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Insecticidal activity of microencapsulated *Schinus molle* essential oil

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ABSTRACT

Microencapsulation of the *Schinus molle* Rev L. (Anacardiaceae) leaves essential oil (EO) has been employed to control the release of active ingredients, protecting them from the external environment, with the concurrent improvement of its insecticidal potential on *Haematobia irritans*. Four microcapsule formulations (EEO1 to EEO4) of *S. molle* EO were prepared by spray-drying, using a mini spray dryer and gum Arabic/maltodextrin (AG/MDX) as the carrier in different proportions, at a ratio of 4:1 (MDX/AG:EO). Encapsulation efficiency (EE: 96–100%), powder morphology and particle size distribution were analyzed as responses. An interesting correlation was found between EO free and microcapsules (EEO) in the preliminary and comparative studies of stability (at 45 °C) and in the insecticide activity on *H. irritans*. In fact, a very slow liberation profile of the microencapsulated EO (EEO4) was observed over a period of 366 h (71% of EO retention), as well as a slower time-dependent insecticide effect (32 and 73% of dead flies at 2 and 4 h of exposure time) compared to the free EO (96% of dead flies at 2 h).

1. Introduction

The horn fly, *Haematobia irritans* (L.) (Diptera: Muscidae), is one of the most important blood-sucking pests of pastured cattle, causing annual economic losses estimated at 1 billion and 150 million dollars in the U.S. and Brazil respectively (Barros et al., 2001; Cupp et al., 2004; Guglielmone et al., 1999, 2001; Oyarzún et al., 2008). This may result in a decreased milk production, reduced weight gains and poor feeding efficiency. Consequently, livestock producers must select an appropriate control method to manage this pest.

The control method used initially was chemical insecticides, but this strategy has led to resistance to most commercially available products (Guglielmone et al., 1999, 2001; Barros et al., 2001). Additionally, the use of those chemicals has negative consequences, such as soil and water contamination, high persistence in the environment, intoxication of people and animals, and residues in foods (Regnault-Roger et al., 2004).

Consequently, the search for new pest controls such as botanical insecticides appears as a more attractive ecological and natural alternative to be explored as integrated pest control.

Schinus molle (L.) (Anacardiaceae) is an highly aromatic evergreen tree native to South America, whose volatile oils have been thoroughly studied (Murray et al., 2005; Rossini et al., 1996), and to which different biological activities are attributed (antibacterial, anti-fungal, anti-inflammatory, cytotoxic, insecticidal) (Atti dos Santos et al., 2009; Abdel-Sattar et al., 2009, 2010). Interestingly, most of these activities correspond to the essential oils protective role the plants have in nature (Bakkali et al., 2008). Specifically, some studies related to the insecticidal and repellent effects of *S. molle* extracts against different insects have been published (Ferrero et al., 2006, 2007; Abdel-Sattar et al., 2009; Deveci et al., 2010).

However, the biological activity of these products can be lost through the volatilization of their components or their degradation (exposure to high temperatures, oxidation and/or UV radiation), making the commercial applications of these oils limited. What is more, essential oils have short residual activity, which reduces the workers' exposure to residues, but this may result in repellent activity and the need for repeated applications in order to obtain insecticidal activity, which may lead to higher phytotoxicity risks (Cloyd and Chiasson, 2007).

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Besides, as an alternative for specific applications, volatile oils can be prepared in a large number of formulations: liquid, semi-liquid or solid forms to be used to control the release of active ingredients and to protect them from the external environment (Miró et al., 2010).

Microencapsulation is one of the most efficient processes for this kind of products. Basically, microencapsulation technology consists in "packing" an active ingredient within a wall material, thus transforming an emulsion into a more stable powder (Tonon et al., 2011). The release of the functional agent occurs by diffusion through the capsule wall and/or rupture of the microcapsules. Therefore, the use of microcapsules could provide a durable repellent and/or insecticidal finish (Miró et al., 2010). At present, different techniques are used for the microencapsulation of food additives (flavors, preservatives, leavening agents, vitamins and minerals). Spray-drying is a low-cost microencapsulation technology commonly used in the industry, which has the attractive advantage of producing microcapsules in a relatively simple, inexpensive and continuous operation, compared to other conventional microencapsulation techniques. Moreover, from a commercial point of view, it has been estimated that the cost of spray-drying is six times lower, per kilogram of water removed, than the cost of freeze-drying (Knorr, 1998). What is more, this technique has been widely used for drying heat-sensitive materials (foods, pharmaceuticals) because of the rapid evaporation of the applied solvent from the droplets (Kolanowski et al., 2005).

Spray-drying involves the atomization of emulsions into a drying medium at a high temperature, which leads to very fast water evaporation. This results in quick crust formation and quasi-instantaneous entrapment of the core material (Gharsallaoui et al., 2007; Tonon et al., 2011; Pulido and Beristain, 2010). Some of the advantages of this method are its ability to handle heat-sensitive materials, the availability and diversity of equipment, the variety of particle sizes produced and an excellent dispensability of particles in aqueous media. Conversely, the main disadvantages of this technology are the high temperature and air exposure necessary for the drying process. Moreover, the product can adhere to the surface of the capsules during drying, thus causing potential modifications of the end product formulation (Kanawija et al., 1992). Spray-drying technology also requires well-adjusted operating conditions, as well as the correct composition of the solution that contains the active principles (Vaidya et al., 2006; Gallo et al., 2011; Soliman et al., 2013).

In this work we describe the preparation and characterization of microcapsules of *S. molle* essential oil (EEO) using the spray-drying technology. Operational parameters such as concentration of wall material on loading capacity and encapsulation efficiency (EE) were studied. The microcapsules were evaluated for the content and stability of EO, and scanning electron microscopy was used to observe the powder morphology and particle size distribution. Insecticide activity was also studied using the *H. irritans* bioassay model. The experimental model developed should provide valuable insight in order to correlate the insecticide efficacy of the microencapsulated essential oil with storage and application times.

2. Materials and methods

2.1. Chemicals

Laboratorio Uruguay S.A. (LUSA, Montevideo, Uruguay) kindly donated technical-grade diazinon (87.9% AI). Gum Arabic and maltodextrin were purchased from Parafarm (Saporiti, Argentina).

2.2. Plant material and isolation of the essential oil

The selection of the natural active material used (*S. molle* essential oils, EO) was carried out by means of an in vitro screening of insecticide activity by using the bioassay previously developed and reported against *H. irritans* (Andina et al., 2012). Samples of fresh leaves and stems, representing the entire population of *S. molle*, were collected randomly at the Centro de Extensión y Capacitación en Plantas Aromáticas y Medicinales (Monte Vera, Santa Fe, Argentina) during the full flowering period (September to November). The samples were representative of the species and were chosen in order to be representative of the same pedoclimatic and collection conditions; the extraction conditions were also identical for all samples. The essential oil was obtained from fresh leaves and stems by classical steam distillation for 2 h at normal atmospheric pressure in pilot-scale stainless steel equipment.

2.3. Analysis and identification of EOs

The composition of the oil was determined by GC using a Shimadzu (Tokyo, Japan) model 14 B gas chromatograph equipped with a FID and Shimadzu EZ-Chrom data-processing software. Analyses were conducted following the IRAM-ISO/TC 54 Norm, with minor modifications. Chromatography was performed using a DB-WAX (Agilent J&W, USA) fused-silica capillary column (30 m × 0.250 mm i.d.), coated with polyethylene glycol (0.25 μm phase thickness); the oven temperature program was 40 °C for 8 min, raised to 190 °C at 15 °C/min, and then to 230 °C at 30 °C/min, finally maintained at 230 °C for 20 min; the temperature of the injector was 210 °C; the temperature of the flame ionization detector (FID) was 230 °C; the gas carrier was nitrogen (100 kPa); the injection mode was split with a split ratio of 1:40.

The components of the oil were identified by comparing their linear retention indices (LRIs) to those of pure standards or as reported in the literature (Atti dos Santos et al., 2009). The percentages of each component were reported as raw percentages without standardization. Repeatability of the measuring system showed variation coefficients under 5% for all the components.

2.4. Preparation of microcapsules by spray-drying

2.4.1. Preparation of emulsions

Four different suspensions (1–4) were prepared using maltodextrin (MDX) and gum Arabic (AG) as carrier (wall material) in different proportions (4:1, 3:2; 2:3; and 1:1, respectively). MDX and AG were previously dissolved in distilled water at 50 °C for 2 h and left to stand for 12 h at room temperature. For the emulsion preparations, the EO of *S. molle* was incorporated into the wall material suspension using an Ultraturrax T18 homogenizer (IKA(R) T18 basic, Staufen, Germany) at 24,000 rpm for 30 min. The emulsions obtained (50–60 mL) were stored at room temperature until use, and were examined with optical light microscopy applying 100× magnification using an Olympus microscope (BX41, Tokyo, Japan).

2.4.2. Spray-drying

Spray-drying was performed using a laboratory-scale Mini Spray Dryer (Büchi B-290, Büchi Labortechnik AG, Flawil, Switzerland). The samples were atomized with a hot air stream in the drying chamber, thus making it possible to obtain solid microparticles where the EO was trapped within a film of encapsulating material. A two-fluid nozzle of 0.5 mm cap orifice diameter was used. The following parameters were fixed: pump, 15%; aspirator %, 100; Q-flow, 600 l/h; inlet temperature, 160 °C; outlet temperature, 100 °C.

2.4.3. Analysis of spray dried microcapsules

To evaluate the ability of the selected carrier, the spray-dried microcapsules were analyzed for total essential oils (EO_t) using the following protocol: to a known quantity of microcapsules (450 mg), 1.37 g of water in a 15-mL polypropylene tube was added. The tube was sonicated for 2 min, and it was then shaken vigorously for 2 min (vortex mixer), with subsequent manual agitation (3 min). An aliquot of 0.5 g was taken in a 15-mL tube (by triplicate) and then 5 mL of internal standard solution in chloroform (IS: camphor, 1 mg/mL) was added. The tube was shaken (2× 3 min), then sonicated for 30 min and centrifuged at 3500 rpm for 10 min. An aliquot of organic layer (2 mL) was taken, and 0.5 g of Na_2SO_4 was added, shaken for 1 min and finally centrifuged. The organic layer was then separated and analyzed by GC to determine EO_t. One microliter was injected in the column of the GC under the experimental conditions described above (Section 2.2). The EO content was then estimated using a calibration curve.

The calibration curve was built using appropriate dilutions (five with three replicates) of EO (range of 0.277 mg/mL to 5.47 mg/mL) in chloroform, using sabinene (major component 48% of EO, Atti dos Santos et al., 2009) as the chromatographic tracer of the EO mixture, and camphor as IS (1 mg/mL final concentration). The analytical procedure was validated according to the following criteria: linearity was established from a calibration curve applying least-squares linear regression analysis and correlation coefficient (r); accuracy and precision were evaluated by processing replicates of samples ($n = 6$) expressing the results as relative standard deviation (RSD). Recoveries were estimated for three different loads of EO microcapsules, and with different solvents (acetone, chloroform, ethyl acetate). The best recoveries (72–87%) were obtained using chloroform. The matrix effect was evaluated for three concentrations of EO (5.47, 2.73 and 1.36 mg/mL) following the same procedure described above for the preparation of microcapsules (but without EO). EO determination was conducted, but after microcapsules were ruptured with chloroform, and the sample was centrifuged, 2 mL of organic layer was taken and then evaporated. The residue was recovered with appropriate dilutions of EO in chloroform and analyzed through GC as it was described. Response factors (sabinene area/IS area) were compared to those corresponding to the calibration curve in chloroform.

2.4.4. Analysis of surface components of spray-dried microcapsules

Surface oil (EO_s) was measured by adding 5 mL of IS solution in chloroform (camphor, 1 mg/mL) to a known quantity of microcapsules (0.225 g) and shaken with a vortex mixer for 1 min at room temperature (Tonon et al., 2011). The mixture was filtered, 0.5 g of Na_2SO_4 was added to the filtrate solution, and then it was centrifuged at 3500 rpm. The supernatant was analyzed by GC to determine EO_s.

2.5. Encapsulation yield (EY) and encapsulation efficiency (EE)

The EY was calculated as the ratio of the total oil (EO_t) obtained at the end of the process, and the initial essential oil added (EO_i).

$$EY\ (\%) = \frac{(EO_t - EO_s)}{EO_i} \times 100$$

The encapsulation efficiency (EE) was calculated as the ratio of the total oil obtained at the end of the process and the total essential oil added (EO_t) without prior washing of the microcapsules with the organic solvent (Riyajan and Sakdapipanich, 2009).

$$EE\ (\%) = \frac{(EO_t - EO_s)}{EO_t} \times 100$$

Total oil (EO_t) was assumed to be equal to the total oil encapsulated as the encapsulation efficiency (EE) was 96–100% (preliminary tests revealed that all the initial EO was retained).

2.6. Comparative studies of stability of essential oil free (EO) and microencapsulated (EEO)

A known quantity (two replicates) of EO (35 mg) or EEO (0.550 g) mixed in 15-mL polypropylene tubes with 0.550 g of water was incubated (45 ± 2 °C, 75% relative humidity) over a period of 24 h. Similarly, a second experiment was conducted for EEO over a longer period of time (24 h to 15 days). The tubes (two replicates) were removed from the incubator at 20 min, 1 h, 2 h, 5 h and 24 h, and at 1, 2, 6, 11 and 15 days, and were conditioned as described above (Section 2.4.3 for EO analysis). The percentage retention of EO was calculated using the formula: EO at elapsed time × 100/EO at zero time (Vaidya et al., 2006). For the preliminary studies of stability and biological activity, the microcapsule preparation selected was the one with the highest proportion of gum Arabic (EEO4) (see below).

2.7. Scanning electron microscopy (SEM)

Particle size and morphology of spray-dried microcapsules were evaluated using scanning electron microscopy (SEM, JEOL JSM-5900LV, SEMTech Solutions, North Billerica, MA, USA) operated at 5 kV with magnifications of 5000×. The microcapsules were attached to a double-sided adhesive tape mounted on the SEM stubs, coated with 3–5 mA gold/palladium under vacuum (by sputtering in a Dentom Vacuum Desk II for 120 s, and 40 mA).

2.8. Biological assays

2.8.1. Horn fly collecting

Hereford cows (24–36 months old, body weight (BW) 243–248 kg) naturally infected by *H. irritans* were selected from the herd belonging to the School of Medicine, University of the Republic, Montevideo, Uruguay (UdelaR) (Institutional Animal Care and Use Committee; exp. 071140-000611-10) (Breijo et al., 2013). Flies on animals were captured using an insect net, and they were immediately transported to the laboratory (controlled temperature of 25 ± 2 °C), and anesthetized (previously standardized in a chamber of CO_2 at a flow rate of 5 L/min, for 10 min).

2.8.2. Insecticide bioassay

2.8.2.1. Susceptibility control of flies. The bioassay technique (Sheppard and Marchiondo, 1987), and more specifically its adaptation (Marcon et al., 1997) with minor modifications we described before (Andina et al., 2012), was first conducted in order to test, and control the pharmacological susceptibility of captured horn flies, as well as to validate the model, using Diazinon (organophosphate insecticide commercially available) as reference drug. It is noted that the population of wild flies captured are not homogeneous (i.e.: age, sex), then so the standardization of the bioassay using a reference drug, is a good tool to test and confirm the pharmacological susceptibility preservation of flies used in each bioassay.

Briefly, anesthetized flies were divided into groups of 25 ± 2 unsexed adult flies, and placed in Petri dishes previously conditioned. An aliquot of 1 mL of dilutions of Diazinon (six replicates at least) made in acetone (0.1, 0.3 and 0.6 μg/mL final concentration) was applied in a fume hood. Petri dishes treated only with acetone were used as untreated controls. Acetone was allowed to evaporate from the conditioned Petri dishes for 2 h before placing the flies. The mortality of horn flies in each Petri dish was determined 2 h after the introduction of flies by counting live and dead

flies. Diazinon (0.6 μg/mL) was used as positive control (reference) to test susceptibility of flies used in every experiment within this method. This bioassay was used for the preliminary screening of natural samples at 1 mg/mL (final concentration) among which *S. molle* (EO) was selected for its activity.

2.8.3. Comparative insecticide studies

Comparative studies of activity between free essential oil of *S. molle* (EO) and microcapsulated (EEO4) were conducted using plastic cups (9 cm high × 7 cm in diameter) covered with an elastic mesh, above which previously conditioned cotton towels were placed. An aliquot of 1 mL of appropriate dilution in acetone allowing final concentrations of: EO (at 30 mg/mL), EEO (equivalent to 100 mg/mL of EO), Diazinon (reference drug at 10 μg/mL), or untreated control (acetone) were applied onto cotton towels (six replicates at least). Acetone was allowed to evaporate for 2 h before placing the flies into plastic cups, and the same procedure described above was followed. The mortality of horn flies in each cup was determined at different elapsed times of incubation for 4 h (15, 30, 60, 90, 120, 150, 180, 210 and 240 min). Two independent experiments, with horn flies collected on different days, were also conducted to evaluate the reproducibility of the bioassay using EO (at 30 mg/mL).

2.8.4. Data analysis

The ANOVA test and subsequently Dunnett's test were used to analyze the data ($P < 0.05$). The regression lineal GraphPad Prism 6 (trial version, GraphPad Software Inc., La Jolla, CA, USA) program was used to analyze the dose-mortality response to Diazinon bioassays. To analyze the comparative response of EO and EEO bioassays, multiple comparisons of means were made with the Tukey test ($P < 0.05$), and the differences were labeled as significant ($P = 0.05$) when their 95% confidence intervals (CI) did not overlap.

3. Results and discussion

3.1. Analysis and identification of EOs

The composition of the oil was previously communicated and determined by GC–MS analysis (Atti dos Santos et al., 2009) which was equipped with MS reference libraries (McLafferty and Stauffer, 1991; Adams, 2007). In this regard, sabinene (48.63–51.74%) and limonene (10.20–16.98%) were described as the main components of the oils obtained from the leaves of *S. molle*, while bicyclogermacrene (18.12%) was also important in the leaves oil, in accordance with our results (not shown) as it was confirmed before (using GC–MS analysis) to select sabinene as chromatographic tracer for the adapted method (GC-FID) described before.

3.2. Emulsion droplet size

Emulsions were prepared with the same EO concentration, but with different composition of wall material (GA-MDX ratio). Fig. 1 shows light microscopic images of the four emulsions produced (emulsion 1–4). The diameter of the droplets in all prepared emulsions ranged from 10 μm to 40 μm. The highest content of GA, emulsion 4, showed the smaller droplets for the same oil concentration, contrary to emulsion 1 (smaller content of GA). These results agree with those exposed in literature (Jafari et al., 2008). Among wall materials, GA has been the most popular and common ingredient for spray drying encapsulation of oils and flavors, since it has emulsifying properties and provides excellent volatiles retention during the drying process. However some authors reported further success when using a blend of GA with other wall materials like MD, which proved to be effective for the encapsulation of: cardamom oil, citral and linalyl acetate, citrus oils, soy oil, rice flavor, fatty acids, pine flavor, and bixin. In these cases MD can successfully replace a part of GA as wall material (Jafari et al., 2008; Vaidya et al., 2006).

3.3. Preparation and analysis of microcapsules

The encapsulation yield (EY) after the spray-drying procedure was estimated at 75–90% (Fig. 2), and the encapsulation efficiency (EE) was about 100% for the four microcapsule preparations (EEO1 to EEO4). Total oil (EO_t) was assumed to be equal to the total oil encapsulated, since the encapsulation efficiency (EE) was practically 96–100%, calculated as defined above. Microcapsule preparation EEO4 showed the best encapsulation yield (90%) according to the higher GA content in it. The analytical method for

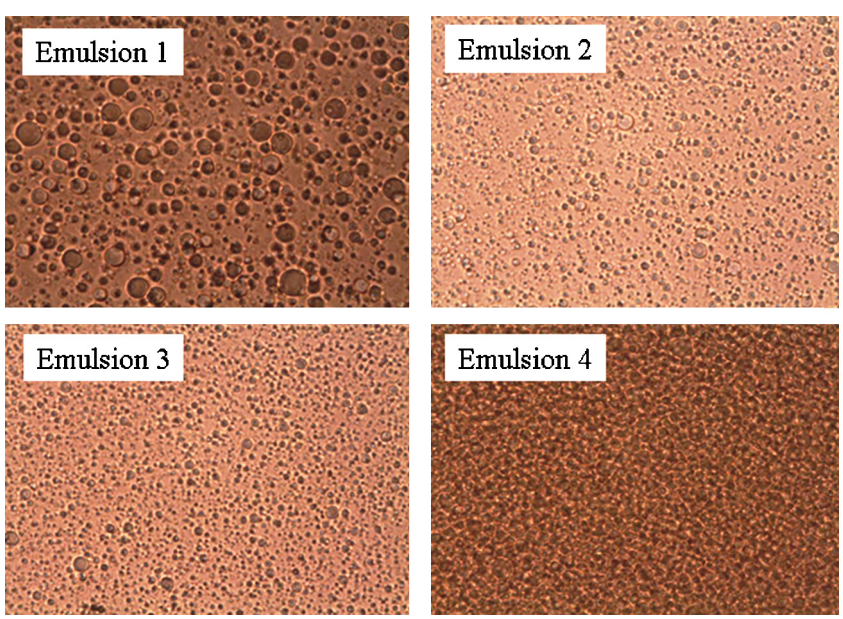

Fig. 1. Light microscopic emulsions (1–4) images produced with a ratio of carrier–water 3:7 and carrier–essential oil 4:1 constant. Emulsion 4 showed the highest content of AG (MDX: AG, 1:1). The droplet mean diameter of droplets in all prepared emulsions ranged from 10 μm to 40 μm.

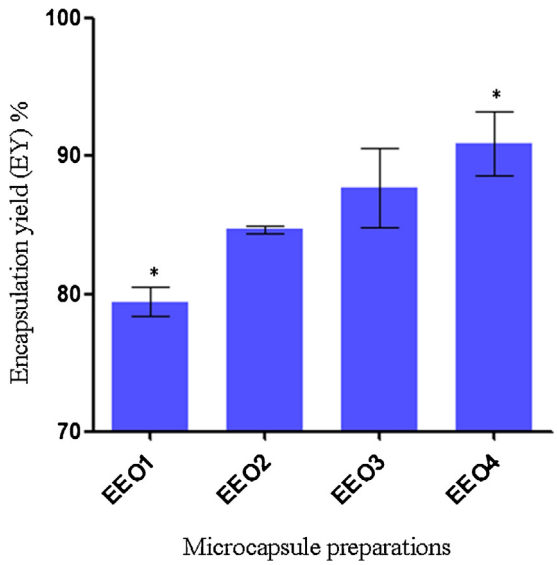

Fig. 2. Encapsulation yield (EY) of microcapsule preparations (EE1 to EEO4). EY was calculated as the ratio of the weight of the total oil content obtained at the end of the process and the initial essential oils added (EO_i). EY (%) = $((EO_t - EO_s) \times 100)/EO_i$. *Significant differences according to Tukey test ($p < 0.05$)

EO and EEO was validated according to the following parameters: accuracy (RSD), 2.9%; linearity (r), 0.998; and adequate recoveries (72–87%) using chloroform as the extraction solvent. (A typical gas chromatographic profile of a point of the calibration curve (3.43 mg/mL of EO), is included as supplementary material). The matrix effect was evaluated for three EO concentrations (5.47, 2.73 and 1.36 mg/mL), and the response factors (sabinene area/IS area) were compared with those corresponding to the calibration curve in chloroform. No significant differences were found (ANOVA test, $p < 0.05$) (a gas chromatography profile of a standard curve point, EO at 3.34 mg/mL is included as supplementary information).

3.4. Characterization of microcapsules

Comparative studies of stability for free and microencapsulated essential oil (EO and EEO4 respectively) were conducted during 24 h of monitored time at 45 °C. The percentage retention of EO in microcapsules was calculated as: $EO_{\text{at elapsed time}} \times 100/EO_{\text{at zero time}}$. Under these experimental conditions, a very rapid liberation of free EO was demonstrated (Fig. 3)

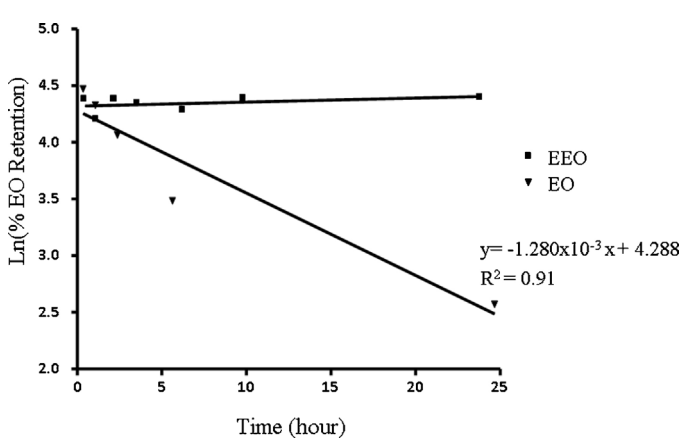

Fig. 3. Comparative study of stability for total essential free oil (EO) and microencapsulated essential oil (EEO 4) prepared from gum Arabic–maltodextrin carrier (monitored time, 0–24 h at 45 °C). The percentage retention of EO in microcapsules was calculated as: EO at elapsed time × 100/EO at zero time.

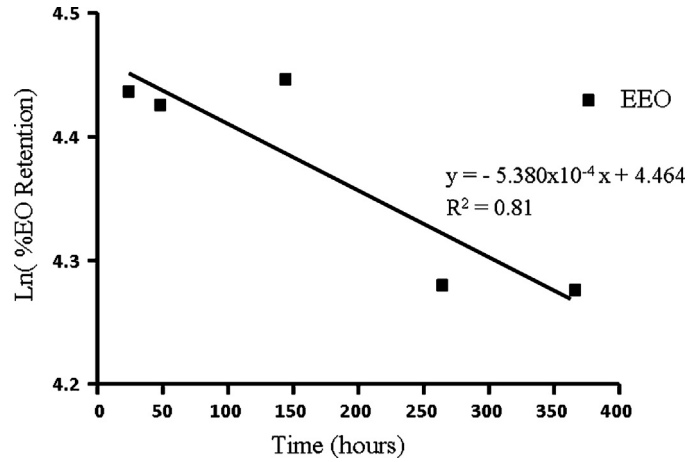

Fig. 4. Stability of total essential oil (EO) in microcapsule (EEO4) prepared from gum Arabic–maltodextrin (monitored time, 25–366 h at 45 °C). The percentage retention of EO in microcapsules was calculated as: EO at elapsed time × 100/EO at zero time.

(less than 15% retention at 24 h of monitored time), contrary to EEO, which showed a very slow liberation during the monitored time. In fact, in the second experiment (monitored time: 25 h to 366 h) a percentage of retention of 71% of essential oil in EEO microcapsules was determined (Fig. 4). A semi-log plot of EO percentage vs. storage time showed a sharp linear decrease indicating the reduction in sabinene constituent could follow first order kinetic. Even when a longer period (weeks) of stability studies could be conducted in order to obtain the rate constant (slope of the graph) for calculating the half-life, $t_{1/2}$ (time required for the reduction of a value to 50%), the slow liberation of EO was confirmed in this preliminary study. The structures of dried microencapsulated *S. molle* EO samples (EEO) as well as microcapsules without EO (wall material) observed by SEM are shown in Fig. 5. The powders showed different particles sizes, which agrees with the results obtained for the particle size distribution measured (range, 0.2–40 μm; mean, 9.5 μm) using a laser light diffraction instrument (COULTER® LS Particle Size Analyzer, Beckman Coulter, Brea, CA, USA). Regarding the shape, the powders showed most of the particles with rounded external surface, and continuous wall, which is important to provide low permeability to gases and core retention. Surfaces were concave and shriveled, which is described as typical of microcapsules produced by spray drying. Hollow particles were also observed, which could be explained by the formation of vacuoles inside the particles after crust development (Nijdam and Langrish, 2006).

3.5. Biological assays

The results of probit analysis of dose–mortality response of horn flies with Diazinon are shown in Fig. 6 as it was communicated previously by our group (Andina et al., 2012). The results were in agreement with those reported previously (dose-mortality response) for horn fly *H. irritans* susceptible strains (Li et al., 2007), using the same method. Diazinon susceptibility of horn flies used in each experiment was tested using the Petri dish method, which was also conducted for the screening of natural and synthetic new products. When *S. molle* EO was assayed at 1 mg/mL using this preliminary screening, a number of 14.16 ± 1.58 dead flies was observed at 2 h post incubation (59.4% of death), allowing to select it among the natural samples tested by this promising activity.

There were slight modifications to the Petri dish method in order to compare the EO and EEO insecticide activities, i.e. to compare the essential oil free sample with microcapsules of EO prepared through spray-drying. In fact, EEO suspension (equivalent to 1 mg/mL of EO) in acetone produced a sticky residue when acetone

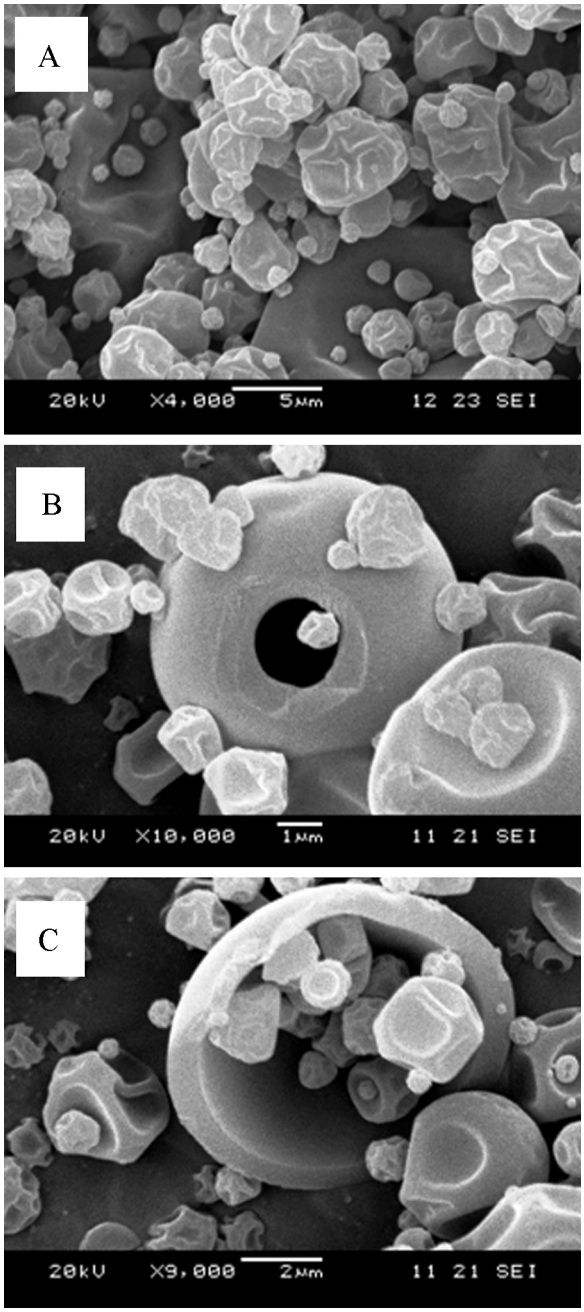

Fig. 5. SEM of EEO 4 microcapsules prepared from maltodextrin–gum Arabic (1:1) as carrier of *S. molle* essential oil (4:1, carrier:EO): (A) EEO4 microcapsules and (B and C) EEO4 microcapsules without essential oil (wall material).

was evaporated in the Petri dishes, making it difficult to manage the anesthetized flies. Then, the biological assays were carefully modified according to the nature of the specific compounds here involved.

The use of plastic cups covered with elastic mesh, above which previously conditioned cotton towels were placed, resulted in a more appropriate method to perform the comparative study of the activity described above. Diazinon was also used as a reference drug at 10 μg/mL (final concentration in the cotton towels) to test the susceptibility of flies using this method. This concentration was selected among a range of serial dilutions of Diazinon in acetone (data not shown). As it is shown, higher concentrations of Diazinon as well as EO, were necessary to obtain insecticidal response under these experimental conditions compared to Petri

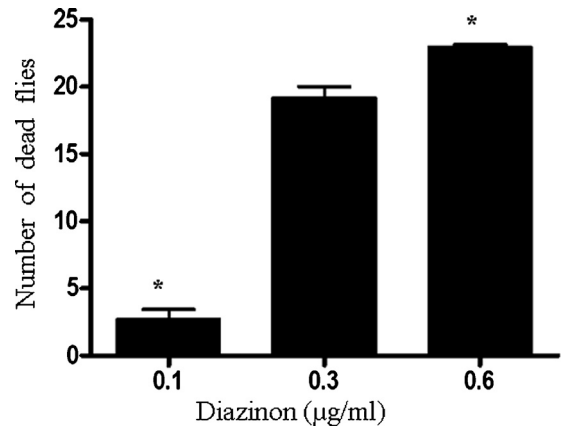

Fig. 6. Dose–mortality responses to Diazinon on *H. irritans* using Petri dish bioassay. The average number of dead flies (±SD) with Diazinon at different concentrations after 2 h of incubation time (*significantly different from Dunnett's test, $P < 0.05$).

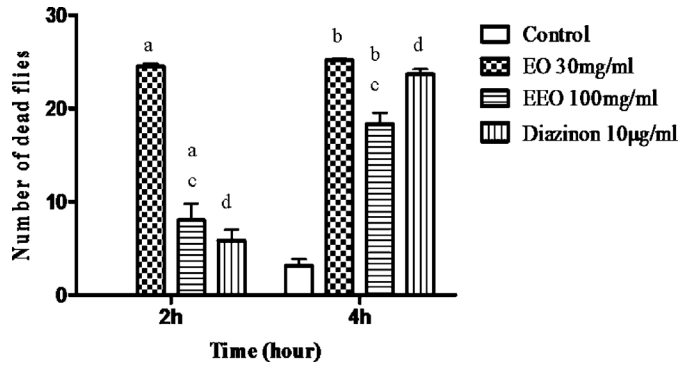

Fig. 7. Comparative study of insecticide activity of *S. molle* essential oil (EO at 30 mg/mL) free and microencapsulated (EEO at 100 mg/mL) on *H. irritans*. Number of dead flies (±SD) at 2 h and 4 h of incubation time. Bars with letters are significantly different (Tukey test, $p < 0.05$).

dish method mentioned before (0.6 μg/mL and 1 mg/mL for Diazinon and EO, respectively). As shown in Fig. 7, a time dependent effect for Diazinon at 10 μg/mL was observed at 2 and 4 h of incubation (23 and 94.8% of dead flies, respectively). Two independent experiments using EO (30 mg/mL) were also conducted, on different days, in order to study the bioassay reproducibility, as well as to select the appropriate bioassay duration. As Fig. 8 shows, a very rapid response which allowed for a plateau (maximum response) prior 2 h of post incubation was observed in both independent

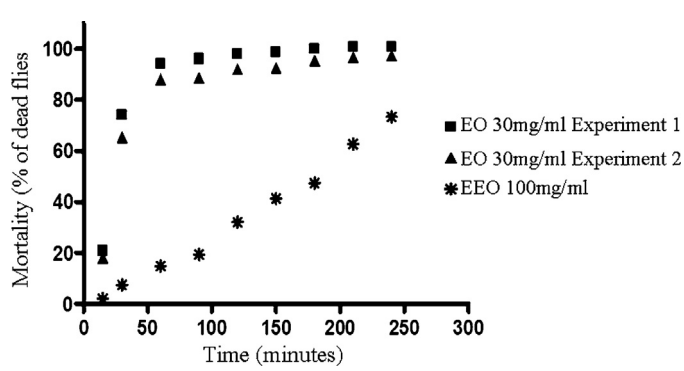

Fig. 8. Average mortality (%) in comparative insecticide activity of EO and EEO on *H. irritans* along 4 h of monitored incubation time. Two independent experiments (1 and 2) using *Schinus molle* essential oil (EO free) were conducted to study reproducibility of the bioassay.

experiments for EO (30 mg/mL). By contrast, a slower time dependent response was observed along the time for EEO (100 mg/mL).

Fig. 7 summarizes comparative results of insecticide activities at 2 and 4 h for EO, EEO, reference drug (diazinon) and untreated control (vehicle). Significant differences were observed for EO and EEO treatments at 2 h (96 and 32% of dead flies, respectively) and 4 h post incubation time (100 and 73% of dead flies, respectively). Control untreated (acetone) experiments showed 13% of dead flies at 240 min, which determined the end of experimental time duration.

The effect of most plant extracts used in pest control is insectistatic (inhibiting normal pest development) more than insecticidal, as it is the case of some plants that inhibit feeding in different ways. In this context, a broad spectrum of toxicity and repellence activities of different *S. molle* leaves extracts (methanolic, ethanolic, water, acetone) have been described against different insect pest species. In this regard, mortalities of 55–90% at 100 mg/mL were described for: *Chrysoperia externa*, *Trichogramma pintoi* and *Copidosoma koehleri* (Iannacone and Lamas, 2003); 80% of mortality at 50 mg/mL against *Xanthogaleruca luteola* Mûller (Huerta et al., 2010); 53% of mortality at 150 mg/mL against *Blatella germanaica* L. (Ferrero et al., 2007), among others.

Differences in susceptibility of pest species, the presence and amount of diverse active substances in the leaves extracts of *S. molle* (Abdel-Sattar et al., 2010) as well as their relative abundance (depending on the extraction method used) could be some of the multiple reasons that explain the diversity of dose-responses found. This context makes it necessary to standardize herbs and phytotherapics, and therefore the content of the active principles. The proposed GC method described herein, using sabinene as phytochemical marker, is simple, rapid and reliable, and can be successfully applied in the industry for quality control of both raw plant material and microcapsules. In addition, monoterpenes as sabinene, among the volatile compounds of plants, are described with a plant defense role against herbivores and plant pathogens, showing antimicrobial and larvicide activity. Even when the mechanism of action involved to explain those activities is not fully understood, it is speculated that the membrane disruption caused by these lipophilic compounds could produce inhibition of electron transport, protein translocations and other enzyme-dependent reactions (Chenga et al., 2013).

In conclusion, our results showed that the microencapsulation procedure here developed could be an interesting strategy to obtain a botanical insecticide to control the release of active ingredients (*S. molle* essential oil). In this way, essential oils are protected from the external environment during product application and storage time, providing a more persistent insecticidal effect.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2013.12.038.

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