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Valorization of Plant Extracts by Encapsulation in Lipid Nanosystems for Application as Potential Insecticides †

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Abstract: Plants have been used for centuries to treat diseases and are considered an important source of new antimicrobial agents. Plant extracts can be obtained and their composition determined, being widely employed in the pharmaceutical and cosmetic industries. A less explored and potential application is the use as green insecticides/insect repellents, as an alternative to current pesticides. Despite the desirable properties, many of the isolated components (phytochemicals) present limitations on their use, due to high volatility and easy degradation when exposed to air. Nanoencapsulation techniques arise as promising strategies to allow the preservation and controlled release of plant extracts. In this work, a series of plant materials, *Phytolacca americana* L., *Tagetes patula* L., and *Ruta graveolens* L., were subjected to *Soxhlet* extraction using various solvents and times of extraction. The extracts obtained were submitted to biological studies, to assess their potential against the insect cell line Sf9. Encapsulation assays in lipid nanosystems were carried out, with encapsulation efficiencies higher than 70%.

Keywords: plant extracts; nanoencapsulation; liposomes; green insecticides

1. Introduction

Many plant extracts are known for their antimicrobial action, as well as analgesic, sedative, anti-inflammatory, spasmolytic and locally anaesthetic properties [1]. Despite their high interest, many of the components isolated from plant extracts present some limitations for their potential use, due to the high volatility and easy degradation when exposed to air. Considering these drawbacks, nanoencapsulation techniques that allow preservation and controlled release of plant components arise as strategies of great interest [2,3]. Among all the encapsulation vehicles, lipid-based nanosystems have been widely employed in pharmaceutical and cosmetic industries [4].

A less explored and potential application of plant extracts is the use as green insecticides/insect repellents, as an alternative to current pesticides. In this regard, nanoencapsulation also represents a promising way for pesticides safe application [5,6].

In this work, a series of plant materials, *Phytolacca americana* L., *Tagetes patula* L., and *Rute graveolens* L., were subjected to *Soxhlet* extraction using various solvents and times of extraction. The vegetable material used was dried according to standard procedures. The extracts were purified

by silica gel column chromatography or by successive washes with solvents of increasing polarity. After characterization, these extracts were submitted to biological studies, to assess their potential against an insect cell line (*Sf9*). Extracts from *Ruta graveolens* L. leaves have been reported to act as natural fungicides for plant disease control [7], while *Phytolacca americana* L. and *Tagetes patula* L. also exhibit well-known insecticidal properties [8,9].

Encapsulation assays in lipid nanosystems were carried out, using both the thin film hydration and ethanolic injection methods for the preparation of extract-loaded nanovehicles. Here, the encapsulation efficiencies obtained for the extracted plant material point to a promising application of encapsulated extracts, namely as green pesticides.

2. Materials and Methods

2.1. Plant Materials Extraction

The plant material was separated according to different parts (leaf, stem, fruit, and flowers). In the case of flowers, the flower was also separated from the receptacle. Herbal material such as fruit, which contains sugars and is more humid, was previously lyophilized. In this case, pokeweed berries and French marigold flowers (yellow, orange, and red) were lyophilized for seven days in a lyophilizer (VirTis, SP Scientific, St. Louis, Missouri, USA) at a condenser temperature of −40.0 °C and a vacuum of 248 mT. The less humid vegetable material was dried in an oven (Heraeus, Hanau, Germany) at a temperature of 40–45 °C for 24 h.

The dried plant material was grounded with a chopper (Moulinex, Écully, France) and the resulting powder passed through a sieve. Only the powder of plant material less than 910 μm was transferred to a new container properly identified and stored under vacuum for further use.

Soxhlet equipment was used for extraction of plant material. Plant material was extracted with various solvents (DCM, Water/EtOH 1:1, EtOAc). The crude extracts were freed from solvents by a rotary vacuum evaporator and then air dried. The extracts obtained were used for the assessment for further studies.

2.2. Assays in Insect Cell Line Sf9

The insect cell line Sf9 (Spodoptera frugiperda) was maintained as a suspension culture with Grace's Insect Medium supplemented with 10% FBS and 1% antibiotic. Cells were plated at a density of 30,000/well and exposed to the extracts for 24 h, after which cells were incubated with a solution of MTT (0.5 mg/mL, final concentration). Three independent experiments were conducted, each one in triplicate.

2.3. Nanoencapsulation Studies

For nanoencapsulation studies, the extracts obtained from *Phytolacca americana* L., *Tagetes patula* L., and *Ruta graveolens* L. were used. Liposomes were prepared using a commercial lipid mixture used in the food industry, soybean lecithin [10] (Sternchemie), containing 22% phosphatidylcholine, 20% phosphatidylethanolamine, 20% phosphatidylinositol, and 10% phosphatidic acid as major components at 1 mM concentration. In the thin film hydration method [11], a lipidic film was obtained from the evaporation of ethanolic lipid solution and addition of the extract, followed by hydration, bath sonication, and extrusion (Lipex™ Extruder, Northern Lipids, Burnaby, BC, Canada) through polycarbonate membranes (200 nm pore size). In the ethanolic injection method [12], simultaneous injection of the extract and lipid were carried out under vigorous vortexing in an aqueous buffer solution.

2.4. Encapsulation Efficiency

For each extract, concentration dilutions of $5 \times 10^{-3} - 2 \times 10^{-1}$ mg/mL were performed to determine a calibration curve (absorbance vs. concentration). Loaded liposomes were subjected to centrifugation at 11,000 rpm for 60 min using Amicon® Ultra centrifugal filter units 100 kDa. Then,

the filtrate (containing the non-encapsulated compound) was pipetted out, the water was evaporated, and the same amount of ethanol was added. After vigorous agitation, its absorbance was measured, allowing the determination of compound concentration using a calibration curve previously obtained in the same solvent. Absorption spectra were performed in a Shimadzu UV-3600 Plus UV-vis-NIR spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Three independent measurements were performed for each system. The encapsulation efficiency, $EE(\%)$, was obtained through Equation (1):

$$EE(\%) = \frac{Total\ amount\ -\ Amount\ of\ nonencapsulated\ extract}{Total\ amount} \times 100 \quad (1)$$

3. Results and Discussion

3.1. Extraction and Characterization

Phytolacca americana L. (c), *Tagetes patula* L. (French marigold), and *Ruta graveolens* L. (common rue) were collected in the north region of Portugal from September 2017 to May 2018. The material was dried in an oven at 40–45 °C for 24 h. The dried material was crushed using a shredder and then sieved through a sieve with a porosity of less than 910 μm. Berries, leaves, and stem of *Phytolacca americana* L., and yellow, orange, and red leaves of the flowers of *Tagetes patula* L., and leaves from *Ruta graveleons* L. were used.

The *Soxhlet* extraction technique with either ethanol/water (1:1), dichloromethane (DCM) and ethyl acetate (EtOAc) was used for obtaining the extracts of the different parts of the mentioned species (Table 1). The obtained extracts were further subjected to a dry procedure, either by solvent removal under vacuum or by lyophilization, and kept in a desiccator under nitrogen atmosphere.

Table 1. *Soxhlet* extraction conditions and colours of the obtained extracts.

Species	Part of the Plant	Solvent	Extraction Time (h)	Colour
Phytolacca americana L. (american pokeweed)	Berries	ethanol/water (1:1)	9	brownish
			4	pink
			2	dark pink
		DCM	9	green
			4	green
			2	green
	Leaves	ethanol/water (1:1)	9	green
		DCM	4	green
Tagetes patula L. (French marigold)	Leaves (yellow, orange, and red)	ethanol/water (1:1)	9 (yellow)	brown
			9 (orange)	brown
			9 (red)	brown
		DCM	4 (yellow)	dark green
			4 (orange)	dark green
			4 (red)	dark green
	Flowers (yellow, orange, and red)	ethanol/water (1:1)	9 (yellow)	yellow
			9 (orange)	yellow
			9 (red)	burgundy
		DCM	4 (red)	orange yellow
Ruta graveolens L. (common rue)	leaves	DCM	4	green
		ethanol/water (1:1)	9	brownish
		EtOAc	4	green

3.2. Assays in Sf9 Insect Cell Line

The extracts obtained from *Phytolacca americana* L. and *Tagetes patula* L. were submitted to preliminary biological tests, namely viability assessment with the insect *Sf9* cell line, a clonal isolate of *Spodoptera frugiperda Sf21* cells (IPLB-Sf21-AE). The results are shown in Figures 1–4.

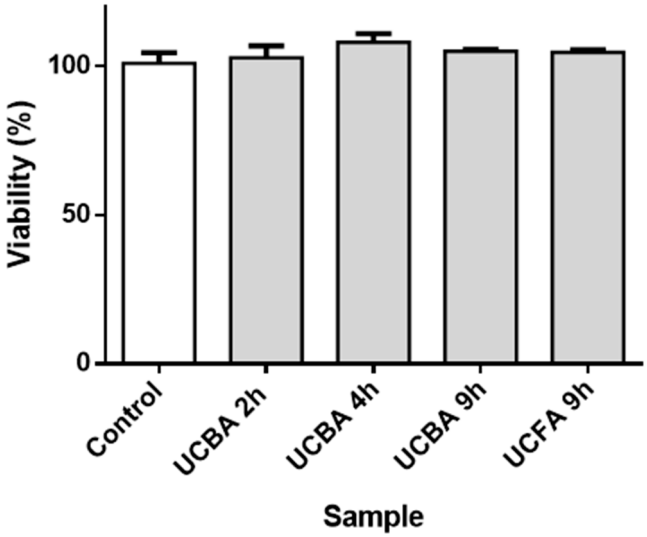

Figure 1. Effect on viability of membrane of *Sf9* cells of ethanol/water (1:1) extracts of *Phytolacca americana* L. (berries and leaves). UCBA 2h: berries at 2 h extraction; UCBA 4h: berries at 4 h extraction; UCBA 9h: berries at 9 h extraction; UCFA 9h: leaves at 9 h extraction. Cells were incubated with concentrations of 0.25 mg/mL for 24 h. Results correspond to the mean ± SD of at least 3 independent experiments.

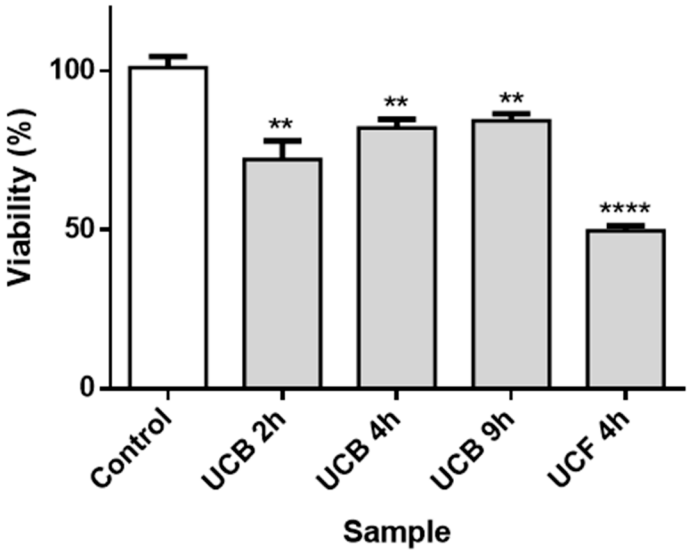

Figure 2. Effect on viability of membrane of *Sf9* cells of dichloromethane extracts of *Phytolacca americana* L. (berries and leaves). UCB 2h: berries at 2 h extraction; UCB 4h: berries at 4 h extraction; UCB 9h: berries at 9 h extraction; UCF 4h: leaves at 4 h extraction. Cells were incubated with concentrations of 0.25 mg/mL for 24 h. Results correspond to the mean ± SD of at least 3 independent experiments. ** $p < 0.01$; **** $p < 0.0001$.

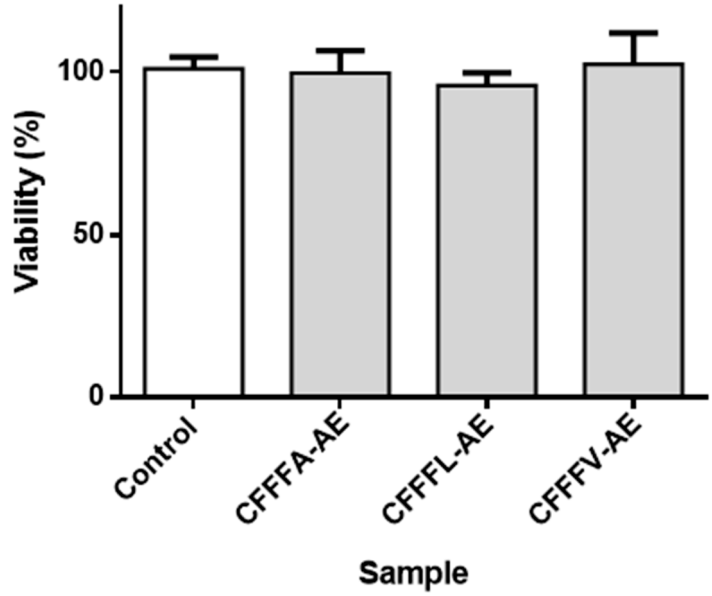

Figure 3. Effect on viability of membrane of *Sf9* cells of ethanol/water (1:1) extracts of *Tagetes patula* L. (9 h extraction). CFFFA-AE: leaves of yellow flowers; CFFFL-AE: leaves of orange flowers; CFFFV-AE: leaves of red flowers. Cells were incubated with concentrations of 0.25 mg/mL for 24 h. Results correspond to the mean ± SD of at least 3 independent experiments.

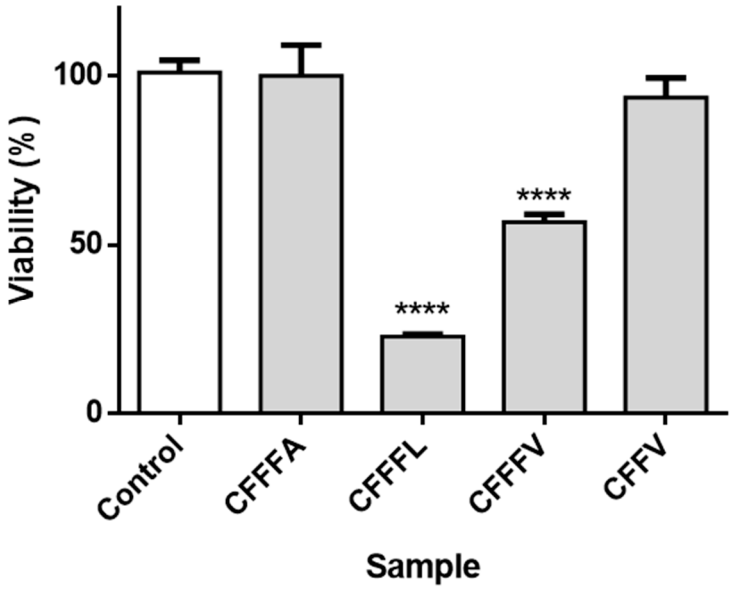

Figure 4. Effect on viability of membrane of *Sf9* cells of dichloromethane extracts of *Tagetes patula* L. (4 h extraction). CFFFA: leaves of yellow flowers; CFFFL: leaves of orange flowers; CFFFV: leaves of red flowers; CFFV: red flowers. Cells were incubated with concentrations of 0.25 mg/mL for 24 h. Results correspond to the mean ± SD of at least 3 independent experiments. **** $p < 0.0001$.

In ethanol/water extracts, the cell viability was not significantly affected by the extraction time (Figures 1 and 3). Considering that the extract composition includes chlorophylls, their removal will lead to lower viability in insect cell cultures at lower concentrations of the extract. The results obtained show a lower viability in the case of extracts obtained from dichloromethane in both species (Figures 2 and 4), with the best results related to leaves of both *Phytolacca americana* L. and *Tagetes patula* L. (leaves of orange flowers).

3.3. Nanoencapsulation Assays

Encapsulation assays in lipid nanosystems were carried out, using both the thin film hydration and ethanolic injection methods for the preparation of extract-loaded nanocarriers. Thin film hydration is one of the simplest ways to prepare liposomes, affording homogeneous small vesicles after extrusion, being especially suitable for hydrophilic compounds [11]. The ethanolic injection method has been shown to be adequate for enhanced encapsulation of poorly water-soluble compounds [12]. Table 2 shows the determined encapsulation efficiencies for both preparation methods and the three extracts (for *Tagetes patula* L. extracts, thin film hydration was not tested).

Table 2. Encapsulation efficiency, *EE(%)* ± *SD(%)*, of plant extracts in liposomes prepared by the two methods (SD: standard deviation).

Species	Thin Film Hydration	Ethanolic Injection
Phytolacca americana L.	77.5 ± 6	73.3 ± 5
Tagetes patula L.	---	97.1 ± 3
Ruta graveolens L.	73.0 ± 9	93.3 ± 6

The encapsulation efficiencies are higher for the ethanolic injection method, except for *Phytolacca americana* L., which can be due to a higher hydrophilicity of this extract. Nevertheless, the high *EE(%)* values obtained (Table 2) point to promising future applications of the extract-loaded soybean liposomes as green insecticides, with the possibility of controlled release of the encapsulated compounds.

4. Conclusions

A series of extracts of different plant species were obtained by *Soxhlet* extraction with dichloromethane and ethanol/water (1:1). Preliminary biological assays using an insect cell line revealed lower viability in the case of extracts obtained from dichloromethane in both species, with the best results related to leaves of both *Phytolacca americana* L. and *Tagetes patula* L. (leaves of orange flowers). Further studies will be carried out in order to ascertain the best concentration for each plant, including the removal of components that may cause biological activity reduction. The encapsulation assays in lipid-based nanosystems point to promising future applications of the encapsulated extracts as green insecticides with the possibility of controlled release.

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Conflicts of Interest: The authors declare no conflict of interest.

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