Article

J. Mex. Chem. Soc. **2024**, *68*(2)
Regular Issue

ISSN-e 2594-0317

Nanoencapsulation of Antifungal *Piper schlechtendalii* Extract in Poly(lactide-co-glycolic) Acid to Enhance Photostability

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Received Febraury 3rd, 2023; Accepted September 4th, 2023.

DOI: http://dx.doi.org/10.29356/jmcs.v68i2.1964

Abstract. Synthetic fungicides are used to control fungal diseases in plants, such as those caused by members of the Fusarium genus. Nonetheless, the over-application of synthetic formulations can generate other problems, like phytotoxicity, or the pollution of water and soil. In this context, botanical extracts with antifungal properties can represent an environmentally friendly alternative to control fungal infections. The application of natural products in the form of crude extracts still requires the incorporation of toxic organic solvents to be used as vehicle. Nanotechnology allows the dispersion of hydroalcoholic extracts in water simply by the nanoencapsulation of the active molecules in a biodegradable polymer, with the advantage that no organic solvents are required while, at the same time, this polymer may protect the extract against photodegradation. The present study aims to encapsulate an antifungal Piper schlechtendalii crude extract in poly(lactide-co-glycolide) acid nanospheres, conferring good dispersion in water while protecting the active ingredients against degradation by solar irradiation. The particle size, zeta potential, and encapsulation efficiency obtained were 170 nm, -37 mV, and 33.7 %, respectively. The system obtained showed good dispersion in water, in the form of a colloidal suspension of polymeric nanospheres. After 24 h of exposure to UV-A radiation, crude extract only retained 58.35 % of its original Fusarium solani growth inhibition capacity, while the nanoencapsulated extract retained 70 %. The study concluded that the biodegradable polymer does confer photoprotection to the active ingredients in the antifungal Piper extract while simultaneously removing the necessity of organic solvents as vehicles, potentially reducing the environmental impact.
Keywords: Plant extract; biodegradable polymer; fusarium; pesticide; colloidal suspension.

Resumen. Los fungicidas sintéticos son usados para controlar enfermedades fúngicas en plantas, como aquellas causadas por el género Fusarium. Sin embargo, la aplicación desmedida de formulaciones sintéticas puede generar otros problemas, como fitotoxicidad, o contaminación de agua y suelos. En este contexto, extractos botánicos con propiedades antifúngicas representan una alternativa ecológicamente amigable para controlar infecciones fúngicas. La aplicación de productos naturales en forma de extractos crudos aún requiere la incorporación de disolventes orgánicos tóxicos para ser utilizados como vehículos. La nanotecnología permite la dispersión de extractos hidroalcóholicos en agua simplemente nanoencapsulando las moléculas activas en polímeros biodegradables, con la ventaja de que no requiere disolventes orgánicos mientras que, a la vez, dicho polímero protege al extracto contra fotodegradación. El presente estudio busca encapsular un extracto crudo antifúngico de Piper schlechtendalii en nanoesferas de poli(ácido láctico-co-glicólico), brindando buena dispersión en agua

J. Mex. Chem. Soc. **2024**, *68*(2)
Regular Issue

ISSN-e 2594-0317

mientras se protege a los ingredientes activos contra degradación por luz solar. El tamaño de partícula, potencial zeta, y eficiencia de encapsulación obtenidos fueron 170 nm, -37 mV, y 33.7 %, respectivamente. El sistema obtenido mostró buena dispersión en agua, en forma de una suspensión coloidal de nanoesferas poliméricas. Después de 24 h de exposición a radiación UV-A, el extracto crudo solo retuvo 58.35 % de su inhibición de crecimiento de Fusarium solani original mientras que el extracto nanoencapsulado retuvo el 70 %. El estudio concluyó que el polímero biodegradable logra brindar fotoprotección a los ingredientes activos del extracto de Piper mientras que, simultáneamente, remueve la necesidad de usar disolventes orgánicos como vehículo, reduciendo potencialmente el impacto ambiental.
Palabras clave: Extracto vegetal; polímero biodegradable; fusarium; suspensión coloidal.

Introduction

One of the most common alternatives to control fungal infections on plants, like those caused by fungi of the *Fusarium* genus, is the application of synthetic fungicides, but such pesticides have a few problems associated with them. Over 90% of the applied pesticides are unable to reach the target area required for effective pest control. [1] This is due to different factors including the application technique, physicochemical properties of the pesticides, and environmental conditions like wind speed, humidity, and temperature, influence the extent of loss during application. [2,3] The remaining losses are attributed to leaching, evaporation, deposition, being washed away, and degradation of the active ingredient by photolysis, hydrolysis, and microbial activity. [4] Given all these accumulated losses, the amount of pesticide in the target area is below the minimum effective concentration, and to achieve the desired biological response within a given period, it is necessary to increase the quantity of product applied, as well as the number of applications. This does not only increase the cost of the treatment, but also brings unfavorable outcomes either to plants or to the environment, including soil and water pollution, ultimately endangering human health. [4,5] In addition to the previously mentioned, there are reports that indicate that some synthetic fungicides, like the triazole fungicides, can provoke phytotoxicity in economically important crops, affecting the growth and development of seedlings and the root system, reducing the number of primary roots. [6]

As an environmentally friendly alternative, the search for natural products with applications related to pest management is currently very active. Plant extracts with antimicrobial properties that contain a wide spectrum of secondary or specialized metabolites, whose concentration of bioactive compounds and synergistic effects depend on the environmental conditions, have generated interest from the field of plant disease control. [7–9] In this regard, natural products in the form of extracts, fractions or pure compounds obtained from plants of the genus *Piper* have shown promising antifungal activities. [10] For example, extracts of *Piper capense* and *P. eriopodon* showed antifungal properties against filamentous fungi, such as mycotoxigenic species of the genus *Aspergillus*, *Fusarium*, and *Penicillium*. [11] *Piper divaricatum* also showed antifungal activity against *Fusarium* infection on black pepper, reaching up to 100% of growth inhibition.

[12] At the same time, hexanic extracts of *P. auritum* and *Piper holtonii* showed a relatively good antifungal activity against different phytopathogenic fungi, including *Collectotrichum acutatum, Collectotrichum gloeosporioides,* and *Botryodiplodia theobromae.* [13] Furthermore, isolated compounds obtained from *Piper spp.* extracts have demonstrated fungicide properties against *Fusarium spp.* [14] This biological activity of some of the genus *Piper* species is attributed to the presence of a wide variety of secondary metabolites, like alkaloids, amides, propenyl phenols, lignans, terpenes, flavonoids, among others. [15,16] Regarding the fungicidal activity (resistance to *Fusarium solani f sp piperis*), it is attributed to phenolic compounds specifically found in American *Piper* species. [17] Biological history says that American Piper had developed a mechanism of defense known as Systemic Acquired Resistance (SAR). [18,19] Phenols and flavonoids are overexpressed and act in the fungal wall and the plasmatic membrane, resulting in the disruption of the cell. [20] Some phenols and flavonoids with these characteristics present in Piper are: safrole, apiole, dillapiole, asebogenin, apigenin, quercetin, kaempferol, myricetin and epicatechin. [21]

However, the usage of plant extracts can be hampered by the high volatility and easy degradation of many of the active compounds. Furthermore, in many cases it is necessary to use toxic organic solvents as

J. Mex. Chem. Soc. **2024**, *68*(2)
Regular Issue

ISSN-e 2594-0317

vehicles to incorporate these extracts in the final formulation. So, the encapsulation in suitable nanostructures is a strategy for the preservation and controlled release of bioactive compounds, allowing a better dispersion in water and safer application. [22] Among the materials used to encapsulate the plant extracts, it was found that poly(lactide-co-glycolide) (PLGA) is a very promising one, due to being a biocompatible and biodegradable polymer that degrades by hydrolysis of the ester backbone into non-harmful and non-toxic compounds, and it has been used to enhance the antimicrobial activity of fruit extracts. [23-27] As an additional advantage, PLGA has been proven to bring photoprotection to nanoencapsulated active ingredients. [28] This is relevant given UV light may represent negative effects on natural extracts. The ethanolic extract of *Euphorbia herita* presented antifungal and antibacterial activity against experimental pathogens such as *Escherichia coli* and *Aspergillus niger*, but overexposure to ultraviolet light caused a gradual decrease in the antimicrobial capacity of this plant. [29]This study aims to encapsulate an antifungal crude extract obtained from the *Piper schlechtendalii,* a plant without previous antifungal activity studies and that is endemic to Mexico (with presence in the state of Veracruz), in PLGA nanospheres to protect the active ingredients from photolysis and to preserve their bioactivity when exposed to UV light, as well as to gain dispersion in water without the need of organic solvents.

Experimental

Reagents

G Hydrochloric acid (reagent grade, Sigma-Aldrich, USA), potato dextrose agar (PDA) (Difco, USA), ethanol (analytical-grade, Quimica Rique, Mexico), dichloromethane (DCM) (industrial-grade, Pochteca, Mexico), poly (lactic-co-glycolic) acid (PLGA) 85:15 (reagent grade, Mw 190,000- 240,000, Sigma-Aldrich, USA), acetone (industrial-grade, Pochteca, Mexico), poly vinyl alcohol (PVA) (reagent grade, Mw 89,000-98,000, Sigma-Aldrich, USA), sodium citrate tribasic (reagent grade, Sigma-Aldrich, USA), and type 1 distilled water, obtained by a Genpure water purification system (Thermo Scientific, USA). All organic solvents were purified by simple distillation before use. The industrial-grade solvents were previously distilled before being used in the experiments. The nanoencapsulation of the extract was performed using either the emulsification and solvent evaporation, or the diffusion and solvent evaporation techniques. [30] DCM and acetone were tested as the solvents for the organic phase, PLGA was tested as the biodegradable carrier, and sodium citrate and PVA were tested as the emulsion stabilizer.

Biological material

Plant material (stem and leaves) was collected in Zozocolco, Veracruz, Mexico (Lat 20.14186 Long -97.587901), on April 13, 2019. Only phenotypically similar specimens with healthy appearance were selected. For the bioassays, a phytopathogenic strain of *Fusarium solani* was used, donated by Dr. Mauricio Luna Rodríguez, researcher from the Faculty of Agronomy of the Universidad Veracruzana, Xalapa, Veracruz, Mexico. The strain was cultivated in Petri dishes with potato dextrose agar (PDA) at 27 ± 1°C with periodic reseeding.

Piper schlechtendalii crude extract obtention

The vegetable material was dried on the stove at 45 °C and then pulverized. The hydroalcoholic extract was obtained by maceration in ethanol solution/water solution (70:30), using a mass/volume (m/v) proportion of 1:10, and constant stirring (90 rpm) for 48 h at room temperature. Using only water as solvent could yield a much lower extraction when compared to ethanol or methanol, given these substances increase the solubility of organic material with lower polarity. [31] The solvent was then separated by vacuum filtration and eliminated by rotary evaporation (Büchi RII, Büchi, Switzerland), leaving a completely dry extract (PsE) that was stored in refrigeration until use.

Preparation of stock aqueous solutions

The aqueous phase in the experiments consisted of the dissolution of an emulsion stabilizer in type 1 distilled water. Two different stabilizers were tested, using either a 1 mM sodium citrate solution, or a 1 % w/v PVA solution, respectively. To avoid precipitation of the PLGA nanospheres during the washing process,

J. Mex. Chem. Soc. **2024**, *68*(2)
Regular Issue

ISSN-e 2594-0317

washing solutions were prepared by diluting the aqueous phase ten times, obtaining a concentration of 0.1 mM sodium citrate washing solution, and a 0.1 % w/v PVA washing solution.

Preparation of stock organic solutions

The organic phase consisted of the dissolution of PLGA 85:15 in an organic solvent, with a concentration of 5 mg/L. Two solvents were tested separately: acetone and DCM.

Emulsification and solvent evaporation technique

For each emulsion prepared, 10 mg of the extract previously dissolved in 1 mL of ethanol were added to 5 mL of the PLGA in DCM solution to conform the organic phase. Once homogenized by magnetic stirring, the organic phase was added to 15 mL of the aqueous phase, consisting in a solution of either sodium citrate, or PVA. The emulsion was obtained using an ultrasonic processor (Sonics & Materials, model VCX750-110V) applying 70 % of amplitude in three intervals of 10 seconds. To avoid boiling the DCM, the emulsion was rested in an ice bath for 10 seconds between each interval. Finally, the DCM was removed by rotary evaporation, obtaining solid nanospheres of PLGA suspended in water. Each encapsulation process was repeated 3 times.

Diffusion and solvent evaporation technique

Similarly to the steps performed with the emulsification and solvent evaporation technique, 10 mg of the extract was previously dissolved in 1 mL of ethanol and added to 5 mL of the PLGA solution, the difference being that acetone was used as the organic solvent instead of DCM. Once homogenized by magnetic stirring, the organic phase was poured to 15 mL of the aqueous phase (solution of either sodium citrate, or PVA) under heavy stirring. Since acetone is miscible with water, there was no need for the ultrasonic processor to homogenize both phases. Finally, the acetone was removed leaving the mixture in an extraction hood overnight. Each encapsulation process was repeated 3 times.

Washing of nanospheres

To remove the fraction of extract and stabilizer that remained outside of the PLGA, the nanospheres were washed by centrifugation at 5,000 x g for 30 minutes. Once the supernatant was discarded, the nanospheres were re-suspended in a similar volume of the washing solution, obtaining a colloidal suspension of PLGA nanospheres. This process was performed 3 times.

Characterization of nanospheres

The size and zeta potential were obtained using the dynamic light dispersion technique (DLS), while scanning electron microscopy (SEM) was used to observe the form of the nanoparticles, obtaining the images with a FEI Quanta 250 FEG (United States of America) scanning electron microscope in the Advanced Microscopy Unit (INECOL) To measure the amount of encapsulated extract, it had to be released from the nanospheres and then quantified. To achieve this, a known volume of nanospheres suspension was centrifuged at 5,000 x g to remove the water. Then, a 1 mL of ethanol was added, and the suspension was left in an ultrasonic bath for 30 minutes. After that, the nanospheres were centrifuged at 16,000 x g for 30 minutes. Finally, the ethanol was collected, and the amount of released extract was measured by UV-vis spectrophotometry (Thermo Scientific, Genesys 10S UV-Vis, United States of America). Given the chlorophyll presented the highest absorbance peak, and assuming that all the chemical species in the extract were captured and released in the same proportion, chlorophyll was used as the tracer to measure the concentration of the extract, at a wavelength of 279 nm.

The encapsulation efficiency (EE) was calculated using Equation 1.

$$EE(\%) = (EX_m / EX_0) * 100 \quad (1)$$

where EX_0 is the extract mass used in the encapsulation process and EX_m is the extract mass measured in the ethanol after being released.

J. Mex. Chem. Soc. **2024**, *68*(2)
Regular Issue

ISSN-e 2594-0317

P. schlechtendalii extract release tests

The release profile was obtained by dialyzing 1 mg of extract (either encapsulated or in crude form) in 100 mL of a water/ethanol mixture (90:10), using 10 cm segments of semipermeable cellulose tubes (45 mm flat, 12-14 kD). The pH of the release medium was adjusted to 5 with concentrated HCl, as this was the pH obtained for the PDA used in the antifungal activity tests. The extract was incorporated in 5 mL of the water/ethanol mixture and placed inside the tube (donor medium), while 95 mL of the same mixture was used as the receiving medium, adding a total volume of 100 mL. The dialyzes were performed in amber capped bottles, with constant magnetic stirring (60 rpm), shielded from light by using a cardboard box at room temperature. Samples were taken at different time lapses, and the volume withdrawn was replaced with fresh water/ethanol mixture. Finally, the extract concentration was measured by UV-Vis spectrophotometry. The experiment was performed 3 times for both the crude extract and the nanoencapsualted extract, respectively.

Photodegradation assay

To measure if there is any photoprotection given by the PLGA, 1 mg of extract in crude and nanoencapsulated forms, respectively, were placed in individual glass vials and then irradiated using a UV-A lamp (λ = 354 nm), receiving an irradiance of 13.73 ± 0.035 mW/cm^2 for 24 h.

Antifungal activity of the crude and encapsulate *P. schlechtendalii* extracts

For antifungal characterization of the extracts, 3 µL of conidia suspension of *F. solani* were inoculated in the center of the wells of 12-well culture plates with 1 mL of solid PDA, adjusting the final concentration to 5x10^6 spores/mL, approximately. To test the treatments, the *Piper* extract, either in the crude form or nanoencapsulated form, was incorporated to the PDA up to a concentration of 1 mg/mL. The plates were incubated at 28 ± 1 °C in complete darkness for 5 days. PDA culture media with 5% of ethanol was used as negative control (C-), while solid PDA culture media supplemented with commercial fungicide (Prozan ®) 0.5 µL was used as positive control (C+). The 5% of ethanol present in the control is due this solvent was used as a vehicle for the crude extract. Empty PLGA nanoparticles were tested to evaluate the antifungal activity of the encapsulating polymer, both before and after being exposed to UV-light for 24 h (P and UP, respectively). The concentration of empty PLGA nanoparticles was the same as that of the PLGA present in the nanospheres that contain 1 mg of encapsulated extract. The treatments tested consisted in the extract in its crude and nanoencapsulated forms, before and after been exposed to UV light for 24 h; crude extract (E), nanoencapsulated extract (N), UV irradiated extract (UE), and UV irradiated nanospheres with extract (UN). A treatment consisting in the encapsulation of previously irradiated extract was tested as well (N-UE). The experiments were performed with 5 replicates. The antifungal activity was recorded at 7 days after the inoculation and the area of fungus growth were obtained with image analysis software Image J®. The growth inhibition percentage (GIP) was calculated using Equation 2.

$$GIP\ (\%) = 100 - (A_i/A_c) * 100 \quad (2)$$

where A_c is the growth area of the fungus in the negative control well (full growth), and A_i is the growth area of the fungus in the well exposed to the treatment.

Comparative chemical profiling of the extract

An untargeted metabolomic analysis was performed on the extract before and after been exposed to the 24 h of UV-light ($n \geq 6$), using an ultra-high performance liquid chromatography (UPLC) system (Waters, model Acquity class I), coupled to a quadrupole time-of-flight (QTOF) mass spectrometer (Waters, model Synapt G2-Si HDMi), as proposed by Monribot-Villanueva et al. [32] The chromatography was carried out on an Acquity BEH column (1.7 µm, 2.1 x 50 mm) with a column and sample temperatures of 40 °C and 15 °C, respectively. The mobile phase consisted of (A) water and (B) acetonitrile, both with 0.1 % of formic acid (SIGMA). The gradient conditions of the mobile phases were 0-20 min a linear gradient from 1 to 99 % of B, 20-24 min 99 % of B, 24-25 min a linear gradient from 99 to 1 % of B (total run time 30 min). The flow rate was 0.3 mL/min and 5 µL of extract was injected. The mass spectrometric analysis was performed with an electrospray ionization source in positive mode with a capillary, sampling cone and source offset voltages of 3000, 40 and 80 V, respectively. The

J. Mex. Chem. Soc. **2024**, *68*(2)
Regular Issue

ISSN-e 2594-0317

source temperature was 100 °C and the desolvation temperature was 20 °C. The desolvation gas flow was 600 L/h and the nebulizer pressure was 6.5 Bar. Leucine-enkephalin was used as the lock mass (556.2771, $[M+H]^+$). The conditions used for MSe analysis were mass range 50-1200 Da, Function 1 CE, 6 V, function 2 CER 10-30 V, scan time 0.5 sec. The data were acquired and processed with MassLynx (version 4.1) and MarkerLynx (version 4.1) software respectively. All statistical analysis (Hierarchical clustering, t-test and Fold Change analysis) were performed in the bioinformatic MetaboAnalyst platform. [33]

Results and discussion

Characterization of nanospheres and selection of encapsulating systems

Table 1 shows the hydrodynamic particle diameter, zeta potential, and encapsulating efficiency for the four encapsulating systems tested. Both particle size and zeta potential were obtained using a Malvern Zetasizer, model Nano ZS90 (England).

Table 1. Parameters of the different encapsulating systems.

Solvent	Stabilizer	Size (nm)	Zeta potential (mV)	Encapsulating Efficiency (%)
Acetone	PVA	201.5 ± 5.82	25.63 ± 3.18	20.02 ± 2.54
Acetone	Sodium Citrate	147.7 ± 3.05	37.67 ± 4.93	24.95 ± 3.49
DCM	PVA	269.73 ± 3.75	27.77 ± 0.63	4.19 ± 1.36
DCM	Sodium Citrate	259.16 ± 3.31	35.37 ± 1.5	2.93 ± 0.81

It can be observed that the emulsion stabilizer used in the encapsulation influences the size and the zeta potential of the nanospheres, resulting in both a smaller diameter and a higher stability of the particles when using sodium citrate. Fig. 1 shows the SEM images of the particles of each of the four encapsulating systems, where spherical particles with smooth surfaces can be observed.

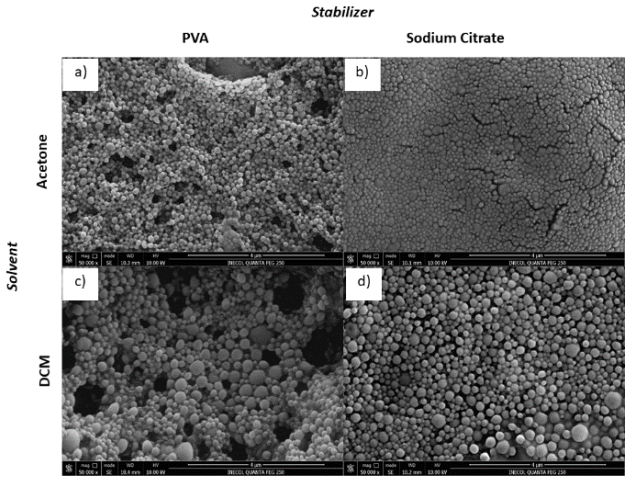

Fig. 1. Morphology of the nanospheres obtained using different solvents and stabilizers at 50 000 X: **(a)** Acetone + PVA, **(b)** Acetone + Sodium Citrate, **(c)** DCM + PVA, **(d)** DCM + Sodium Citrate.

J. Mex. Chem. Soc. **2024**, *68*(2)
Regular Issue

ISSN-e 2594-0317

Since smaller particles can achieve better penetration of the active substance into tissues with reduced consumption rates, and a higher zeta potential denotes better dispersion of the particles in water for longer times, sodium citrate was chosen as the best stabilizer to use in the nanoencapsulation of the extract. [34] On the other hand, the solvent presents an important effect on the encapsulation efficiency, given that the systems that used DCM only achieved percentages under 5 %, while the systems that used acetone managed to reach encapsulations over the 20 %, making the acetone the better solvent to use in the extract encapsulation. In addition to the previously mentioned, the system that used acetone and sodium citrate was the one that yielded the smallest particle size, higher zeta potential, and higher encapsulation efficiency, making it the best encapsulation system for the extract. The largest amount of extract is captured during the nanoemulsion step, with a capture percentage of 0.5 to 20 %, depending on the type of nanosystem. The capture efficiency values in PLGA nanoparticles are related to the solubility of the trapped material in water, since the hydrophilic compounds during the synthesis tend to pass from the organic phase to the aqueous phase, presenting weaker interactions with the polymer and resulting in lower capture efficiencies than the hydrophobic compounds. [29] Regarding the polymer matrix, PLGA degrades *in vivo* into harmless products. Its final degradation products are lactate (lactic acid salt form) and glycolate (glycolic acid salt form), and in *in vitro* systems it does so through hydrolysis of ester bonds, obtaining lactic acid and glycolic acid as final degradation products. [35]

Extract release profiles

It has been reported that the lipases from fungi of the *Candida, Mucor,* and *Rhizopus* geniuses have a significant effect on PLGA degradation by enzyme-catalysed cleavage of ester bonds of the polymer. [36] More specifically, studies have demonstrated that fungus of Fusarium genius would favor the degradation of PLGA copolymers by destroying the crystallization of poly lactide acid (PLA) segments. [37] However, this process was completed over a relative long time (15 weeks) compared to the 6 days that took the release profile experiment. Given this, it is safely to asume that the extract is releases mainly by molecular difussion and not by erotion of the polymer matrix. Preliminary results showed that the extract would not be released into distilled water due to its low solubility, while a liberation in pure ethanol proved to be too fast, dialyzing 100 % of the extract in a few hours, regardless of whether it was in its crude or nanoencapsulated form. Fig. 2 shows the release profiles obtained for the crude extract and the nanoencapsulated extract in water/ethanol (90:10) medium, adjusted at pH 5.

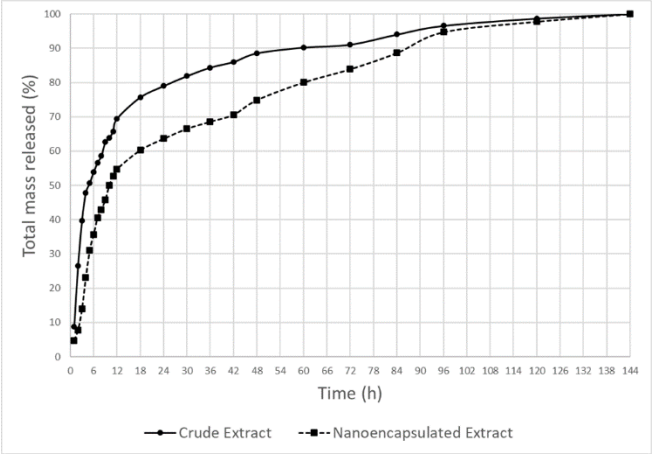

Fig. 2. Percentage of mass of *Piper* extract released in the water/ethanol (90:10) receiving medium.

At first sight, the nanoencapsulated extract is dialyzed slower than its crude form. This is congruent with the literature, and is explained by the molecular diffusion, a relative slow mechanism of mass transport that the extract must endure to travel from the PLGA polymer matrix of the nanospheres to the donor medium inside the cellulose tube, and finally, from the donor medium to the receiving medium. [16] While the crude extract has dialyzed 50 % of its mass in 5 hours, the nanoencapsulated extract took 11 hours to reach that

J. Mex. Chem. Soc. **2024**, *68*(2)
Regular Issue

ISSN-e 2594-0317

percentage. Another important point is at hour 48, having almost reached 90% of the crude extract mass release while the nanospheres still conserve 25 % of the extract load.

Photodegradation assay

In order to determine the effect of UV exposition on PsE, an untargeted metabolomic analysis was performed comparing PsE exposed and non-exposed to UV radiation. The UV exposition clearly changes the chemical profile of the PsE (Fig. 3**(a)**). The hierarchical clustering by the Ward algorithm displayed in the top of Fig. 3**(a)** shows that UV treatment consistently affect the chemical composition of the *Piper* extract. The compounds tentatively identified in the PsE exposed and non-exposed to UV radiation were grouped mainly in seven metabolic pathways (Fig. 3**(b)**) which exhibited the highest pathway impact values. Detailed information about all metabolic pathways and the tentatively identified compounds is shown in Supplementary Material. When it is performed a comparative fold change (FC) analysis using a threshold value of 1.5 joined to a *t*-test analysis between PsE exposed and non-exposed to UV radiation, the intensity of 294 features (mass/charge-retention times pairs) remain unchanged, while the intensity of 20 and 58 features increased and decreased after UV treatment, respectively (Fig. 3**(c)**). The compounds methyl-hexyl caffeate (FC=3.63) and linolenic acid (FC=3.03) increased their content after UV treatment. On the other hand, the compounds feruloyltyramine (FC=0.63), pyridoxine (FC=0.59), pantetheine phosphate (FC=0.59), apiole (FC=0.50), aceteugenol (FC=0.50), methyl-trimethoxycinnamate (FC=0.48), eugenyl benzoate (FC=0.48), (2E,6E)-piperamide-C7:2 (FC=0.48), sphinganine (FC=0.44), methyl 3-(3,4-dimethoxyphenyl)propanoate (FC=0.30), 3-hydroxy-4-methoxy-2-(3,7,11-trimethyldodeca-2,6,10-trienyl)benzoic acid (FC=0.26), ethyl 3-(3,4-dimethoxyphenyl)propanoate (FC=0.25) and acetoxychavicol acetate (FC=0.08) decrease their content after UV exposition (Fig. 3**(c)**). Detailed information about the tentatively identified compounds is shown in Supplementary Material.

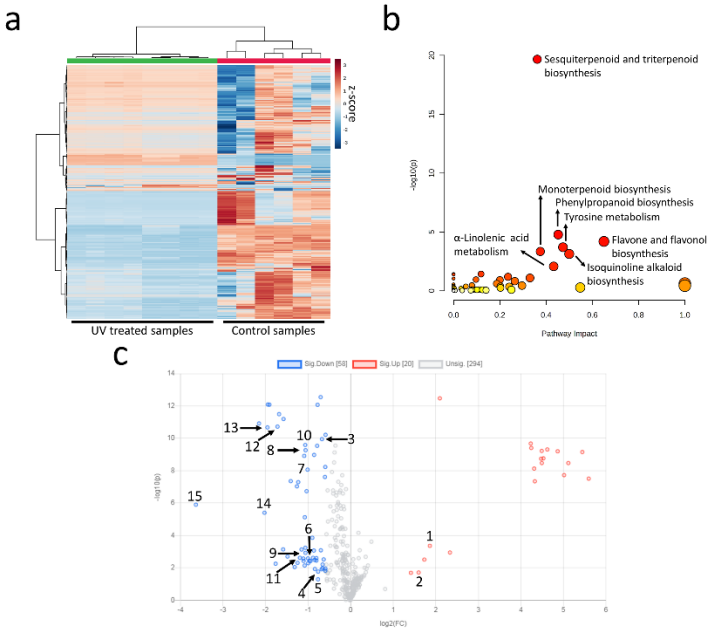

Fig. 3. Comparative metabolomic analysis of PsE before and after UV treatment. **(a)** Heatmap showing the 372 features detected in the extract before (Control) and after UV treatment (UV treated). **(b)** Pathway impact of the tentatively identified compounds. **(c)** Volcano plot of the features intensity of UV treated/Control samples. 1: Methyl-hexyl caffeate; 2: Linolenic acid; 3: Feruloyltyramine; 4: Pyridoxine; 5: Pantetheine phosphate; 6: Apiole; 7: Aceteugenol; 8: Methyl-trimethoxycinnamate; 9: Eugenyl benzoate; 10: (2E,6E)-Piperamide-C7:2; 11: Sphinganine; 12: Methyl 3-(3,4-dimethoxyphenyl)propanoate; 13: 3-Hydroxy-4-methoxy-2-(3,7,11-trimethyldodeca-2,6,10-trienyl)benzoic acid; 14: Ethyl 3-(3,4-dimethoxyphenyl)propanoate; 15: Acetoxychavicol acetate.

J. Mex. Chem. Soc. **2024**, *68*(2)
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ISSN-e 2594-0317

Antifungal activity

The fungal growth in the well plates at the time the experiment was concluded is shown in Fig. 4. It can be perceived that the PLGA nanospheres, both before and after exposure to UV light, have very little antifungal activity, and it's close to the one presented by the negative control. Concerning the antifungal activity of the crude PsE, it is comparable to the one reported for other plants of the *Piper* genius when tested *in vitro* against fungi of the *Fusarium* genius. For example, *Piper sarmentosum* extracts exhibited inhibition activity against *F. graminearum* at concentrations of 1 and 2 mg/mL. [38] This behavior is not limited to the *Piper* extracts, since experiments using essential oils, such of the obtained from *Piper auritum,* demonstrated growth inhibition against *Fusarium oxysporum* and *Fusarium equiseti*. [39]

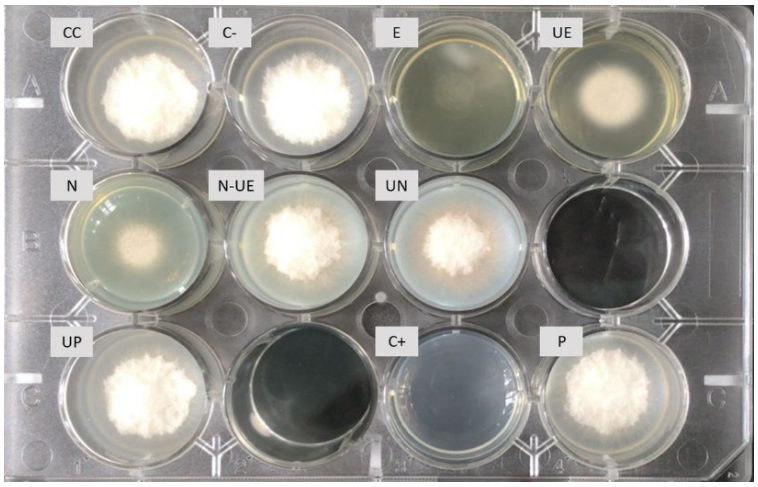

Fig. 4. Representative image of the *F. solani* growth detected in the well plates with the different treatments; crude extract (E), nanoencapsulated extract (N), UV irradiated extract (UE), UV irradiated nanospheres with extract (UN), encapsulation of a previously irradiated extract (N-UE), growth control (CC), positive control (C+), and negative control (C-).

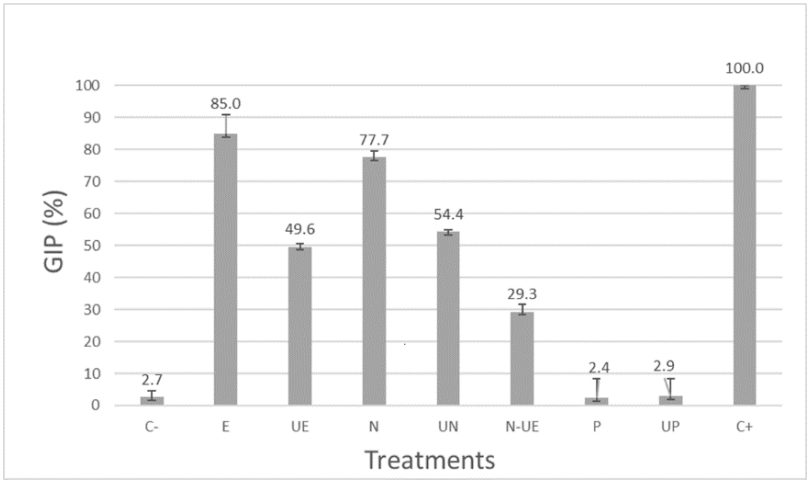

Fig. 5. Growth inhibition potential (GIP) values with standard deviation for the different treatments tested; crude extract (E), nanoencapsulated extract (N), UV irradiated extract (UE), UV irradiated nanospheres with extract (UN), encapsulation of a previously irradiated extract (N-UE), PLGA nanospheres (P), irradiated PLGA nanospheres (UP), positive control (C+), and negative control (C-).

J. Mex. Chem. Soc. **2024**, *68*(2)
Regular Issue

ISSN-e 2594-0317

The GIP for the different treatments tested are shown in Fig. 5. The antifungal activity decrease exhibited by PsE exposed to UV radiation compared to non-exposed treatment (UE vs E) can be explained by the decrease in the content of the metabolites showed previously (Fig. 3**(c)**). Acetoxychavicol acetate, which exhibited the largest decrease (FC=0.08) after UV treatment, has been reported in *Piper cubeba* [40] and it has shown antifungal activity against *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton concentricum*, *Epidermophyton floccosum*, *Rhizopus stolonifer*, *Penicillium expansum*, *Aspergillus niger* and *Candida albicans*. [41] Also, acetoxychavicol acetate inhibited the growth of *Alternaria porri*, *Colletotrichum gloesporioides*, *Fusarium oxysporum* and *Phytophthora nicotianae*. [42]

It is important to mention that the PsE lost some growth inhibition capacity (about 7 %) when encapsulated in the nanospheres. It happens because, at the start of the experiment, there is less availability of the bioactive molecules in the extract, given they must be released from the PLGA matrix to the PDA medium, while the E treatment has the 100 % of the active molecules available from the beginning. Nevertheless, after being exposed from 24 h to UV light, the crude extract growth inhibition capacity was diminished down to 58.35 % of its original, while the nanoencapsulated extract retained up to 70 % of its original. In addition to this, globally, the UN treatment achieved close to a 5 % more GIP than the UE. Finally, the N-UE treatment yielded the lowest GIP, confirming that the PLGA is absorbing a portion of the UV radiation. Previous studies have tested the photoprotective efficiency of PLGA nanoparticles to reduce the adverse biological interactions of photo-degradation products of curcumin upon the exposure of UVA and UVB. The results demonstrated that a significant DNA damage in the cells was due to free curcumin exposed to UV light, while the PLGA nanoparticles with curcumin were totally photosafe. [43] Other studies have used PLA as wall material to protect the photosensitive molecules of spinosad and emamectin benzoate, reaching a significantly higher long-term toxicity to *Plutella xylostella*, as well as a reduction in the photolysis and hydrolysis of these insecticides. [44]

Conclusions

The *Piper schlechtendalii* extract was successfully encapsulated in a PLGA nanospheres system, with particle sizes under 150 nm, having a good dispersion in water, removing the necessity of organic solvents as vehicle for the antifungal natural product. Using acetone as solvent for the organic phase proved to yield the best encapsulation efficiencies, while using the sodium citrate solution as the aqueous phase yielded the best particle size and Zeta potential. The dialysis experiments show that the encapsulated extract is released at a much slower rate as compared to the crude extract. The advantage of this release profile lies in the fact that the whole load of extract would not be exposed to sunlight at the same time (as in the case of the crude extract treatment), but it is released fast enough to be able to control the in vitro growth of the *Fusarium solani* fungus tested. *In vivo,* tests are needed in order to determinate if there is a decrease in the antifungal activity due to a very slow release of PsE in a medium lacking any organic solvent. Finally, even when the nanoencapsulated extract was not as effective, compared to its crude form at the beginning of the experiment, once the treatments were exposed to UV light, the PLGA probed to partially absorb the radiation, protecting the active ingredients in the extract, and reaching a higher inhibition than the one yielded by the crude extract. This difference was only 5 % but could grow if the treatments were exposed to the UV radiation for longer times. This nanoencapsulated system has potential as a mean to protect the extract from photodegradation and perhaps represents, in the future, an environmentally friendly alternative for the application of the antifungal *Piper schlechtendalii* extract.

Acknowledgements

This work was supported by the FORDECyT (Fondo Institucional de Fomento Regional para el Desarrollo Científico, Tecnológico y de Innovación) of CONAHCyT (Consejo Nacional de Humanidades, Ciencias y Tecnologías) under Grant 292399 and PRONACES-CONAHCyT under Grant 316998.

J. Mex. Chem. Soc. **2024**, *68*(2)
Regular Issue

ISSN-e 2594-0317

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