chitosan and arabic gum nanoparticles can efficiently retain loaded products in the gastric medium. In other words, in an acidic environment after 180 minutes, the release profile of stingless bee propolis indicates that there is a release of 36% of LMW-chitosan/Arabic gum polymer matrix compared to a 32% release of the same compound, but with HMW. In other words, 11% more release is observed between propolis with different molecular weights of chitosan. After five hours, values of 63% were obtained for LMW-chitosan propolis, while HMW-chitosan recorded 51%.

These results indicate the presence of a strong polymer network that prevented the loss of propolis. In addition, it is highlighted that oral carriers require a slow release in the gastric phase [25]. Regarding the release profile of commercial honey, a value of 44% is observed in both LMW chitosan and HMW chitosan. This indicates that commercial honey faces greater difficulties in its release, possibly due to its composition and the immediate formation of crystals. Conversely, both commercial honey and stingless honey show a difference of 3%, a minimal variation that could be attributed to their acidity and virtually no crystal formation²⁸. Finally, between propolis and honey of the same species, there is a difference of 5%, while with commercial honey, there is a significant disparity of 20% in terms of release²⁹. The Kruskal-Wallis statistical test did not reveal significant differences ($P > 0.05$) in the medians of the groups, and the data in each group show an approximately normal distribution.

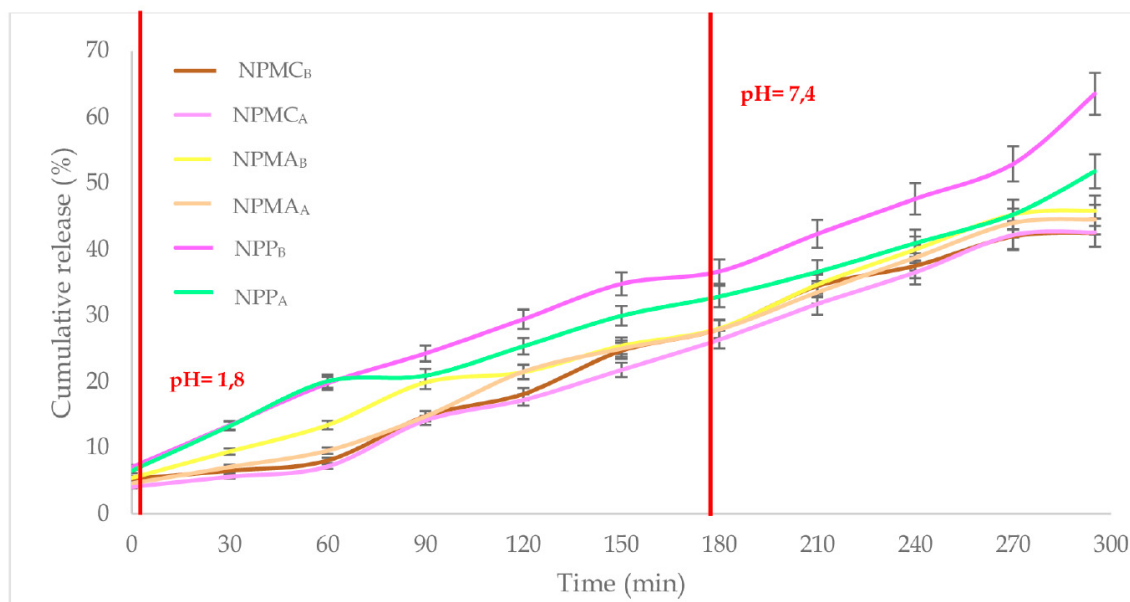


Figure 12. Cumulative release profiles of LMW and HMW chitosan nanoparticles loaded with commercial honey, stingless bee honey, and propolis from *T. angustula* in simulated gastric and intestinal fluids. Results demonstrate gradual release in acidic medium and sustained release in intestinal medium.

DISCUSSION

Effective strategies for the drug delivery of bioactive components, such as propolis, have gained significant relevance in the field of pharmaceuticals¹⁰. Although the therapeutic potential of these components has been demonstrated, the efficacy of their application is limited by challenges in the controlled release and stability of the compounds³⁰. Consequently, the present work addresses the synthesis and characterization of low and high-molecular-weight chitosan nanoparticles that encapsulate commercial honey and stingless bee propolis *Tetragonisca angustula*. The products of this species contain antimicrobial and anti-cancer properties due to the pollination of native Amazonian plants with purely medicinal properties.⁹ In addition, antimicrobial efficacy, controlled release, and cytotoxicity between encapsulated and unencapsulated nanoparticles are compared. In a previous study,³ remarkable physicochemical properties and the successful encapsulation efficiency of a chitosan nanoparticle encapsulated with propolis obtained from Romania were evaluated. Characterization techniques (FT-IR and TGA) allowed us to reaffirm the structures of the polymeric nanoparticles, identifying the most representative functional groups with signals. On the other hand, despite measurements in a concentration range of 10 to 100 µg/mL, no relevant peaks were observed during characterization in the UHPLC analysis. This can be attributed to the previous filtration of the nanoparticles with a filter size of 0.22 µm.

It is noteworthy that the high molecular weight chitosan nanoparticle encapsulated with stingless bee propolis exhibited the highest inhibition, especially against the *S. aureus* ECBI-UIEY strain, followed by the *E. coli* strain ATCC 25922. These results are consistent with previous studies, such as ^{33,34}, which showed the antimicrobial activity of propolis in specific strains. In addition, ³⁵ conclusions support the results by noting the efficacy of propolis against Gram-positive and Gram-negative bacteria, highlighting that this efficacy varies depending on the chemical composition of propolis. As for the other four inhibition halos recorded in this research, three are associated with chitosan nanoparticles, both high and low molecular weight, and one with nanoparticles encapsulated with stingless bee honey. Chitosan's antimicrobial efficacy has been consistently supported by several studies that have evidenced its activity in the formation of HMW and LMW nanoparticles ^{36–38}.

As far as the cytotoxicity assay is concerned, there are no significant differences between the various polymer nanoparticle encapsulations and the control at concentrations of 10 or 100 µg/mL, suggesting that none of the polymeric nanoparticles are harmful at these concentrations ³⁹. Nevertheless, it is noteworthy that both HMW and LMW-chitosan-based nanoparticles, encapsulating propolis, and those encapsulating stingless bee honey, show high optical densities. Specifically, nanoparticles encapsulated with propolis exhibit the highest optical density at both concentrations ⁴⁰. In contrast, LMW-chitosan-based nanoparticles have the lowest optical density at both concentrations, which is consistent with studies suggesting that encapsulating a compound, whether natural or man-made, in chitosan enhances its properties ^{41,42}.

Propolis release profiles plotted as a function of incubation time in FGS (pH=1.8) and FIS (pH=7.4). It can be deduced that polymeric nanoparticles strongly retain encapsulated natural products, in this case, propolis charged in a gastric environment. After 180 minutes in an acidic medium, the release profile showed a release of 36% of stingless bee propolis for the low molecular weight chitosan matrix, compared to 32% for the high molecular weight matrix. Nonetheless, previous studies ³¹, show an 18% release from the chitosan/arabic gum matrix. On the other hand, after transferring the particles to a basic medium, after 300 minutes, release values of 63% were obtained for propolis with low molecular weight chitosan and 51% for high molecular weight, indicating a strong polymeric network that prevents the loss of propolis and a controlled release in the intestinal environment. In this phase, in previous studies, 25, ³, 55% of propolis was released from the matrix.

The robust biopolymer network is considered to inhibit the escape of propolis, and a gradual release into the stomach is highly desirable for use as an oral vehicle. This release process can be attributed to the possible swelling of the polymeric matrix in an acidic environment, resulting from flavonoids and protonation of the amino groups of chitosan.

In our CS-PVP systems with propolis from *Tetragonisca angustula*, the encapsulation efficiency (EE) remained high ($\geq 76\%$) and reached up to $\sim 94\%$ (LMW) and $\sim 89\%$ (HMW), with controlled release that favored the intestinal over the gastric medium (e.g., LMW: 63% vs. 36% at 300 min; HMW: 51% vs. 37%). These values are in the upper range of those reported for nano/microencapsulation of propolis from other bee species and botanical sources. For example, Egyptian propolis (*Apis mellifera*) encapsulated in alginate nanoparticles achieved EE of 84.6–95.9% with good stability and antimicrobial activity ^{44,45}, while chitosan-coated liposomes loaded with stingless bee propolis from Southeast Asia showed EE $\approx 90\%$ and improved resistance to degradation, resulting in sustained release under physiological conditions ^{46,47}. Protein-based systems, such as zein/CMCS nanoparticles with Chinese propolis, reported EE $\sim 83\%$ and gradual release profiles ^{48–50}. By contrast, hydrogel-like matrices obtained by coacervation (chitosan–pectin) with Brazilian propolis showed lower EE ($\sim 19–38\%$) and a strong dependence on the release medium (distilled water vs. artificial saliva), highlighting how the carrier architecture and the surrounding environment strongly influence both retention and release kinetics ^{51–53}. Overall, our EE values and the higher release fraction under intestinal conditions align with the best-performing polymeric systems reported for *Apis mellifera* and stingless bees, suggesting that the CS-PVP combination enhances both the retention of phenolic compounds from propolis and their targeted delivery to the intestine ^{50,52,54,55}.

Differences among studies also reflect the chemical diversity of propolis depending on bee species and local flora. Resins rich in artemillin C (green propolis from *Apis mellifera* in Brazil or Taiwan) tend to show high affinity for cationic matrices (e.g., chitosan) and protein/hydrophobic cores (e.g., zein), supporting high EE and pH-dependent release ^{50,52,55}. In stingless bees from various regions of the world (e.g., *Tetragonula/Heterotrigona* and *T. angustula*), the higher proportion of terpenoids and flavonoids with varied polarity may enhance hydrophobic and electrostatic interactions with CS-PVP or with chitosan-coated liposomes, explaining EE values comparable or superior to those of *Apis mellifera* and release profiles more pronounced in intestinal medium ^{46,47,52,55}. This general pattern, high EE ($\geq 80–90\%$) and release favored at near-neutral pH,

appears consistently in well-designed polymeric and liposomal carriers, whereas less densely cross-linked formulations or softly coacervated systems tend to sacrifice EE and exhibit faster, more medium-dependent release^{51–53}.

CONCLUSIONS

The preparation and characterization of polymeric nanoparticles of natural products based on chitosan/arabic gum, which encapsulate commercial honey, propolis, and honey from native stingless bees *Tetragonsica angustula* (Hymenoptera: Meliponini), was achieved. The physicochemical properties of these nanoparticles were evaluated by FT-IR and TGA spectroscopy. An efficient encapsulation procedure was implemented using glutaraldehyde as the cross-linking agent. FT-IR analysis revealed that the characteristic bands of propolis were maintained during mixing, indicating that bioactive components, such as flavonoids, were stable during the encapsulation process. On the other hand, the TGA analysis showed that the nature and composition of these components influence the thermal properties of the nanoparticles, and that the molecular weight of chitosan plays a crucial role in their degradation profile. The antimicrobial activity of propolis was evidenced in this study, showing variable effectiveness depending on the strain and concentration. Notably, propolis-encapsulated chitosan/arabic gum nanoparticles (NPP_A) were highly effective against *S. aureus* ECBI-UIITEY. Besides, no significant differences in cytotoxicity were observed between the various encapsulations and the control, suggesting the safety of these polymeric nanoparticles at the concentrations evaluated. In this innovative formulation, a proper balance in electrostatic interaction was achieved, which contributes to its effectiveness as a controlled release vehicle. The results of propolis release into simulated gastric and intestinal fluids indicate that this formulation is suitable for pharmaceutical applications, offering greater stability and bioavailability of bioactive compounds, specifically propolis, along with additional nutritional value.

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Additional information: Correspondence should be addressed to hmrodriguez@e-mail.com

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