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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, *Tamus communis* 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 anesthetic 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, *Tamus communis* 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 *Tamus communis* 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, which contained sugars and is more humid, such as fruits, has previously been lyophilized. In this case, pokeweed berries and French marigold flowers (yellow, orange and red) were lyophilized for 7 days in a lyophilizer (Virtis.SP.SCIENTIFIC) at a condenser temperature of -40.0 °C and a vacuum of 248 mT. The less humid vegetable material was dried in an oven (Heracus) at a temperature of 40-45 °C for 24 h.

The dried plant material was grounded with a chopper (Moulinex) and the resulting powder passed through a sieve. Only 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 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) were 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 hours, 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 *Tamus communis* L., *Tagetes patula* L. and *Ruta graveolens* L. were used. Liposomes were prepared using a commercial lipid mixture used in 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 thin film hydration method [11], a lipidic film was obtained from evaporation of ethanolic lipid solution and addition of the extract, followed by hydration, bath sonication and extrusion (Lipex™ Extruder, Northern Lipids, Canada) through polycarbonate membranes (200 nm pore size). In 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 2×10^{-5} - 50×10^{-4} mg/mL were performed to determine a calibration curve (absorbance *vs.* concentration). Loaded liposomes were subjected to centrifugation at 11000 rpm for 60 minutes 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 to determine 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),

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

3. Results and Discussion

3.1. Extraction and characterization

Tamus communis L. (dog grape), *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 hours. Then, 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 *Tamus communis* L. and yellow, orange and red leaves of flowers of *Tagetes patula* L. and leaves from *Ruta graveleons* L. were used.

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
Tamus Communis L. (dog grape)	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 *Tamus communis* 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.

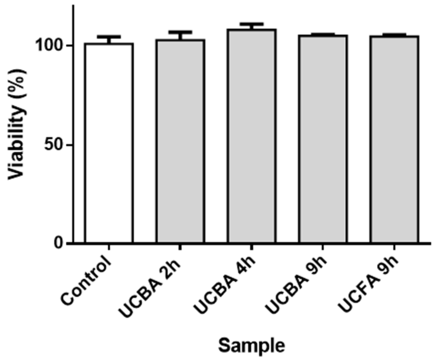

Figure 1. Effect on viability of membrane of *Sf9* cells of ethanol/water (1:1) extracts of *Tamus communis* L. (berries and leaves). UCBA 2h: berries at 2h extraction; UCBA 4h: berries at 4h extraction; UCBA 9h: berries at 9h extraction; UCFA 9h: leaves at 9h extraction. Cells were incubated with concentrations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

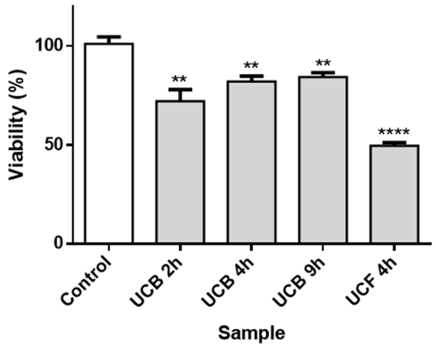

Figure 2. Effect on viability of membrane of *Sf9* cells of dichloromethane extracts of *Tamus communis* L. (berries and leaves). UCB 2h: berries at 2h extraction; UCB 4h: berries at 4h extraction; UCB 9h: berries at 9h extraction; UCF 4h: leaves at 4h extraction. Cells were incubated with concentrations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

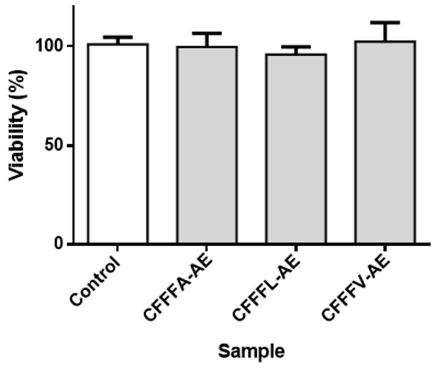

Figure 3. Effect on viability of membrane of *Sf9* cells of ethanol/water (1:1) extracts of *Tagetes patula* L. (9h extraction). CFFFA-AE: leaves of yellow flowers; CFFFL-AE: leaves of orange flowers; CFFFV-AE: leaves of red flowers. Cells were incubated with concentrations of 0.25 mg/mL for 24 hours.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

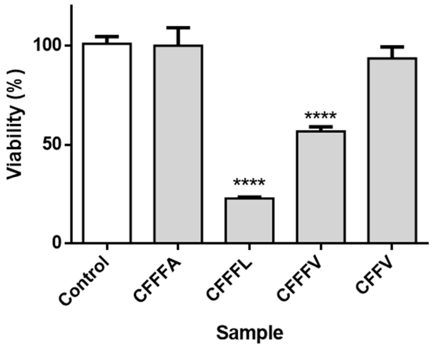

Figure 4. Effect on viability of membrane of *Sf9* cells of dichloromethane extracts of *Tagetes patula* L. (4h extraction). CFFFA: leaves of yellow flowers; CFFFL: leaves of orange flowers; CFFFV: leaves of red flowers; CFFV: red flowers. Cells were incubated with concentrations of 0.25 mg/mL for 24 hours. 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 *Tamus communis* 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 *Tagete 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
Tamus communis 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 *Tamus communis* 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 Tamus communis* 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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