AAPS PharmSciTech

Essential Oil Formulations Useful as a New Tool for Insect Pest Control

Submitted: February 14, 2002; Accepted: June 2, 2002

Mario D.L. Moretti[1], Giovanni Sanna-Passino[1], Stefania Demontis[2], and Emanuela Bazzoni[2]
[1]Dipartimento di Scienze del Farmaco, Università di Sassari, Via Muroni 23/a, 07100 Sassari, Italy
[2]Istituto per lo Studio degli Ecosistemi (ISE, CNR), Sez. Ecologia Applicata e Controllo Biologico, Via De Nicola, 1, 07100 Sassari, Italy

ABSTRACT This study investigated the effects of some essential oils on *Limantria dispar* (Lepidoptera: Lymantridae, gypsy moth) larvae, one of the most serious pests of cork oak forests. The essential oils were first formulated as oil in water (o/w) emulsions and used in laboratory bioassays to assess their lethal concentration (LC50). Microcapsules containing the most promising oils (*Rosmarinus officinalis* and *Thymus herba-barona*) were then prepared by a phase separation process, followed by freeze-drying. The formulations thus obtained, characterized in terms of essential oil content and composition, morphology, storage stability, and release profile, were tested on gypsy moth larvae. The results showed that the tested oils possess interesting larvicidal effects that make them suitable for application in integrated control strategies. The microencapsulation process gave high encapsulation yields (over 98%) with both essential oils, which have different chemical compositions. The microcapsules had toxic effects at a concentration similar to that usually employed for localized treatments with microgranular synthetic pesticides. Toxicity appeared to be maximized when the microparticles adhered to the typical hair structures of several defoliator families. These formulations seem to be able to protect the core material against environmental agents and could be considered for use in controlled drug release systems. The natural active principles they contain could provide an alternative system in insect pest control.

***Corresponding Author:** Mario D.L. Moretti, Dipartimento di Scienze del Farmaco, Università di Via Muroni, 23/a 07100 Sassari, Italy; E-mail:dsfgigi@ssmain.uniss.it

Key Words: essential oils, microencapsulation, in vitro release, *Limantria dispar* L., larvicidal effect

INTRODUCTION

In the past few years, several studies have focused on the potential use of essential oil applications in biological control of different insect pests. The essential oils may be more rapidly degraded in the environment than synthetic compounds, and some have increased specificity that favors beneficial insects [1]. Their action against stored product insects has been extensively studied [2]. Moreover, these natural derivatives are considered to be an alternative means of controlling harmful larvae of Lepidoptera. Recent research has demonstrated their larvicidal and antifeeding effects [3-5], their capacity to delay development and adult emergence and cause egg mortality [6], their deterrent effects on oviposition [7], and their arrestant and repellent action [8]. Despite these most promising properties, problems related to their volatility, poor water solubility, and aptitude for oxidation have to be resolved before they are used as an alternative pest control system [9]. Pesticides are usually formulated as both conventional liquid (aqueous and nonaqueous solutions or dispersions) and solid (wettable powders and water-dispersible granules) systems or as controlled-release systems [10]. The choice of formulation is influenced by several factors, such as the physical, chemical, and biological properties of the pesticide; the mode of application; the crop to be treated; and agricultural practices. Economic considerations also have to be taken into account,

particularly when repeat field applications are necessary to maintain pest control. Controlled-release formulations allowing smaller quantities of pesticide to be used more effectively over a given time interval seem to be the best choice to meet these multiple demands of efficacy, suitability to mode of application, and minimization of environmental damage [11].

The aim of this study was to assess the activity of some essential oils on *Lymantria dispar* L. (Lepidoptera: Lymantridae, gypsy moth) larvae, one of the most destructive pests of cork oak forests in the eastern United States, Europe, and Asia. *L dispar* is frequently responsible for heavy infestations leading to the total defoliation of large areas of forest oak trees. The quantity of the damage is related to larval density. Until a few years ago, defoliators were controlled exclusively with chemical products that had negative effects on the beneficial entomofauna and the environment. Currently, the use of *Bacillus thuringiensis*-based insecticides is the most common control tactic for the gypsy moth [12]. The newest *B thuringiensis*-based formulations consist of matrix-type microcapsules in which envelopes are produced from natural or synthetic polymers through a phase separation process [13].

In this work, 7 essential oils of different chemical composition were first formulated as oil in water (o/w) emulsions and tested in bioassays to determine their toxicity on IInd and IIIrd instar *L dispar* larvae. The most promising oils were then formulated as microcapsules and assessed as a potential new tool for insect pest control using *L dispar* larvae as a model.

Of the different encapsulation processes, spray drying is the one most frequently used to encapsulate flavors [14], despite its limitations due to the relatively high temperature used (coalescence of oil droplets, nonuniform size of microcapsules, and loss of core material). Taking into account the limitations of this process and the physicochemical characteristics of essential oils, we decided to use a coacervation process, well known as a general method for encapsulating active principles. Freeze-drying, widely used for dehydrating mixtures containing heat-sensitive ingredients, was chosen as a method for dehydrating wet microparticles, which are usually air-dried or dehydrated by treating with chemical agents [15].

MATERIALS AND METHODS

Gelatin 120 Bloom (Cruciani, Rome, Italy), anhydrous Na_2SO_4 (Merck, Milan, Italy), glutaraldehyde 50% solution (Sigma Aldrich, Milan, Italy), sodium hydroxide 1M solution (Merk), Tween 80 (Sigma Aldrich), and silicon oil (ICN Pharm, Milan, Italy) were used as received, without further purification. *Cinnamomum zeylanicum* Nees ex Blume (Lauraceae) oil (Ulrich) was purified by steam distillation before use.

Thymus herba-barona Loisel, *Rosmarinus officinalis* L, *Myrtus communis* L, *Eucalyptus globulus* Labill, *Salvia officinalis* L, *Helichrysum italicum* sub *microphyllum* G Don essential oils were obtained from the corresponding aromatic plant species, as reported in the Methods section.

Essential Oil Extraction

Fresh plant materials were collected in Sardinia during the balmy period and distilled within 24 hours in a steam distiller with an aqueous phase recycling system (Albrigi, Stallavena-Verona, Italy), using a plant material:water ratio of 2:1. The distillation time was about 2 hours, and the oil obtained was separated from the aqueous solution and dried by treating with anhydrous Na2SO4. Each essential oil was transferred into a dark glass flask filled to the top and kept at a temperature of 4°C until used.

Oil Yield

Oil yields were determined using a Clevenger-type apparatus (Soffieria Vetro, Sassari, Italy) in conformity with European Pharmacopoeia standards [16]. The values reported are the mean of at least 3 distillations.

Oil Analyses

The composition of the examined essential oils was determined by gas chromatography (GC) and mass spectra (MS) analyses. GC analyses were performed using a Carlo Erba HRGC 5300 Mega series gas chromatograph with Flame Ionization Detector (FID) (Carlo Erba, Milan, Italy). Two columns with different polarity were employed. A Supelco Inc. fused silica SPB-5 (5% diphenyl, 94% dimethyl, 1% vinylpolysiloxane bonded phase) column (30 m x 0.32 mm, film thickness 0.25 µm) (Supelchem, Milan, Italy) was used with a temperature program of 60°C to 150°C at 5°C/min, with 20 minutes final hold, then 150°C to 220°C at 5°C/minute, holding the final temperature for 20 minutes. The carrier gas was helium (1 mL/min). The detector temperature was 250°C. The injection system was "on column." A Supelcowax 10 (Carbowax 20M bonded phase) column (15 m x 0.25 mm, film thickness 0.25 µm) (Supelchem, Milan, Italy) was used with a temperature program ranging from 50°C to 180°C at 3°C/min, holding the initial temperature for 8 minutes and the final temperature for 20 minutes. The carrier gas was helium (0.6 mL/min). Injector and detector temperatures were 200°C and 220°C respectively. The injection was performed as "split mode" (ratio 1:40). All GC analyses were performed by injecting 1% solutions of the compound of interest into redistilled hexane. The retention indices of the oil constituents were calculated as described in the literature [17], and peak identities were determined by comparison of their retention indices with authentic standards, and by peak enrichments with standard compounds. Quantitative data were obtained using ethylene glycol monobutyl ether as an internal standard. Data are expressed as the mean values of 3 analyses.

GC/MS analyses were done on a Hewlett Packard 5890 gas chromatograph directly coupled to a Hewlett Packard HP 5971 A (70 eV) mass selective detector (Hewlett Packard, Milan, Italy). A 30 m x 0.25 mm fused silica column coated with 0.25 µm of methyl siloxane bonded phase (DB-5MS, Fisons, Milan, Italy) was employed, using helium as the carrier. The oven temperature program was 60°C (3 minutes), then 3°C/min to 150°C (5 minutes), and finally 10°C/min to 200°C (20 minutes). Injector and detector temperatures were 70°C and 280°C respectively. Sample injection was made on column by a Hewlett Packard 7673 autosampler. Component identification was carried out by comparing the obtained MS data with those reported in Library Wiley on MS-ChemStation HP v. C.00.07.

Preparation of Oil Formulations

Essential Oil Emulsions

Liquid formulations were prepared by dispersing 0.25, 0.5, and 1.0% wt/wt of active principle in an aqueous mucilage containing 1.0% of Tween 80 using a turboemulsifier (IKA Labortechnik T25 basic, Staufen, Germany) coupled with a cooling apparatus (Haake GH-D8, Karlsruhe, Germany) at 13 500 rpm for 10 minutes at 5°C.

Essential Oil Microcapsules

An aqueous dispersion containing 10% wt/wt of gelatin in purified water was prepared at 40°C using a glass vessel apparatus with an external jacket connected to a circulating thermostatic bath (Haake GH-D8). A suitable amount of the selected essential oil was then added and emulsified using a high shear mixer (turbine) at 1200 rpm. The coacervate phase was obtained by adding a suitable amount of Na_2SO_4 (as 20% wt/wt water solution) and cooling the system to 5°C, stirring for 1 hour. Glutaraldehyde solution (1 mmol/g gelatine) was then added, and pH was regulated to 8 by adding a suitable amount of NaOH 1N. The resulting mixture was maintained at 5°C and stirred (750 rpm) for 3 hours. The hardened microparticles were filtered, rinsed with cold water, and finally dehydrated by freeze-drying using a LIO-5P apparatus (CinquePascal, Trezzano SN, Milan, Italy). The aqueous phase, separated from the hardened microparticles, was collected and transferred in the steam distiller to measure the amount of nonentrapped oil. The encapsulation yield was determined using the following formula:

$$\text{Encapsulation Yield (\%)} = (C - N/C) \times 100$$

where:
C = amount of loaded essential oil (g)
N = amount of nonentrapped essential oil (g)

A more detailed description of the quantities of the starting materials and run cycle parameters used appears in Tables 1 and 2 respectively. Results are reported as the mean values of 3 determinations on the same batch.

Empty Microcapsules

Empty microcapsules, used as a control in the biological assays, were prepared by removing the essential oil, by steam distillation, from the freshly prepared wet microparticles. After oil distillation, the microparticles were filtered, rinsed with distilled water, and dehydrated by freeze-drying as described above.

Table 1. Microencapsulation of Essential Oils: Starting Materials

Components	Amount (g)
Gelatin	15
Essential oil	30
Water	135
Na_2SO_4 20% solution	200
Glutaraldehyde 50% solution	3
NaOH 1N	3

Table 2. Microencapsulation of Essential Oils: Run Cycle Parameters

	Values
Emulsification step	
Temperature, °C	40
Stirring rate, rpm	1200
Time, h	0.5
Coacervation step	
Temperature, °C	From 40 to 5
Stirring rate, rpm	1200
Time, min	60
Hardening step	
Temperature, °C	5
Stirring rate, rpm	750
Time, h	3
Dehydration step	
Sample temperature, °C	–80
Condenser temperature, °C	–54
Residual pressure, mbar	From 0.1 to 0.05
Time, h	4

Characterization of the Formulations

Emulsions were characterized in terms of essential oil content and composition as follows: the essential oil content was determined in a Clevenger-type apparatus, previously cited, by distilling the active principles from the liquid formulation in the presence of 0.5% silicon oil as an antifoam agent. The essential oil obtained was measured and analyzed by GC.

The essential oil content and composition, particle size distribution, shape, and surface characteristics of the wet and dried microcapsules were analyzed as follows:

- Essential oil content: The content was determined by extracting the encapsulated oil by steam distillation of an accurately weighed amount of wet and dried microcapsules. The values reported are the mean of at least 3 distillations. For the wet product, data refer to the dried mass.
- Composition of the encapsulated oil: The composition was determined by GC analyses of the essential oil obtained by steam distillation from the prepared microcapsules (wet and dried). The values reported are the mean of 3 analyses.
- Particle size distribution: The particle size distribution was determined in a LS100 particle size analyzer (Coulter Corporation, Miami, FL) capable of measuring particles from 0.4 μm to 900 μm. Data are reported as both the mean diameter and distribution expressed by d_{10}, d_{50}, and d_{90}.
- Shape and surface characteristics: These characteristics were studied by optical microscopy (Zeiss Standard Universal, Zeiss, Germany) and scanning electron microscopy (SEM) (Zeiss DSM 962, Zeiss). For SEM images, samples of microparticles were placed on a double-sided tape that had previously been secured on aluminium stubs and then analyzed at 20 kV acceleration voltage after gold sputtering under an argon atmosphere.

The percentage of aqueous phase of wet microcapsules collected after filtration was calculated, taking into account, for each sample, the essential oil content and the amount of dry residue obtained after oil extraction. Data are reported as percentage of humidity (mean values of 3 replications).

In Vitro Release Study

The release profile of encapsulated essential oils was evaluated at 25 ± 1°C and 60 ± 5% relative humidity (rh) using samples containing 2.00 g of the microparticles, placed in 90-mm diameter glass petri dishes covered with a nylon net. At set time intervals, the amount and composition of the residual content of encapsulated essential oil were determined as previously described. Each experiment was replicated 4 times; only mean values are reported. Data are reported as both cumulative release profiles and release rate profiles.

Storage Stability

This was assessed as reported in the literature [10]. Essential oil content and composition were determined after exposure of dried microcapsules in sealed glass containers at 54°C for 2 weeks under daylight cycle.

Bioassays

Insects

Egg masses of *L dispar* were collected in Sardinia and stored at 4°C. The eggs were separated by hand from each mass and cleaned of hairs. They were then left to hatch in an air-conditioned environment at 25 ± 1°C, 60% ± 5% rh, and 12:12-hour light:dark regime. The newly hatched larvae were fed with the ICN-Biomedicals (Milan, Italy) artificial diet (the Gypsy Moth Diet, Catalog No. 960294). The larvae were reared up to the IInd and IIIrd instar and used in the bioassays.

In the same laboratory conditions, *Plodia interpunctella* Hubner (Lepidoptera: Pyralidae, Indian meal moth), a hairless Lepidoptera, was reared on maize flour and utilized as a control to verify the body adhesion of the microparticles tested.

Toxicity Tests

Essential oil toxicity was assessed using liquid formulations as follows: groups of 20 gypsy moth larvae were fed with 0.5 g of ICN artificial solid diet modified, after preparation, by injecting 35 µL of the selected oil emulsion in the gelled material, cube-shaped, 5-mm thickness; a 50-µL syringe was used. The number of dead larvae was counted after 24, 48, and 72 hours. After 24 hours, the residual amount of artificial diet was weighed to determine the quantity eaten by each group, considering also larval mortality. Each test was replicated 3 times on IInd and IIIrd instar larvae.

Control was considered both artificial diet with water (blank control) and essential oil vehicle (1.0% Tween 80 mucilage, Tween control).

Lethal concentration (LC_{50}) was calculated by the Trimmed Spearman-Karber method, as reported in the literature [18].

Toxicity tests with encapsulated oils (*Thymus* and *Rosmarinus* oils only) were performed with open glass dishes (90-mm diameter) containing 10 larvae and 0.3 mg/cm^2 of the selected formulation, using both the gypsy moth and the Indian meal moth. The upper surface of each dish was covered with a nylon net. During the tests, larval behavior was observed and mortality was recorded at set time intervals from 0 to 10 hours. Each test was replicated 4 times. Control tests were carried out with empty microparticles.

Statistical Analyses

A 1-way analysis of variance was performed on transformed mortality data (arcsine of the square root); mean values of treatments were separated from the control values using Student-Newman-Keuls test. Results of all statistical tests were considered significant if $P < .05$. Untransformed data

were corrected using Abbott's formula [19] as follows:

$$[(A - B)/A] \times 100$$

where:
A = number of surviving larvae in the control
B = number of surviving larvae in the test

RESULTS AND DISCUSSION

Table 3 reports the essential oil yield of the plant species and the main constituents of each oil used. The essential oil yield is related to the plant species, ranging from 0.3% (*Helichrysum*) to 3.6% (*Rosmarinus*). The composition of the examined oils is very complex since they are made up of several compounds of different characteristics. Briefly, *R officinalis* and *Myrtus* oils contain high levels of monoterpene hydrocarbons, mainly α-pinene and camphene. *Salvia* oil is mainly composed of monoterpene ketones (camphor and α-thujone). Neryl acetate and its corresponding alcohol are the main constituents of *Helichrysum* oil, while cinnamic aldehyde is the principal component of *Cinnamomum* oil. Carvacrol and thymol are the main constituents of *Thymus* oil, while eugenol, another phenolic derivative, is present in *Cinnamomum* oil. Finally, the epoxide derivative 1,8 cineol is the principal component of *Eucalyptus* oil and is also present in *Myrtus* and *R officinalis* oils.

Table 3. Oil Yield and Percentage of the Main Chemical Classes of Examined Essential Oils

Essential Oil	Oil Yield*	Hydrocarbons	Alcohols	Ketones	Esteres	Phenols	Aldehydes	Oxides
Cinnamomum zeylanicum	—	4	2	—	1	12	81	—
Eucalyptus globulus	2.8	14	1	—	—	—	—	86
Helichrysum italicum	0.3	24	18	—	59	1	—	1
Myrtus communis	0.8	64	6	—	7	3	—	29
Rosmarinus officinalis	3.6	64	6	11	8	—	—	9
Salvia officinalis	2.4	20	11	54	2	—	—	12
Thymus herba-barona	2.2	27	< 1	< 1	< 1	68	—	< 1

Microencapsulation Process and Characteristics of Microcapsules

The process used led to a high encapsulation yield (over 98%) with both *Rosmarinus* and *Thymus* oils (see Table 4). If dried mass is considered, the essential oil content of the wet microparticles was fairly similar for both oils. On the other hand, data relating to the dried products show that the percentage of entrapped *Rosmarinus* oil was always higher than that of *Thymus* oil. This difference could be due to the better hydrophilic characteristics of *Thymus* oil components compared to those of *Rosmarinus* oil, which could favor aqueous phase entrapment during coacervation. Consequently, a higher loss of essential oil occurred during the final dehydration stage, because oil volatility depended on the water concentration in the evaporating system.

The particle size distribution showed no marked differences between the essential oils examined. Size distribution of wet microparticles was almost the same as that of the corresponding dried products, indicating that the drying system used had no noteworthy influence on particle size.

Figure 1 shows the microparticles at the end of the phase separation process: the soft microparticles are composed of a matrix-type core phase, entrapped in a shell material of a fairly constant thickness. The coacervate phase is made up, to a large extent, of aggregates. Aggregation is considered a major problem in the phase separation process and should be solved by either controlling pH and temperature or adding a suitable surfactant agent [20]. As shown in the SEM images (Figures 2 and 3), the hardening process did not modify the structure of aggregates. At the highest magnitude used, dried microcapsules appear to be made up of single spherical units of about 0.2-μm diameter, linked to each other to form a blackberry-like structure. The external surface of each unit is almost regular and smooth, showing that the gelatin forms a continuous film surrounding the essential oil droplets.

Table 4. Encapsulation Yield and Particle Size Distribution (Mean Values, Standard Deviation Within 5%)

	Rosmarinus Oil	*Thymus* Oil
Wet microcapsules		
Encapsulation yield	99.2	98.6
Essential oil content (%wt/wt)	68.4	66.5
Percentage of humidity	70.2	86.5
Particle size (μm)		
Mean diameter	58.5	56.7
d_{10}	13.2	12.6
d_{50}	51.0	48.9
d_{90}	100.9	96.5
Dried microcapsules		
Loss on drying (%wt/wt)	68.6	83.5
Essential oil content (%wt/wt)	65.2	55.6
Percentage of oil recovery*	95.3	83.6
Essential oil/gelatin ratio	1.9	1.7
Particle size (μm)		
Mean diameter	57.9	54.9
d_{10}	11.8	11.5
d_{50}†	50.4	51.2
d_{90}	100.6	97.3

*Refers to the amount of oil charged.

†Median value.

The methods used in the preparation process did not cause any appreciable modifications in the composition of either essential oil. No marked changes in the oil content and composition were recorded during the stability test, indicating that the encapsulated oils are quite stable when stored in the absence of humidity (see Tables 5 and 6).

Release Profile Studies

The patterns of essential oil release from the microcapsules are reported in Figures 4 and 5. Despite the different composition of the core materials, the release profiles of both preparations were almost the same. When exposed to a moist environment, microcapsules initially absorbed water. This absorption led to some modifications in the shell material, which became rubbery and sticky in appearance. After 24 hours of exposure, no further increase in the sample weight was recorded: at this time point, the release rate increased, rapidly reaching a maximum of about 45 mg/*die*. It then decreased up to 10 mg/*die* during the following 48 hours, becoming

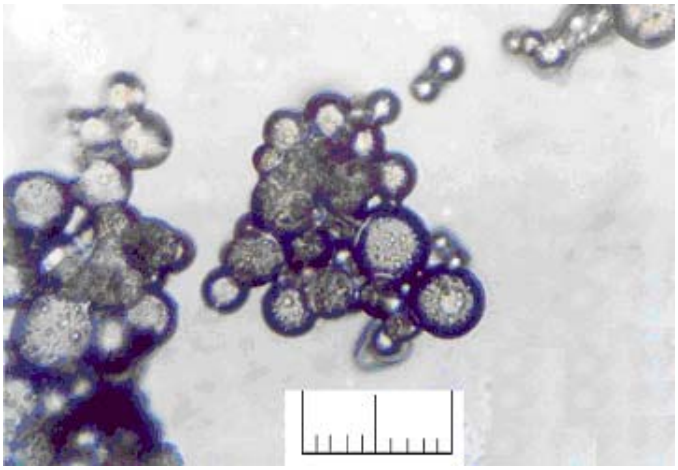

Figure 1. Image of *R officinalis* essential oil microparticles at the end of the coacervation process (contrast phase micrograph; magnitude 100 x 1.25; scale bar: 100 mm).

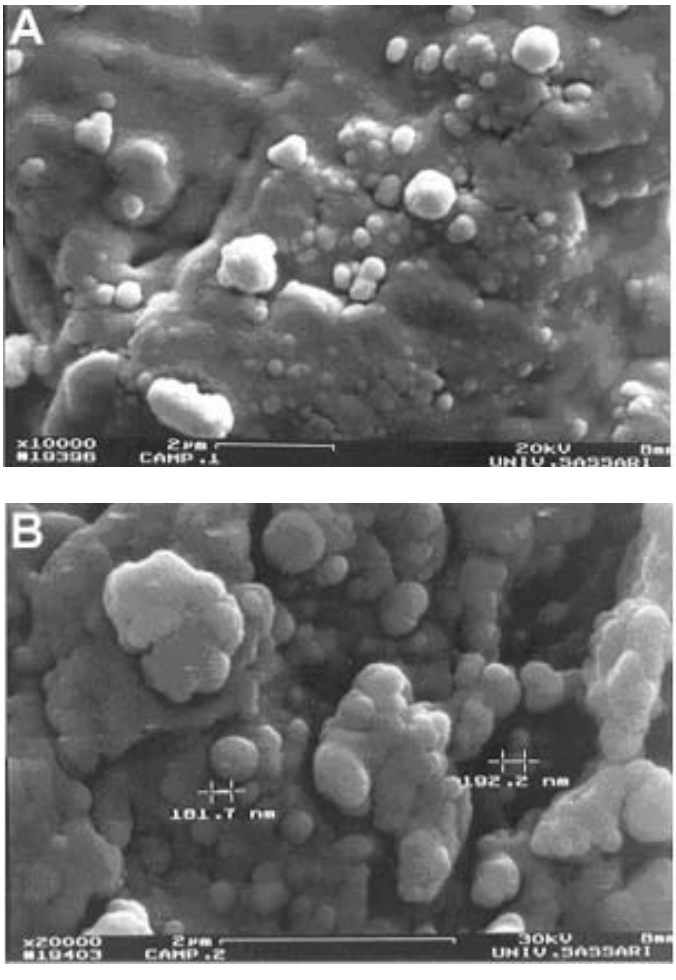

Figure 2. Scanning electron micrographs showing the aggregation of wet (A, x10 000) and dried (B, x20 000) *R officinalis* oil microcapsules.

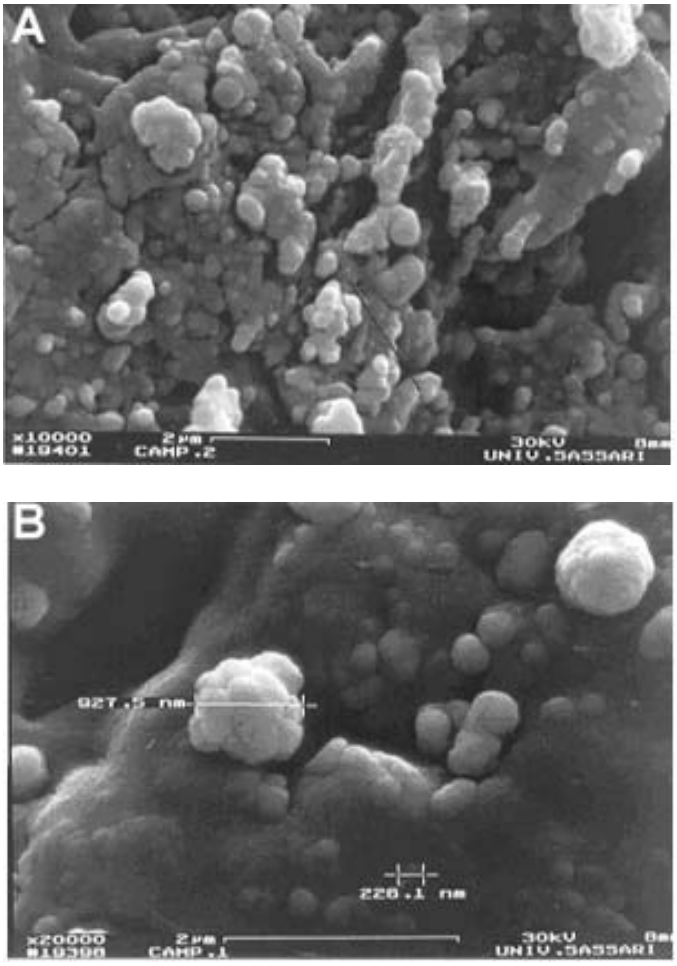

Figure 3. Scanning electron micrographs showing the aggregation of wet (A, x10 000) and dried (B, x20 000) *T herba-barona* oil microcapsules.

almost constant in the last stage of the tests. After 30 days of exposure, the microcapsules still contained about 20% of active principles, and their weight was almost constant, indicating that environmental humidity progressively replaces the internal oil phase. Although further studies are required to determine the mechanism of drug release, our experiments lead us to hypothesize that the microcapsules act as a diffusion-based delivery system.

Bioassays

Data concerning the larvicidal effects of the examined liquid formulations, expressed as percentage of mortality and LC_{50}, are reported in Table 7. After 24 hours, no oil formulation determined mortality values statistically different from the control (data

Table 5. Composition of *Thymus herba-barona* Essential Oil (Mean Values Expressed as %wt/wt, Standard Deviation Within 3%)

Components	A	B	C
α-thujene	1.6	1.6	1.6
α-pinene	0.7	0.7	0.7
Camphene	0.2	0.2	0.2
β-pinene	0.1	0.1	0.1
β-myrcene	2.1	2.1	2.1
α-phellandrene	0.1	0.1	0.1
Δ-3-carene	1.5	1.5	1.5
p-cymene	6.2	6.1	6.1
Limonene	0.9	0.9	0.9
γ-terpinene	6.3	6.2	6.1
Terpinolene	1.1	1.1	1.1
Terpinen-4-ol	0.2	0.2	0.2
α-terpineol	0.1	0.1	0.1
Bornyl acetate	0.1	0.1	0.1
Thymol	10.4	10.3	10.4
Carvacrol	58.0	57.8	57.7
β-caryophyllene	5.0	4.9	4.8
Caryophyllene oxide	0.2	0.2	0.2

*A indicates before encapsulation; B, after encapsulation; C, encapsulated oil after stability test

Table 6. Composition of Rosmarinus officinalis Essential Oil (Mean Values Expressed as %wt/wt, Standard Deviation Within 3%)*

Components	A	B	C
α-pinene	38.9	38.8	38.7
Camphene	8.0	7.9	7.8
β-pinene	1.1	1.1	1.1
β-myrcene	4.7	4.6	4.5
α-phellandrene	0.6	0.6	0.6
Limonene	4.2	4.2	4.2
1,8 cineol	8.6	8.6	8.6
γ-terpinene	0.5	0.4	0.4
p-cymene	1.6	1.5	1.5
Camphor	7.0	6.9	6.8
Linalool	1.0	0.9	0.9
Bornyl acetate	8.3	8.3	8.3
β-caryophyllene	0.1	0.1	0.1
Verbenone	3.0	2.9	2.9
Borneol	5.0	4.9	4.9
Geraniol	0.2	0.2	0.2

*A indicates before encapsulation; B, after encapsulation; C, encapsulated oil after stability test

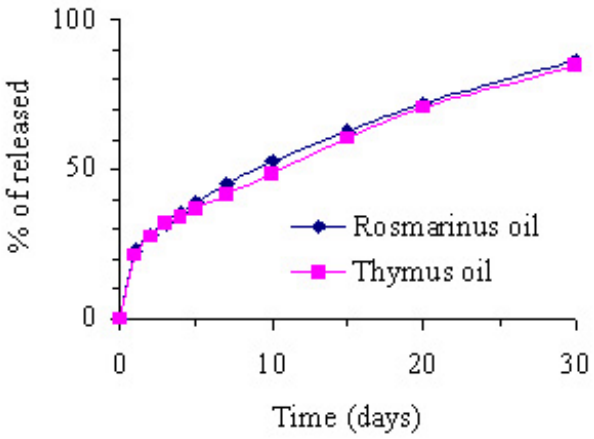

Figure 4. Percentage of essential oils released from microcapsules (mean values; standard deviation within 3%).

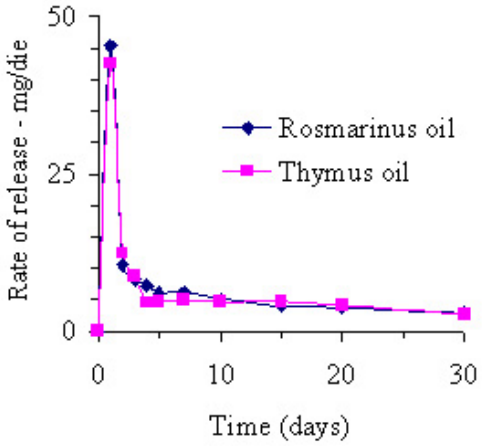

Figure 5. Rate of release of essential oils from microcapsules (mean values; standard deviation within 3%).

not reported). The mortality at 1.0% of active principle was statistically significant after 48 and 72 hours of exposure only. LC_{50} calculated after 72 hours of exposure ranged from 0.35 mL/g (*Thymus* oil) to 0.60 mL/g (*Eucalyptus* oil).

Figure 6 shows the percentage of mortality at the end of each test, corrected using the Abbott formula. Mortality of over 60% was recorded for *T herba-barona*, *R officinalis*, *M communis*, *C zeylanicum*, and *H microphyllum* oils. The highest value was obtained with *T herba-barona* oil (about 84% at 1.0% concentration). According to the literature, the toxic effect found in our study could be attributed to its phenolic content, in particular carvacrol, known to have insecticidal activity [21, 22].

Previous in vitro studies [23] demonstrated that carvacrol interacted with the cellular membranes of pathogenic bacteria, changing their permeability toward H^+ and K^+. This mode of action, similar to that found in the *B thuringiensis* δ-endotoxins [24], would determine the dissipation of ion gradients, leading to impairment of essential processes in the intestinal cells of larvae, and finally cell death.

Table 8 shows the diet intake of *L dispar* larvae during the first 24 hours of the test. No statistically significant differences were found by comparing data obtained from *Salvia* and *Eucalyptus* oils with the blank control. Data obtained with *Helichrysum*, *Rosmarinus*, *Myrtus*, and *Cinnamomum* oils do not statistically differ from those for the Tween control, while the corresponding data for *Thymus* oil were statistically different compared with the other treatments.

As regards tests carried out using microparticles of *T herba-barona* and *R officinalis* oils, first it must be pointed out that the microparticles were entrapped quickly in the body hairs of free-moving *L dispar* larvae. During the observations, larval motility decreased progressively until it collapsed after about 2 hours. This effect could be attributed to toxic action of active principles on the motor system, and it seems enhanced by microparticle adhesion to hairs of the larval body. In fact, when hairless *P interpunctella* larvae were used in the same experimental conditions, no effect on spontaneous motility was recorded.

Table 7. Percentage of Mortality (Untransformed Data)* at 48 and 72 Hours After Ingestion of Diet Modified by Different Essential Oil Emulsions

	48 Hours			72 Hours			
	0.25%	0.5%	1.0%	0.25%	0.5%	1.0%	LC_{50} (µL/g)
Cinnamomum zeylanicum	11.6	20.0	53.0†	20.0	33.0†	75.0†	0.45
Eucalyptus globulus	5.0	10.0	27.0	13.3	22.0	58.0†	0.60
Helichrysum italicum	13.3	20.0	48.0†	25	38.0†	73.0†	0.42
Myrtus communis	8.3	18.0	43.0†	18.3	33.0†	73.0†	0.46
Rosmarinus officinalis	13.3	15.0	33.0†	26.6	40.0†	78.0†	0.40
Salvia officinalis	8.3	13.0	15.0	20	30.0†	28.0	—
Thymus herba-barona	20.0	25.0	53.0†	31.6	45.0†	88.0†	0.35
Control	8.3	10.0	15.0	20.0	20.0	28.0	—

*Standard deviation < 5%.
†Statistically significant difference to the control (P < .05 Student-Newman-Keuls test).

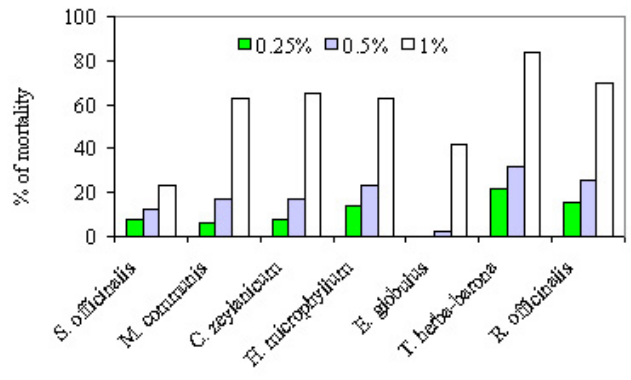

Figure 6. Percentage of corrected mortality obtained from emulsions tested on *L dispar* larvae.

Table 8. Diet Modified With 1% Emulsions Eaten After 24 Hours (mg)*

	Treated groups† (Mean Values ± SD)	Surviving larvae† (Mean Values ± SD)
Cinnamomum zeylanicum	62.6 (± 4.5)^{bc}	3.8 (± 0.2)
Eucalyptus globulus	85.3 (± 3.6)^{a}	4.6 (± 0.8)
Helichrysum italicum	59.3 (± 4.4)^{c}	4.2 (± 0.6)
Myrtus communis	61.5 (± 4.3)^{bc}	3.8 (± 0.5)
Rosmarinus officinalis	61.8 (± 2.4)^{bc}	3.6 (± 0.1)
Salvia officinalis	86.5 (± 1.3)^{a}	4.8 (± 0.2)
Thymus herba-barona	46.4 (± 4.9)^{d}	3.4 (± 0.3)
Tween control	71.3 (± 2.9)^{b}	3.7 (± 0.1)
Blank control	88.3 (± 2.1)^{a}	4.5 (± 0.2)

*SD indicates standard deviation.
†Referred to 3 groups of 20 larvae. The same letter indicates no significant difference ($P < .05$, Student-Newman-Keuls test).
‡Referred to a living larva at 24 hours.

The mortality trend, reported in Figure 7, shows almost equal values for both formulations tested on *L dispar*. Mortality increased progressively, reaching the maximum over 7 hours. No mortality was recorded for the controls, though the same adhesion of the microparticles to the hairs of the larval body was observed. In *P interpunctella* the level of mortality was low (about 5% after 10 hours).

CONCLUSIONS

The results obtained so far show that the encapsulation process is a suitable method for entrapping essential oils of a very different chemical composition. This method reduces loss of the active principles, leading to high-loaded microparticles

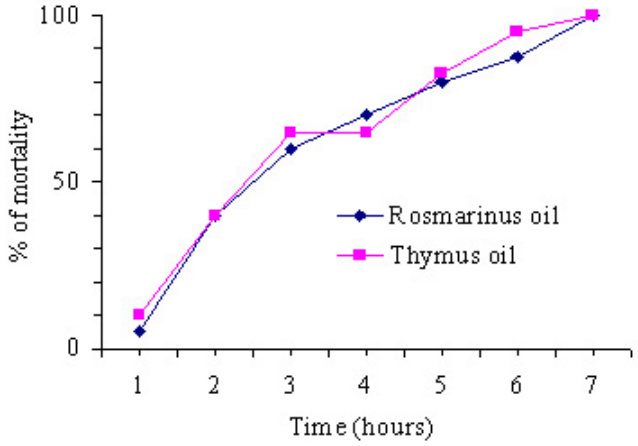

Figure 7. Percentage of mortality obtained from microcapsules tested on *L dispar* larvae.

that offer protection against environmental agents; it also offers the possibility of controlled drug release. Data obtained in the release tests show that loss of active principles occurs when microcapsules are exposed to environmental humidity; consequently, these formulations must be stored in closed containers. At a concentration similar to that usually employed in localized treatments with microgranular synthetic pesticides, they could be used to control the release of active principles in ways suitable for both acute and long-term treatments.

Of the essential oils tested, *R officinalis* and *T herba-barona* seem to be the most promising, since the plants are easily cultivated and give a high oil yield. Their action seems attributable to the progressive paralysis of *L dispar* larvae, which become motionless and consequently stop feeding until death occurs. These effects appear maximized by microparticle adhesion to the hair structures typically present in some of the main defoliator families (Lymantridae, Lasiocampidae, Thaumatopoeidae, and Arctidae).

Given these encouraging results, further experiments are in progress to assess the suitability of natural active principle formulations for application as a new tool in integrated control of different pest larvae.

REFERENCES

1. Pillmoor JB, Wright K, Terry AS. Natural products as a source of agrochemicals and leads for chemical synthesis. Pestic Sci. 1993;39:131-140.

2. Bazzoni E, Sanna-Passino G, Moretti MDL. Essential oils and other control techniques against stored product insects. In: Majumdar DK, Govil JN and Singh VK, eds., Recent Progress in Medicinal Plants, Vol. 8 Phytochemistry and Pharmacology II.. Houston, TX: Sci Tech Publishing LLC; 2002: 313-342.

3. Bathal SS, Singh D, Dhillon RS. Effect of crude root oils of *Inula racemosa* and *Saussurea lappa* on feeding, survival and development of *Spodoptera litura* (Lepidoptera: Noctuidae) larvae. Eur J Entomol. 1993;90(2):239-240.

4. Park SJ, Lee SG, Shin SC, Lee BJ, Ahn YJ. Larvicidal and antifeeding activities of oriental medicinal plant extracts against four species of forest insect pests. Appl Entomol and Zool. 1997;32(4):601-608.

5. Larocque N, Vincent C, Belanger A, Bourassa JP. Effects of tansy essential oil from *Tanacetum vulgare* on biology of oblique-banded leafroller, *Choristoneura rosaceana*. J Chem Ecol. 1999;25(6):1319-1330.

6. Marimuth S, Gurusubramanian G, Krishna SS. Effect of exposure of eggs to vapours from essential oils on egg mortality, development and adult emergence in *Earias vittella* (F.) (Lepidoptera: Noctuidae). Biol Agr Horticult. 1997;14(4):303-307.

7. Naumann K, Isman MB. Evaluation of neem *Azadirachta indica* seed extracts and oils as oviposition deterrents to noctuid moths. Entomol Experimental Appl. 1995;76:115-120.

8. Landolt PJ, Hofstetter RW, Biddick LL. Plant essential oils as arrestants and repellents for neonate larvae of the codling moth (Lepidoptera, Tortricidae). Environ Entomol. 1999;28(6):954-960.

9. Moretti MDL, Peana AT, Franceschini A, Carta C. In vivo activity of *Salvia officinalis* oil against Botrytis cinerea. J Essent Oil Res. 1998;10:157-160.

10. Markus A. Advances in the technology of controlled-release pesticide formulations. In: Benita S, ed., Drugs and the Pharmaceutical Sciences, Vol. 73, Microencapsulation: Methods and Industrial Applications. New York, NY: Marcel Dekker; 1996:73-91.

11. Kydonieus AF. Controlled Release Technologies: Methods, Theory, and Applications. Vols. 1 and 2. Boca Raton, FL: CRC Press; 1980.

12. Luciano P, Lentini A, Prota R. Un quinquennio di prove di lotta microbiologica alla *Lymantria dispar* (L.). Paper presented at: Atti XVII Congresso Nazionale Italiano di Entomologia 1994, Udine, Italy.

13. Margalit J, Markus A, Pelah Z. Effects of the persistence of *Bacillus thuringiensis* var. israelensis serotype H-14. Appl Microbiol Biotechnol. 1984;19:198.

14. Greenblatt HC, Dombroski M, Klishevich W. Encapsulation and controlled release. London, England: Special Publicationof-Royal Chemical Society; 1993.

15. Ribeiro A, Arnoud P, Frazao S, Venacio F, Chaumeil JC. Development of vegetable extracts by microencapsulation. J Microencapsul. 1997;14:735-742.

16. Determination of essential oils in vegetable drugs. European Pharmacopoeia. 4th ed. Strasbourg, France: European Directorate for the quality of Medicines, Council of Europe Editions; 2002: 183-184.

17. Van Den Dool H, Kratz P. A generalisation of the retention index system including linear temperature programmed gas-liquid partition chromatography. J Chromatogr. 1963;11:463-471.

18. Hamilton MA, Russo RC, Thurston RV. Trimmed Spearman-Karber Method for estimating median lethal concentrations in toxicity bioassays. Environ Sci Technol. 1977;11(7):714-719.

19. Abbott WS. A method of computing the effectiveness of insecticide. J Econ Entomol. 1925;18:265-267.

20. Duquemin SJ, Nixon JR. The effect of sodium lauryl sulphate, cedrimide and polysorbate 20 surfactants on complex coacervate volume and droplet size. J Pharm Pharmacol. 1985;37:698-702.

21. Lee SG, Kim SI, Ahn YJ, Kim JB, Lee BY. Effectiveness of carvacrol derived from *Thujopsis dolabrata* var. hondai sawdust against *Thecodiplosis japonensis* (Diptera: Cecidomyiidae). Pestic Sci. 1997;49(2):119-124.

22. Karpouhtsis I, Pardali E, Feggou E, Kokkini S, Scouras ZG, Mavragani-Tsipidou P. Insecticidal and genotoxic activities of oregano essential oils. J Agric Food Chem. 1998;46(3):1111-1115.

23. Ulthee A, Kets EPW, Smid EJ. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. Appl Environ Microbiol. 1999;65(10):4606-4610.

24. Knowless BH. Mechanism of action of *Bacillus thuringiensis* δ-endotoxins. Adv Insect Physiol. 1994;24:275-308.