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Development of a New Method To Prepare Nano-/microparticles Loaded with Extracts of *Azadirachta indica*, Their Characterization and Use in Controlling *Plutella xylostella*

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ABSTRACT: Biodegradable nanoparticles have been widely explored as carriers for controlled delivery of therapeutic molecules; however, studies describing the development of nanoparticles as carriers for biopesticide products are few. In this work, a new method to prepare nanoparticles loaded with neem (*Azadirachta indica*) extracts is presented. In this study, nanoparticles were formulated as colloidal suspension and (spray-dried) powder and characterized by evaluating pH, particle size, zeta potential, morphology, absolute recovery, and entrapment efficiency. A high-performance liquid chromatography method was used for nanoparticle characterization. The best formulations presented absolute recovery and entrapment efficiencies of approximately 100% and a release profile based on swelling and relaxation of the polymer or polymer erosion. The biological data of the formulated products against *Plutella xylostella* showed 100% larval mortality. The nanoparticle information improved the stability of neem products against ultraviolet radiation and increased their dispersion in the aqueous phase.

KEYWORDS: *Azadirachta indica, nanoparticles, spray-dried powders, stability, Plutella xylostella*

INTRODUCTION

Although chemical pesticides are useful for insect pest control, their indiscriminate use has led to a number of environmental problems, including serious health hazards to humans and animals, development of pesticide resistance in insects, destruction of beneficial insects as parasites and predators of harmful insects, and pesticide residues.[1] In recent years, natural products have gained interest as a promising substitute for conventional pesticides in insect control.[2,3] In general, these phytochemicals are small molecules existing in a solid matrix and consist of a complex mixture of compounds.[4]

Azadirachta indica A. Juss (neem) is an evergreen tree with insecticidal qualities, which shows promise as a replacement for neurotoxic products such as organophosphates and carbamates in insect control.[5] Neem and its products repel pest insects and also affect their biology, feeding, and sexual communication.[6] Neem has several of the most important desirable features in a botanical insecticide. The tree is perennial, and not invasive, and there is no requirement to destroy the plant to obtain the extract. The extract itself possesses a multiplicity of components, which makes it more difficult for the insects to acquire resistance, and the concentration of the active components is high, making extraction an easy and low-cost process. In its active form, neem is more toxic to pest insects than to their natural enemies.[1,7] These properties make neem a potentially useful material for pest management programs.

The neem tree is a source of many triterpenoids. The main active ingredient in neem pesticides is a compound called azadirachtin;[8] therefore, the effectiveness of neem products (neem extracts and oil) is dependent on their azadirachtin content. Unfortunately, commercial use of neem products is limited by the sensitivity of azadirachtin to light, humidity, temperature, and acid or alkaline media.[2,8,9] These problems limit use of neem in agriculture because an insecticide should persist long enough to cause the death or control of the pest insect. Therefore, for neem products to be commercially effective, their secondary metabolites such as azadirachtin must first be stabilized through the development of new formulations.

Among the current trends in the development of natural product formulations is the control of release rate and targeting of compounds by nanoencapsulation. This new technique may increase the stability and solubility of natural products and thereby increase their efficacy.[10,11] Generally, nanoparticles (NPs) have been obtained from biodegradable and biocompatible polymers.[12] Many methods have been developed for preparing NPs,[10,11,13,14] and the commonly utilized techniques for NP preparation are nanoprecipitation, emulsification−solvent evaporation, emulsification−solvent diffusion, and double emulsification−solvent evaporation.[12,15]

Colloidal delivery systems such as polymeric NPs (nanocapsules and nanospheres) show great potential as a means of efficiently delivering one or a mixture of active molecules (e.g.,

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herbal extracts) to their site of action. Moreover, NPs may also minimize any unwanted toxic effects and improve the physical stability or photostability of active molecules.[16]

In this work, we present a new technique to prepare nanoparticles containing neem products in colloidal suspensions, using poly(ε-caprolactone) (PCL), named by nanoemulsion/solvent displacement and interfacial deposition. We also characterized the nanocapsules, and we describe the preparation of microparticles in powder produced by spray-drying. Formulations were characterized with regard to their particle size distribution, zeta potential (ZP), pH, entrapment efficiency (EE) of azadirachtin, and morphology. In vitro photostability studies of azadirachtin were also performed for the most promising formulations. The neem NP powder was tested against diamondback moth, and the results were compared with natural neem oil.

MATERIALS AND METHODS

Reagents and Chemicals. Neem seed kernels (1 kg) were provided by Baraúna Ind. Co. Ltd. (Catanduva, SP, Brazil). The herbal materials, neem extracts, and neem oil enriched with azadirachtin were obtained as described by Forim et al.[17]

PCL (average molecular weight 65000), sorbitan monostearate (Span60), and polysorbate 80 (Tween80) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Colloidal silicon dioxide was purchased from Importadora Química Delaware Ltd. (Porto Alegre, Brazil), and all organic solvents [of high-performance liquid chromatography (HPLC) grade] were purchased from J. T. Baker (Ecatepec, Mexico). Ultrapure water was produced in our laboratory using a Milli-Q System (18 MΩ) (Millipore Corp., Bedford, MA, USA).

Preparation of NPs. We developed a new method to prepare nanocapsules containing neem products. This technique is a modification of the solvent displacement method first described by Fessi et al. in 1989.[14] The general scheme and average quantities of applied products for this process are as follows (Figure 1). In step 1, a

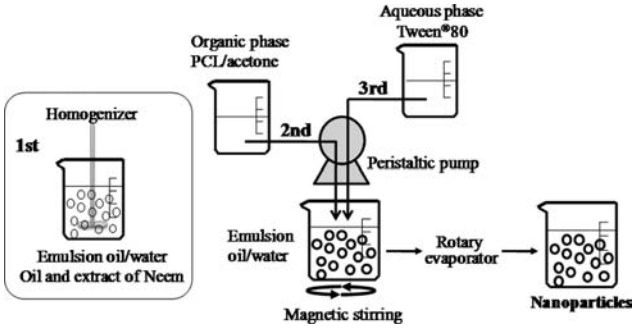

Figure 1. General scheme for the nanoemulsion/solvent displacement and interfacial deposition nanoencapsulation process.

nanoemulsion (phase I) was prepared by vigorous homogenization of neem oil (4.0 g), neem extracts (1.0 g), and Span60 (1.0 g) (internal constituents) in water (200 mL), using an Ultra-Turrax homogenizer (IKA T 10 basic Ultra-Turrax; Ika-Werke, Germany) at 27167*g* for 2 min. This step 1 produced nanodrops of oil in the aqueous phase. Because of the difference of polarity between oil and water, limonoids (active compounds of neem) were solubilized in the oil phase. After a brief period of stabilization, the second phase (phase II) was prepared with the polymer (1.0 g of PCL) and a partially water-soluble organic solvent (acetone; 200 mL) and poured into phase I, under moderate magnetic stirring, using a peristaltic pump at 10% of speed (PumpPro TPM 600 55 rpm; Waton-Marlow, Wilmington, UK). After 10 min of stirring, a third aqueous phase (phase III) was prepared by dissolving 1.0 g of Tween80 in water (100 mL) and pouring this into phase II, under moderate magnetic stirring, again using a peristaltic pump at 10% of speed. A standard nanoformulation in the dispersion medium was defined as follows: neem oil 2% (w/v), neem extract 0.5% (w/v), Span60 0.5% (w/v), polymer 0.5% (w/v), and Tween80 0.5% (w/v). Finally, in step 4, the volume of the NP dispersion fluid was concentrated to 200 mL under reduced pressure (R-21, Büchi, Flawil, Switzerland). In the end, the reaction mixture was stirred for a further 10 min. The third phase is not necessary if the desired final product is a powder.

Following preparation of the nanocapsules, a number of experiments were carried out to evaluate the formulation, including the ratio of oil to polymer, oil to Span60, and polymer to Span60 (Table 1). In these experiments, the quantities of solvent (200.0 mL), PCL (1.0 g), Tween80 (1.0 g), and water (200.0 mL) were kept constant. The best stable formulations were chosen for the biological assays.

Table 1. Parameters Investigated To Evaluate the Final Formulation of Nanocapsules Loaded with Neem Products

	neem (g)			
formulation	oil	extracts	Span60 (g)	final volume (mL)
1	4.0	1.0		200.0
2	4.0	1.0	1.0	200.0
3	2.0	1.0		200.0
4	2.0	1.0	1.0	200.0
5				200.0
6			1.0	200.0

Quantification of Azadirachtin Content in NPs. Azadirachtin was assayed by HPLC using an Agilent 1200 series liquid chromatography apparatus (Agilent Technologies, Santa Clara, CA, USA), configured with a G1322A degasser, a G1311A quaternary pump, a G1329A autosampler, a G1316A column oven, and a G1314B ultraviolet (UV) detector. The instrumental configuration is described in Figure 2. Method validation was performed in accordance with the International Conference on Harmonization, FDA, USA.[18] More details about the analytical method have been described by Forim et al.[17]

Pretreatment and Analyses of Azadirachtin from Polymeric Nanoparticles. The total amount (percentage absolute recovery, % AR) of azadirachtin that could be obtained from NPs was determined using the following procedure. First, 0.9 mL of acetone was added to 0.1 mL of the colloidal NP suspension and left for 2 h. After polymer dissolution, the solution was centrifuged (centrifuge 5810 R; Eppendorf, Germany) at 20800*g* for 30 min at 20 °C. After phase separation, 0.5 mL of the supernatant was dried under vacuum (Savant Speed Vac Plus SC10A, Farmingdale, NY, USA). Subsequently, the azadirachtin was resuspended in 0.5 mL of methanol, and the total amount of azadirachtin was analyzed by HPLC.

The percentage EE (%EE) of azadirachtin in NPs was determined by measuring the concentration of the free unloaded compound in the aqueous phase of the colloidal suspension. Centrifugation was carried out using a tube filter with a 0.22 μm pore cellulose acetate membrane (CostarSpin-X, Corning Inc.). Approximately 0.5 mL of the NP dispersion was placed in the outer chamber of the filter assembly, and the assembly was then centrifuged at 2650*g* for 15 min at 15 °C. The NPs and the encapsulated compounds remained in the outer chamber, whereas the aqueous dispersion medium containing the free unloaded compounds moved to the sample recovery chamber through the filter membrane. After separation, 0.2 mL of the aqueous dispersion medium was dried. The dried product was resuspended in 0.2 mL of methanol, and the amount of the free azadirachtin in the dispersion medium was estimated by HPLC. The %EE was subsequently calculated using the following equation:

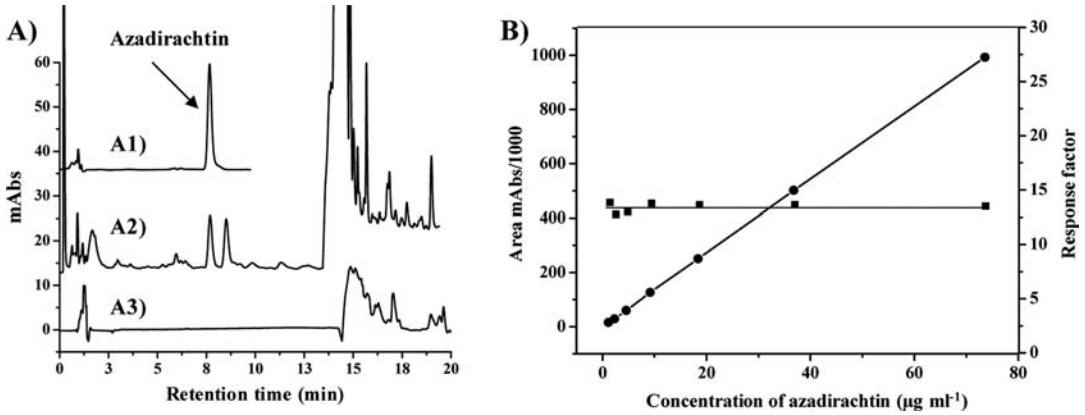

Figure 2. (A) Analytical chromatograms from (A1) azadirachtin standard and nanoparticles loaded (A2) with azadirachtin and (A3) without neem extracts. Analytical parameters: column, Zorbax Eclipse XDBC18 (150 × 4.6 mm i.d., 5 μm particle size); Phenomenex C18 precolumn (4 × 3 mm i.d., 5 μm particle size); mobile phase in a isocratic mode using deionized water and acetonitrile (65:35, v/v); oven temperature at 30 °C with a flow rate of 1.0 mL min^{-1}, injection volume of 10 μL, and UV detector at 217 nm. (B) Linearity for the quantitative method for azadirachtin analysis: (●) calibration curve obtained with luteolin standard solutions (n = 21); (■) azadirachtin response factor versus concentration of azadirachtin standard solution (n = 21).

$$\%\ \mathrm{EE} = [(\text{total quantity of azadirachtin} - \text{quantity of free azadirachtin in the aqueous medium}) / \text{total quantity of azadirachtin}] \times 100$$

Characterization of Colloidal Suspensions. Physicochemical analysis was carried out immediately after preparation of the NPs. The pH values of colloidal suspensions were determined using a potentiometer (B474 Micronal, BR), and particle size (PS) analysis of NPs was performed by photon correlation spectroscopy (PCS). For PCS measurements, 0.1 mL of each evaluated NP suspension was diluted to 10 mL with ultrapure water. Measurements were made at room temperature and a fixed angle of 90° to yield the mean particle hydrodynamic diameter and the polydispersity index (PI). The PI is a dimensionless measure of the breadth of the PS distribution. The ZP was evaluated by laser Doppler anemometry using a ZetaPlus analyzer. Values reported are the mean ± SD of at least three different batches of each NP formulation. Particle size and zeta potential values were produced by Brookhaven Instruments Corp. (USA). The NPs were dried on polished metal supports, sputter-coated with gold, and then examined under scanning electron microscopy (SEM) (Philips XL 30 FEG) at 10 kV, using different magnifications from 1000 to 25000 times.

Preparation of Microcapsule Spray-Dried Powder. Microparticles containing NPs loaded with azadirachtin were prepared by a spray-drying technique, as described by Müller et al.[19] Colloidal silicon dioxide was added to the NP suspension, and then the mixture was fed into a spray dryer (Büchi B-290, Büchi Labortechnik AG, Postfach, Switzerland) equipped with a nozzle atomizer using air as the atomizing gas. The diameter of the nozzle opening was 0.7 mm. The process parameters used were chosen on the basis of pilot experiments. The optimized operational parameters were the feed rate (0.75 mL min^{-1}), atomizing air flow (530 L h^{-1}), aspirator capacity (90%), and inlet and outlet temperatures (125 ± 4 and 50 ± 4 °C, respectively).

Characterization of Microparticle Spray-Dried Powder. The yields of the spray-dried powders were calculated by summing the weights of all components in the colloidal suspensions, discounting the water content. The total amount of azadirachtin in the powder samples was analyzed by dispersing 25.0 mg of the dried powder in 5.0 mL of acetone for 60 min at room temperature with constant stirring. After polymer dissolution, 1.0 mL of this solution was centrifuged at 20800g for 30 min at 20 °C, after which 0.5 mL of the supernatant was dried under vacuum (Speed Vac) to produce the neem extract. This extract was then solubilized in 0.5 mL of methanol, and the total quantity of azadirachtin was analyzed by HPLC.

The powder was also analyzed by SEM. Samples of the powder were sprinkled onto SEM stubs covered with double-sided carbon tape and subsequently sputter-coated with gold prior to examination. The surface morphology was examined by SEM using various magnifications in the range 1000−50000 times.

Photostability Assay of Neem NPs. The NPs in both the colloidal suspension and powder samples were kept during 15 days in a controlled environment. The samples were divided between a number of glass vials, which were then sealed. Nonencapsulated neem oil was used as the positive control at the same treatment. The samples were incubated in a box irradiated with four UV lamps (Philips 15 W actinic lamps; l = 45.0 cm, w = 2.6 cm; Philips, The Netherlands) in a constant temperature of 30 ± 2 °C. The box was built of wood that was totally covered with mirror (l = 60.0 cm, h = 40.0 cm, and w = 60.0 cm). The UV lamps were placed above the samples at a distance of 25 cm. The samples were stirred every day to maintain uniform exposure to humidity and UV radiation. The samples were withdrawn at frequent time intervals (on days 0, 1, 2, 5, 8, 11, and 15) for HPLC analysis.

In Vitro Release Studies of Azadirachtin from Nanocapsules. In vitro release studies of azadirachtin from nanocapsules in colloidal suspension were carried out as previously described by Levy and Benita,[20] with some changes in the dialysis bag diffusion technique. Samples (1 mL) of the neem colloidal suspension were placed into dialysis bags (made from dialysis tubing cellulose membrane, 1.0 cm, Sigma Chemical Co., St. Louis, MO, USA), which were sealed and dropped into 1 L of phosphate-buffered saline (PBS; pH 7.4) that was stirred continuously with a magnetic stirrer.[21] The whole system was kept at 35 °C with a continuous PBS stream at 3.0 mL min^{-1}, controlled by a peristaltic pump. At given time intervals, a dialysis bag was withdrawn from the stirred release medium, and the azadirachtin content of the sample in the bag was analyzed directly by a previously validated HPLC method. Reported values are the mean ± SD of the values corresponding to three different batches. The cumulative release of azadirachtin from the nanocapsules was determined by an empirical equation that was used to estimate the value of the kinetic parameter n, as described previously by Korsmeyer-Peppas:[22]

$$M_t/M_\infty = Kt^n$$

M_t/M_∞ is the released fraction at time t, n is the release exponent, and K is the release factor. From the slope and intercept of the plot of $\ln(M_t/M_\infty)$ against $\ln(t)$, the value of n was calculated.

Biological Assays against *Plutella xylostella*. The insect experiments were carried out in the Insect Plant Resistance Laboratory (Department of Plant Protection, Center of Agronomical Sciences and Veterinary (FCAV), São Paulo State University (UNESP), Jaboticabal

Campus, São Paulo, Brazil, at room temperature (25 ± 2 °C), with 70 ± 10% relative air humidity (RH) and a 12 h photoperiod.

Seeds of kale (*Brassica oleracea* L. var. Acephala) were sown onto a commercial substrate (Plantmax, DDL Agroindústria, Brazil) in a 128 cell polystyrene tray. After 35 days, the plants were transplanted into 5 L pots containing a growing medium of soil, sand, manure, and vermiculite (2.75:1:1:0.25, respectively).

A culture of diamondback moth [*P. xylostella* (L.), Lepidoptera: Plutellidae] was established in a greenhouse. Before that, *P. xylostella* larvae were collected from kales and cabbages in the Jaboticabal region. Before pupation, the *P. xylostella* larvae were reared in plastic cups (15 × 10 × 5.0 cm) with kale leaves that had been previously washed with sodium hypochlorite 0.5% solution and pure water. After the larvae pupated, appropriate numbers of pupae of each sex were removed from the rearing cups and placed in empty cups in large wooden screen cages (44 × 16 cm), where the adults would emerge. The *P. xylostella* used in the assays were randomly chosen.

Before the start of the assay, the kale plants were separately immersed for 3 min in the aqueous phases of the different treatments carried out with different formulations, and control plants were immersed in water. Afterward, the plants were left to dry at room temperature for 30 min. Finally, disks 8 cm in diameter were cut from the kale leaves including the midrib, and placed into individual Petri dishes together with 12 newly hatched first-instar larvae. The dishes were sealed with plastic film and maintained at room temperature (27 ± 1 °C) until the larvae reached the pupal phase. The experimental design was completely randomized, with five treatments and five replications.

Statistical Analysis. Data were analyzed using analysis of variance (ANOVA) with the *F* test, and the means were compared by the Tukey test, with probability set at 5%. The mortality data were transformed to arc-sine $[(x + 0.5)/100]^{1/2}$, with x representing the observed data that were used to obtain the averages and statistical analyses, and the equations were then used to adjust the data to a normal distribution in Gaussian curve and homoscedasticity of variances.

RESULTS AND DISCUSSION

Physicochemical Characterization of Neem Extract-Loaded Nanoparticles. Although the interfacial polymer deposition and solvent displacement technique proposed by Fessi et al.[14] has been used in systemic drug delivery,[10,14,23,24] few papers describe its application to natural products such as neem. Previous studies carried out for us on neem products using this method in our laboratory[14] showed a %AR in colloidal suspension of nearly 100%. However, the %EE of azadirachtin in NPs, using the Fessi et al.[14] method, ranged from 26 to 74%. For this reason, we devised a new method, which we call nanoemulsion/solvent displacement and interfacial deposition. This method produced an increase in both the EE and the stability of azadirachtin dispersed in water.

All prepared colloidal suspensions presented a macroscopic homogeneous appearance of a bluish white milky opalescent fluid, as soon as the organic phase diffused into the aqueous phase (Tyndall effect). This result is in agreement with previous results in the literature for other NP systems.[14,23] This technique proved to be simple as well as easily reproducible, and the result confirmed the formation of NPs.

An important result obtained in this work was the increase in the azadirachtin dispersion in water using the NP formulation. The naturally low solubility of azadirachtin may result in a requirement for large water volumes, many workers, and a long time to introduce the active compound in the field, which increases growing costs. The proposed NP method used in this study allowed formation of a colloidal suspension containing up to 3.4 g of azadirachtin dispersed into 1 L of aqueous phase (4.72 mM). The azadirachtin solubility in NPs was evaluated at 26.0 mg/100 mL (0.361 mM), which was 13 times greater than the natural azadirachtin solubility. If necessary, this dispersion can be improved by using extracts or oils with higher azadirachtin concentrations in the formulations.

Analytical Validation of the HPLC Method. The correct elucidation of NP features, especially the investigation of %AR, %EE, stability, and kinetics release, requires a validated analytical HPLC method. The specificity of the HPLC method used was evaluated by comparing chromatogram analyses of NP samples containing or not containing azadirachtin (Figure 2). No interference peak was observed over a period of 8.6 min in the blank chromatogram (retention time of azadirachtin). The chromatography method showed a separation factor (α) of 1.10 and a resolution (R_s) of 2.09 between azadirachtin and 3-tigloylazadirachtol (Figure 2A2). Hence, the method may be considered to be selective.

The analytical calibration curves (n = 3) were linear over a concentration range from 1.15 to 73.6 μg mL^{-1} (Figure 2B), using the regression equation ($y = ax \pm b$) and the correlation coefficient (r^2) obtained by the least-squares method, where y = 13.52x + 0.1275 and r^2 = 0.9999. The response factor (Figure 2B) showed a 2.98% relative standard deviation (RSD) between all levels of concentration patterns. The correlation coefficient showed a P value of <0.05 (5.32x × 10^{-41}) by ANOVA.

Accuracy and precision were analyzed at three different concentrations (1.38, 31.6, and 69.0 μg mL^{-1}) using the average percentage recoveries and the RSD for azadirachtin, respectively. RSD values for repeatability at each concentration level of standard solutions within a single day (n = 5) and between different days (n = 15) were ≤2.3 and ≤1.7%, respectively. Accuracy and RSD were 101.0 ± 0.06%, showing strong agreement between the experimental and theoretical values (Table 2). The limit of detection (LoD) and limit of quantitation (LoQ) were calculated using the intercept standard deviation (sd = 1.370) and the slope of the calibration curve (S = 13.52) and found to be 0.34 and 1.0 μg mL^{-1}, respectively. This validated method was successfully applied to the characterization of azadirachtin in polymeric NPs.

Table 2. Accuracy and Precision Data Concerning the Validation of the Quantification Method for Azadirachtin by HPLC

	precision				
	intraday (n = 5) RSD (%)				
concn (μg mL^{-1})	day 1	day 2	day 3	interday (n = 15) RSD (%)	accuracy (%) (n = 15)
1.38	2.3	1.6	1.1	1.7	100.9
31.6	1.3	1.4	1.2	1.3	101.0
69.0	0.9	1.7	0.1	0.9	101.0

Characterization of the Colloidal Suspensions. In this study, we used our new process of nanoemulsion/solvent displacement and interfacial deposition for the preparation of NPs loaded with neem products. By using enriched oil and extracts of neem, both previously analyzed by HPLC, it was possible to prepare colloidal suspensions and powder microparticles with high and reproducible azadirachtin content. Good results were obtained with this new method. Analysis of the pH, PS, PI, and ZP (Table 3) showed that the method and

Table 3. Characterization of Colloidal Suspensions of Nanoparticles Loaded with Neem[a]

formulation	pH	particle size (nm)	polydispersity index	zeta potential (mV)	nominal concn (mg L^{-1})	absolute recovery (%)	entrapment efficiency (%)
1	4.87 ± 0.19	231.3 ± 18.89	0.490 ± 0.180	−36.80 ± 4.167	80.0	101 ± 9.35	≥98.0
2	4.84 ± 0.11	243.0 ± 19.03	0.029 ± 0.007	−29.94 ± 4.439	80.0	96.7 ± 5.07	≥98.0
3	4.77 ± 0.17	213.3 ± 11.51	0.321 ± 0.082	−25.22 ± 2.104	40.0	97.6 ± 8.36	≥97.5
4	4.95 ± 0.12	212.5 ± 10.37	0.005 ± 0.002	−28.54 ± 2.961	40.0	98.1 ± 7.12	≥97.5
5	6.53 ± 0.11	177.4 ± 2.38	0.227 ± 0.079	−33.47 ± 2.077			
6	6.69 ± 0.13	244.9 ± 15.87	0.119 ± 0.024	−32.84 ± 2.736			

[a]Values express the average result ± standard deviation (n = 3).

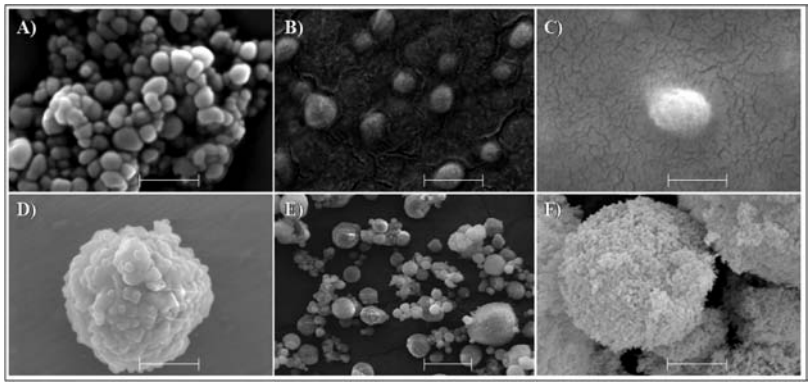

Figure 3. Scanning electron micrographs of both nanoparticles in colloidal suspension loaded with 40.0 (A), 80.0 (B), and 3400.00 (C) mg L^{-1} azadirachtin and nanocapsule spray-dried powder (D, E) and silica spray-dried (F). The bars represent metric scales. In panels A, D, and F (25000×), the width of the scale bar corresponds to 1 μm; in panels B and C (50000×), the width of the scale bar corresponds to 0.5 μm; in panel E (1000×), the width of scale bar corresponds to 20 μm.

formulations were highly reproducible among all formulations using neem oil (P = 0.622). The pH values were directly related to the compounds used in the formulation. For instance, colloidal suspensions prepared with neem oil gave lower pH values. We have previously observed that colloidal suspensions with low values of pH may contribute to degradation of NPs and/or bioactive compounds. In this study, the pH values of the colloidal suspensions were increased by using 0.05 M NaOH solution to bring the pH to 7.0. This process did not change the colloidal stability, but increased the ZP values by approximately 20%.

There was no statistical difference in PS between the various ratios of oil to polymer used in the study (P = 0.053). There was a slight change in the average PS value in formulations with larger amounts of neem oil. It is possible that larger amounts of oil may promote the formation of a larger number of NPs and, thus, colloidal suspensions with larger amounts of oil may be capable of carrying a greater amount of azadirachtin. These PS results are in accordance with those of previous works using nanoprecipitation.[14,23] However, to our knowledge, the current study is the first work to prepare neem products on the nanoscale. The PS is limited by the size of the drops that are immediately formed (oil core) when the neem oil and extracts are transferred to and stirred in the aqueous phase.

With regard to NP size, not only is the average value (nm) important but also the size variability (PI). Formulations 2, 4, and 6 (Table 3), which were prepared using Span60, presented PI values of <0.200, which describe average to good distribution, taking into account that a colloidal suspension is considered to be a homogeneous suspension. Such PI values are necessary to maintain the stability of a colloidal dispersion without formation of microparticles or precipitates. Our colloidal suspensions did not precipitate during 6 months of storage. This narrow PS distribution avoids formation of an azadirachtin concentration gradient between small and large NPs. Such a gradient could lead to the azadirachtin crystallizing into large particles, that is, a crystal growth known as Ostwald ripening.[25] The SEM images (Figure 3A,B) showed a relatively homogeneous system, which is consistent with the observed PI. The morphological analysis for the best formulations selected by SEM identified formation of spherical particles with a regular surface and form, homogeneity, and also lower size dispersion.

The ZP is related to the surface charge of the NPs (electrostatic repulsion). NPs with ZP of > +30 mV or < −30 mV are considered to be very stable in the dispersion medium. All of our formulations had satisfactory values of < −25 mV. Moreover, nanocapsules can also be stabilized by steric effects, depending on the nature of the surfactant. Surfactants are necessary to obtain small and stable oil droplets. In so doing, surfactants may affect the physicochemical properties of the NPs, such as size and drug loading.[26] NPs loaded with neem extracts that were formulated using Tween80 (an amphiphilic nonionic stabilizer) did not appear to have a major influence on the size of particles or on the drug encapsulation for PCL nanocapsules.

The method and formulations described here produced colloidal suspensions with approximately 100% AR and EE (Table 3). It was possible to prepare colloidal suspensions of NPs loaded with a large quantity of azadirachtin and to obtain total dispersion into the aqueous phase. The AR was measured by HPLC, comparing the azadirachtin reference values with the total content quantified in the resulting colloidal suspension. Azadirachtin reference values were calculated by considering the amount of azadirachtin initially present in the quantity of neem oil and extracts that were used in the processes. The EE

was calculated as the ratio between the azadirachtin in NPs and the AR.

To investigate the efficiency of the method for nanoencapsulation, colloidal suspensions of polymeric NPs were prepared with 3400.00 mg L^{-1} azadirachtin. These formulations were prepared as previously described; however, they were prepared using neem extracts and neem oil enriched with large quantities of azadirachtin.[27] In all formulations, the AR and EE were >95% and >98%, respectively.

The spherical morphology of NPs was confirmed by SEM. To obtain good resolution on SEM, it was necessary to dry the colloidal suspension after it was added to the polished metal support. However, this process resulted in clumping of the NPs, and a polymeric pellicle was formed as a result. Nevertheless, it was possible to observe spherical NPs on the polymeric pellicle (Figure 3A–C).

Spray-Dried Nanocapsule Powders Containing Neem Products. The nanocapsules from the formulation with the highest EE of azadirachtin were selected for spray-drying to obtain a nanocapsule spray-dried powder. This process used colloidal silicon dioxide as the drying support and aimed to increase the shelf life of products containing azadirachtin and their stability against photodegradation. This process promotes a reversible aggregation between silicon dioxide and NPs. If the silicon dioxide was not used, we would obtain microparticles with irreversible aggregation of different NPs. Silica may generate a large surface area and good thermal conductivity, qualities that are favorable to water removal. Furthermore, silica is biocompatible and nontoxic, being considered safe even for medication preparation.[28]

The yield from the spray-drying process was dependent on the amount of silica utilized. Better yields were obtained when (a) the ratio of NPs to colloidal silicon was ≤1% (m/m) and (b) the quantity of NPs in water was equal to 1% (m/v), resulting in a yield of 77.5 ± 3.67% (n = 12) and a moisture content of <2.5%.

With regard to the morphological SEM analysis of the spray-dried powder (Figure 3D,E), the microparticles presented a spherical shape and a relatively homogeneous diameter distribution of around 4 μm. At higher SEM magnification, nanostructures were observed on the surfaces of the microparticles. These analyses were confirmed by comparing the nanocapsule spray-dried surfaces (Figure 3D) with the particle surfaces of silica-only spray-dried powder (Figure 3F).

Quantitative analyses of the azadirachtin content in the NP powder were carried out by HPLC as described under Materials and Methods. Formulations were prepared with different amounts of neem extracts or enriched neem oil. These different amounts of extracts or oil allowed preparation of microparticles in the powder with different and controllable azadirachtin contents, which were proportional to the total mass of the extracts/oils used in the formulation. The final amount of azadirachtin in the spray-dried micropowder ranged from 1600.0 to 6800.0 mg kg^{-1}.

Azadirachtin Release Kinetics from NPs. Release assays of UV stability of formulations were carried out in PBS at 35 °C. This aqueous phase was stirred constantly and added in a continuous stream to avoid saturation of the system. In this assay, the azadirachtin was able to pass through the pores of the membrane whereas the NPs could not, hence allowing measurement of azadirachtin diffusion. The release kinetics were obtained by collecting aliquots at pre-established times and quantifying the azadirachtin content by HPLC. The results were expressed in terms of the percentage release (Figure 4).

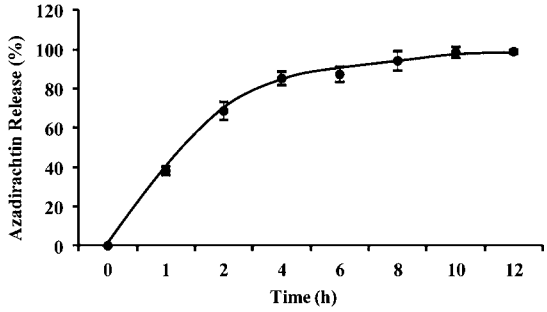

Figure 4. Release profile of azadirachtin from PCL nanoparticles in colloidal suspension (n = 3).

The azadirachtin release profiles were similar for all evaluated formulations. The time required to release 100% of the azadirachtin was 10 h, and approximately 80% of the azadirachtin was released in the first 4 h. It is likely that this behavior occurs because of the rapid migration of azadirachtin adsorbed on the polymeric NP surface or positioned in the superficial polymeric layer,[29] causing a reduction in the release rate. This behavior took place in the continuous stream of PBS at 3.0 mL min^{-1}.

The Korsmeyer–Peppas model considers the release mechanisms of compounds that do not follow Fick's law and that display an anomalous behavior that can be described by the equation $M_t/M_\infty = Kt^n$.[22] This model is commonly used for analysis of release mechanisms that are not well established or when more than one phenomenon is involved. The construction of the graph by using $\ln(M_t/M_\infty)$ as a function of $\ln(t)$ provides the exponent (n) and the release constant (K, time $-n$). The empiric values of n, determined for azadirachtin, were 1.613 and 4.957 for intervals between 1 and 4 h (percentage release < 80%) and between 4 and 12 h (percentage release > 80%), respectively. The release constants were 3.459 and 10.86 day^{-1}, respectively. The value of n identifies the active-compound release mechanism. In accordance with the Korsmeyer–Peppas model, when n values are >0.89, the release mechanism corresponds to first-order release kinetics. In the current study, the azadirachtin release mechanism that was evaluated in the colloidal suspensions presented first-order kinetics, suggesting a transport mechanism originating from the phenomenon of swelling and relaxation of the polymer or a release phenomenon after polymer erosion.[22]

Resistance of Neem Nanoparticles to Photodegradation. The photostability of azadirachtin in the colloidal suspension and spray-dried powder of NPs is shown in Table 4. As a reference, two samples of nonencapsulated neem oil were used, one of which was directly exposed to and the other one protected against UV radiation. During the evaluated period, samples were continuously stirred.

The degradation rates of nonencapsulated neem oil samples, which were exposed to UV radiation, were greater than in all other samples. Nonencapsulated neem oil samples, which were protected against UV radiation, did not show photodegradation signals. Nanoformulations prepared using Span60 presented better UV stability than those prepared with the other chemicals. Comparison of the azadirachtin quantity between NPs formulated with and without Span60 revealed that the colloidal suspension and powder prepared with Span60 were

Table 4. Photodegradation Kinetics Data of Nanoparticles Loaded with Neem Products

sample	photodegradation rate constants[a] (k, days^{-1})	time to 50% photodegradation[b] (days)
neem oil	50.0×10^{-2}	1.39
neem oil (protected against UV light)	0.11×10^{-2}	628
nanoparticle colloidal suspension (without Span60)	9.83×10^{-2}	7.05
nanoparticle colloidal suspension (with Span60)	8.90×10^{-2}	7.79
nanoparticle spray-dried powder (without Span60)	2.01×10^{-2}	34.4
nanoparticle spray-dried powder (with Span60)	1.47×10^{-2}	47.3

[a]Constants for first-order kinetics: $\ln[\text{Aza}]_t/[\text{Aza}]_0 = -kt$. [b]Radiation required time (in days) to reduce the concentration of azadirachtin on 50% from its initial value ($t_{1/2} = \ln 2/k$).

10.4 and 37.4% more stable under UV radiation, respectively. After 7 days of UV exposure, the samples of nonencapsulated neem oil showed 100% azadirachtin degradation, whereas the NPs in colloidal suspension and in powder showed approximately 55 and 10% degradation, respectively. This difference between NPs in colloidal suspension and powder may be explained by the swelling of the polymer and release of azadirachtin in the aqueous medium and also through the ability of silica to absorb or reflect UV radiation.[30]

Biological Efficacy of Nanoparticles Loaded with Neem Products against *P. xylostella*. To evaluate the activity of our neem formulations, NPs in colloidal suspension and in powder were assayed against *P. xylostella*. Larvae of this insect were fed kale leaves that had been previously immersed in the aqueous phases of the different treatments and compared with insects fed leaves that had been immersed in pure water (negative control) or leaves immersed in neem oil emulsion (positive control). Deltamethrin-25 CE was also used as a positive insecticide control. Evaluations on first-instar larvae were started from day 3 of the experiments because of the mining habit of the insect at this stage, and the other evaluations were conducted on days 5, 7, and 9 of the experiment. Third-instar larvae were first assessed 24 h after the start of the experiment, and the other assessments were conducted on days 3, 4, and 7. Results are shown in Table 5.

The experiment with the first-instar larvae showed that the treatment carried out with nonencapsulated neem oil was statistically different from the others only on day 3; by days 5 and 7, all treatments were equally efficient, differing only in the negative control. On day 9, the control neem oil and the colloidal suspension of NPs loaded with neem caused 100% larval mortality, whereas NPs in powder caused 91.7% larval mortality. All neem treatments were more efficient than the control insecticide deltamethrin-25 CE after day 5 of the assays.

Table 5. Different Treatments Assessed To Control *P. xylostella* by Using Various Neem Products

			days after treatments[a]			
treatment	active compound	dose (%)	3	5	7	9
		First Instar				
control			8.33 b	8.3 b	8.33 b	8.33 c
nanoparticles in powder-NC	azadirachtin 5000 mg kg^{-1}	0.10	35.4 ab	70.8 a	77.1 a	91.7 ab
nanoparticles in colloidal suspensions	azadirachtin 5000 mg kg^{-1}	0.05	22.9 ab	87.5 a	95.83 a	100.0 a
neem oil[b]	azadirachtin 4000 mg kg^{-1}	0.05	47.9 a	83.3 a	93.75 a	100.0 a
deltamethrin-25 CE	deltamethrin 25 g ai L^{-1}	0.05	35.4 ab	60.4 a	70.83 a	72.9 b
F (treatment)[c]			4.13*	9.70**	9.55**	45.6**
RDS (%)			29.4	24.1	24.1	11.8
			days after treatment			
treatment	active compound	dose (%)	1	3	5	7
		Third Instar				
control			0.00 b	0.00 b	10.4 b	10.4 b
nanoparticles in powder	azadirachtin 5000 mg kg^{-1}	0.80	4.17 ab	22.9 ab	77.0 a	89.6 a
nanoparticles in colloidal suspensions	azadirachtin 5000 mg kg^{-1}	0.90	6.25 ab	25.0 ab	85.4 a	91.67 a
neem oil-CE	azadirachtin 4000 mg kg^{-1}	0.25	8.33 ab	31.2 ab	89.6 a	95.8 a
deltamethrin-25 CE	deltamethrin 25 g ai L^{-1}	0.50	20.8 a	47.9 a	91.7 a	97.9 a
F (treatment)[c]			3.24**	2.51**	9.05**	28.9**
RDS (%)			5.88	3.78	24.9	14.1

[a]Means followed by the same letter do not differ significantly from each other by Tukey test ($P \leq 0.05$). For statistical analysis, data were converted to arc-sin $[(x + 0.5)/100]^{1/2}$. [b]Nonencapsulated neem oil. [c]*, significant at 5%; **, significant at 1%.

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Torres et al.,[31] evaluating the efficacy of neem extracts against *P. xylostella*, obtained similar results, observing that neem extracts caused 100% mortality of *P. xylostella*. In our study, the difference between the results for NPs loaded with neem products in colloidal suspension and in powder, which were observed regardless of the different treatments, may be due to the release kinetics. Active compounds in NPs in powder depend on hydration of the colloidal silica, and thus NPs in powder need a longer time to release the active compounds.

The third-instar larvae of *P. xylostella* were negatively affected by the control insecticide deltamethrin-25 CE during the first 24 h, and this caused greater mortality than any of the neem treatments during days 1 and 3 of the experiments. However, on days 5 and 7 all of the neem treatments, in different doses and formulations, resulted in high insect mortality that was similar to that produced by deltamethrin. These samples also showed a mortality rate that was significantly higher than that of the larvae treated with the negative control.

All neem products assayed gave similar results; however, the NPs loaded with neem products showed higher stability than the nonencapsulated neem oil. These results suggest that our proposed new method is an efficient way to formulate natural products such as neem. NPs loaded with neem showed a gain in stability and in their ability to disperse in aqueous medium. In addition, the NPs loaded with neem products did not lose their biological efficiency against pest insects during the 6 month period in which they were evaluated. This method may change the manner in which natural products are used for control of agricultural pest insects.

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