AAPS PharmSciTech 2007; 8 (3) Article 67 (http://www.aapspharmscitech.org).

Themed Issue: Oral Controlled Release Development and Technology
Guest Editor - Stephen A. Howard and Jian-Xin Li

Artemisia arborescens L Essential Oil Loaded Beads: Preparation and Characterization

Received: January 22, 2007; Final Revision Received: March 23, 2007; Accepted: April 3, 2007; Published: August 24, 2007

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ABSTRACT

The purpose of this work was to prepare sodium alginate beads as a device for the controlled release of. essential oil for oral administration as an antiviral agent. Different formulations were prepared with sodium alginate as a natural polymer and calcium chloride or glutaraldehyde as a cross-linking agent. Loading capacities of between 86% and 100% were obtained in freshly prepared beads by changing exposure time to the cross-linking agent. Drying of the calcium alginate beads caused only a slight decrease in the loading efficiency. The surface morphology of the different bead formulations were studied using scanning electron microscopy (SEM). Stability studies over a 3-month period showed that glutaraldehyde reacted with some components of *Artemisia arborescens* L essential oil, changing its composition. Calcium alginate beads showed an in vitro controlled release of the essential oil for the investigated 24 hours, while the use of glutaraldehyde as a cross-linking agent was found not appropriate because of the interactions with azulene derivatives and the low degree of matrix cross-linkage.

KEYWORDS: Essential oil, antiviral, controlled release, Artemisia arborescens, beads, sodium alginate.

INTRODUCTION

Essential oils, complex mixtures of volatile, lipophilic substances obtained from different parts of plants by steam distillation, solvent extraction, or cold pressing, have a composition that can vary widely in relation to several factors (eg, source, plant location, cultivation technique, season). Several essential oils, which are secondary plant metabolites with chemical structures with oxygenated compounds such as alcohols, ketones, aldehydes, and esters, have shown therapeutic properties among which antimicrobial activity is one of the most studied.[1]

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Studies on the antimicrobial properties of natural products have always become more important as a consequence of the increasing number of microorganisms that are resistant to synthetic or natural purified drugs. Numerous studies have shown the antimicrobial properties of several essential oils such as Tea tree (*Malaleluca alternifolia*),[2] *Laurus nobilis,*[3] *Origanum compactum*,[4] and *Eugenia caryophyllus*.[5] Recently, *Santolina insularis* and *Artemisia arborescens* essential oils have shown good in vitro antiviral activity.[6,7] Moreover, it has been shown that this property can be affected by the delivery system used to transport them. To preserve the antimicrobial properties, essential oils need to be delivered using carriers able to protect them from environmental exposure.

Because of increasing interest in the formulation of delivery systems capable of improving the performance of active essential oils, this work focuses on the encapsulation of essential oils in alginate beads. To this purpose, *Artemisia arborescens* volatile oil, which had shown good in vitro antiherpetic activity against intracellular herpes simplex virus type 1 (HSV-1),[7] was used as a model essential oil. *Artemisia arborescens* L is an aromatic plant that is endemic in Mediterranean regions. It is an evergreen shrub from the *Asteraceae* family. Components of the *Artemisia* genus have been used for centuries in folk medicine. In particular, *Artemisia arborescens* L, very common in Sardinia, has been used as a treatment for inflammation, headache, and to alleviate urticaria, rheumatic pain, neuralgia, and gastrointestinal pain. It is also used as a bronchodilator and as a lenitive of renal colic and hematic fits caused by favism.[8,9]

The aim of this study was to evaluate alginate beads for the controlled release of essential oils following oral administration. The rationale of this system was to combine the low cost and the simplicity of alginate bead preparation. Moreover, alginate entrapment has been applied to immobilize several types of cells including bacteria, yeasts, molds, algae, plants, and animals.[10] Different formulations of microparticles and nanoparticles were prepared for the controlled release of pharmaceutical and pesticidal substances using divalent cations or glutaraldehyde (GA) as cross-linking agents.[11-19] To this purpose, the *Artemisia arborescens* essential oil was encapsulated in beads, which were prepared with different cross-linking agents, $CaCl_2$ or

GA, and by changing the bead exposure time to the cross-linking agents. Bead formulations were characterized in terms of encapsulation capacity, surface morphology, and in vitro release.

Experimental Methods

Materials

Sodium alginate with a declared viscosity of 250 cps (mannuronic/glucuronic acid moieties ratio 61:39 wt/wt, Sigma code a2158250 g), glutaraldehyde (25% wt/wt), calcium chloride, and methanol were purchased from Sigma-Aldrich, Milan, Italy.

Artemisia arborescens L leaves were collected in the countryside around Usellus, Sardinia, Italy, during full blossom (May-June 2004). The leaves were identified and a voucher specimen was deposited in the Herbarium of the Department of botany and botanical gardens, University of Cagliari, Italy.

Methods

Essential Oil Extraction and Characterization

The fresh aerial parts of the plant (5000 g) were distilled in a steam apparatus with an aqueous phase recycling system for 3 hours. The extracted blue essential oil was separated from the aqueous phase solution and dried over anhydrous sodium sulfate. The oil was stored at 4ºC until used.

Oil yields were determined using a Clevenger-type apparatus in conformity with European Pharmacopoeia standards. The analysis of the major components of the essential oil was performed with gas chromatography/ion trap mass spectrometry (GC/ITMS) and high-performance liquid chromatography (HPLC). A Varian CP 3800 gas chromatograph (Varian Inc, Palo Alto, CA) coupled with a Saturn 2000 ITMS detector (Varian Inc, Palo Alto, CA), a Varian CP 7800 autosampler, a split-splitless injector, and an MS ChemStation were used. The column was a fused silica capillary DB-5MS (5% phenylmethylpolysyloxane, 30 m × 0.25 mm; film thickness 0.25 μm) (J&W Scientific Fisons, Folsom, CA). The injector and interface were at 150°C and 280°C, respectively. The oven temperature was programmed as follows: from 60°C to 180°C (3°C/min), and isothermally held for 15 minutes. Helium was the carrier gas at 1 mL/min; the sample (1 μL) was injected in the split mode (1:20). MS conditions were as follows: ionization mode EI from 50 to 450 atomic mass unit (amu). The oil compounds were identified by comparing their relative retention times with those of authentic samples or by comparing their retention index (RI) relative to the series of n-hydrocarbons, and the computer matching results against commercial library and homemade library mass spectra made up from pure substances and components of known oils and MS literature data.[20,21]

The oil content was also assayed by HPLC at several wavelengths (209, 245, and 284 nm) using a Waters 2690 liquid chromatograph, equipped with a photodiode array detector 996 (Waters Corp, Milford, MA). The mobile phases were methanol and water. Separations were performed using 75% methanol and 25% water as eluent at a flow rate of 1.0 mL/min. The column was an Xbridge C18 5 μm (4.6 × 150 mm, Waters). Appropriate standard methanolic solutions of camphor and α+β-thujon were prepared and tested. All experiments were performed in triplicate. Because of complexity of the essential oil composition, camphor, β-thujon, and chamazulene were used as "lead" components as previously reported.[7] In particular, the camphor was determined at 284 nm and the β-thujon at 209 nm. Retention times for camphor and β-thujon were 1.74 and 2.67, respectively.

Preparation of Beads

The method by Sugawara et al[11] was used with some modifications. In brief, an emulsion (2.4%) of *Artemisia arborescens* essential oil in 2.5% sodium alginate solution was prepared under stirring using a Silverson l4 homogenizer (Silverson Machines Ltd, Waterside, UK) for 5 minutes at 10 000 rpm. The emulsion was then added dropwise into a solution of 10% $CaCl_2$ or 1% glutaraldehyde in 1% HCl in water, using a 25-mL 21-gauge hypodermic syringe under constant stirring (300 rpm). The formed beads were cured for 10, 20, or 30 minutes and then transferred to filter paper, washed with distilled water, and immediately analyzed or dried for 24 hours at room temperature.

Scanning Electron Microscopy

Samples were placed on an aluminum stub with a conductive copper tape and then covered with a gold layer under an argon atmosphere. Observation has been made with a Zeiss DSM 962 (Carl Zeiss, Oberkochen, Germany) operating at 20 kV acceleration.

Drying Rate Study

Just after preparation, a sample of each formulation (0.28-0.37 g) was transferred to filter paper to remove surface adsorbed $CaCl_2$ or glutaraldehyde solution. Masses of the beads were taken at definite intervals of time on a single pan balance until the constant mass was achieved.

Bead size

Ten samples of freshly prepared or dried beads from different formulations were measured using a caliper (DIGI-MET caliper, Preisser, Berlin, Germany) with an accuracy of ± 0.01 mm. The test was repeated 3 times for all formulations.

Encapsulation Capacity

The encapsulation capacity (E%) was determined as a percentage of the total amount of *Artemisia arborescens* L essential oil used for the preparation of the initial emulsion. The fresh or dried beads were homogenized with methanol for 3 minutes using a Silverson N4. The essential oil was extracted from the homogenized beads with methanol for 1 hour in closed conical flasks at room temperature. The essential oil methanol solution was filtered and then analyzed at a wavelength of 284 nm in a UV spectrophotometer (Hitachi U-2000, Hitachi, Tokyo, Japan). The spectrophotometric method results were also compared with those obtained by HPLC, where the most important components of the oil (camphor, β-thujon, and chamazulene) were used as a "lead."[20,21]

In Vitro Release Studies

The in vitro release studies of essential oil from freshly prepared and dried beads were performed at room temperature in a 500-mL closed conical flask containing a 1% Tween 80 phosphate-buffered saline (PBS) solution under magnetic stirring. An amount of fresh or dried beads, corresponding to 72 mg of essential oil, were loaded into gelatin capsules. The capsules were then added to PBS solution. At definite time intervals, the conical flasks were shaken well, and 1 mL aliquot was taken. After diluting with methanol, the content of *Artemisia arborescens* L essential oil was analyzed using an HPLC method previously described.

Stability Studies

Change in encapsulation capacity (R%) and in composition of entrapped essential oil in the different dried bead formulations was studied over a 3-month period.

The dried bead formulations were stored at room temperature in closed conical flasks, and at fixed time intervals they were assayed for their essential oil composition and content as previously described.

Statistical Analysis of Data

Data analysis was performed with the software package Microsoft Excel, Version 2003. Results are expressed as mean ± standard deviation (3 independent samples). Statistically significant difference was determined using the Student *t* test and analysis of variance (ANOVA) with $P = .05$ as a minimal level of significance.

Results and Discussion

Distillation of the aerial parts of *Artemisia arborescens* L gave a blue-green oil with a 0.8% ± 0.1% yield. The most abundant components were camphor, β-thujon, and chamazulene; amounts are expressed as a percentage of peak area (Table 1, Figure 1). Obtained data showed a higher concentration of camphor and chamazulene in comparison to other essential oils of *Artemisia arborescens* L collected in other Mediterranean regions.[22] The presence of azulene derivatives determined the blue-green essential oil color.

Table 1. Main Components of *Artemisia arborescens* L Essential Oil as Determined by GC/ITMS*

Component	Retention Time (R_t)	Area (%)
α-Pinene	5.25	3.17
(-)Canfene	5.66	3.44
β-Thujone	11.3	23.9
Camphor	11.5	35.7
Terpinen-4-ol	13.8	2.20
Muurola-4(14,5 diene) cis	26.3	4.05
Chamazulene (7-ethyl-1,4-dimethylazulene)	35.7	7.65

*GC/ITMS indicates gas chromatography/ion trap mass spectrometry.

The extracted *Artemisia arborescens* L essential oil was successfully encapsulated in sodium alginate beads prepared using 10% $CaCl_2$ or 1% GA (1% HCl) and changing the exposure time to the cross-linking agents. The compositions of the formulations are listed in Table 2.

In general, freshly prepared calcium alginate formulations showed a brilliant blue color independently of exposure time to the cross-linking agent. On the contrary, the use of GA led to an essential oil color change to dark green showing a reaction between this cross-linking agent and the azulene derivatives of the essential oil (Figure 2).

The chemical interactions between GA and the azulene derivatives were confirmed using the GC/ITMS method (Figure 1). In particular, it was shown that 10 minutes after preparation the composition of the incorporated essential oil had not changed if compared with the fresh essential oil when $CaCl_2$ was used as the cross-linking agent. A similar gas chromatogram was obtained for the essential oil extracted from the dried calcium alginate beads, after 3 months of storage. On the contrary, GA beads showed, just 10 minutes after preparation, a change of the main component ratio (β-thujon, camphor, and chamazulene) because of the interaction of GA with chamazulene. After 3 months of storage, disappearance of some peaks, in particular those of azulene derivatives, was evident.

The freshly prepared calcium alginate beads (formulations 1-3) showed a regular, spherical shape that was maintained even after drying (Figure 2). The bead size did not vary significantly when the exposure time to $CaCl_2$ was changed (Table 2). In particular, these freshly prepared

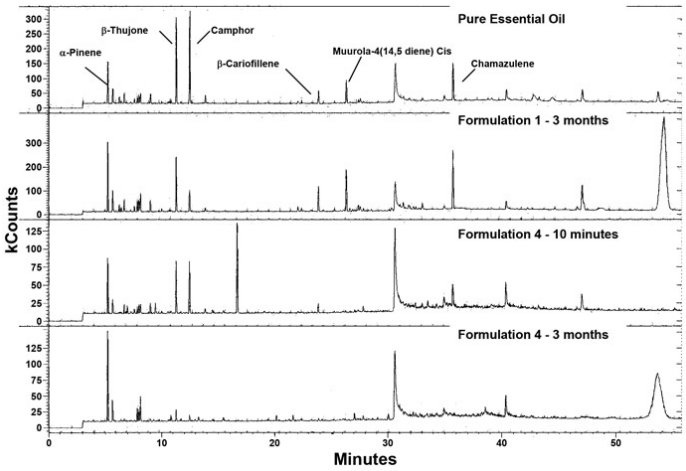

Figure 1. Chromatograms of *Artemisia arborescens* L essential oil, pure or extracted from beads. Essential oil was extracted 10 minutes or 3 months after preparation from formulation 1 ($CaCl_2$) or formulation 4 (glutaraldehyde).

formulations showed a narrow size range between 3.24 ± 0.71 mm for formulation 3 and 3.35 ± 0.92 mm for formulation 2.

The loss of water during the drying process led to a decrease in the bead size. Dried formulations prepared using longer exposure times were larger, with formulations 2 and 3 having a size of 2.32 ± 0.42 mm and 2.40 ± 0.51 mm, respectively.

In general, fresh beads prepared with glutaraldehyde were 1.5-fold larger than the corresponding calcium alginate beads (Table 2, Figure 2). Moreover, GA beads showed an irregular shape, which retained the droplet form, and this was particularly evident when the beads were dried (Figure 2, formulation 6).

During this work, drying rate was also studied to evaluate the transport rate of water through the alginate matrix and thus to have information on the influence of the cross-linkage on this transport and also on drug release. Results obtained from the drying process are shown in Figure 3. It can be seen that the longer the exposure time to the cross-linking agent,

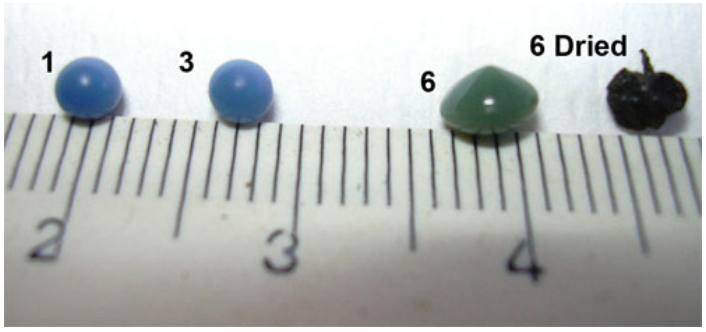

Figure 2. Photo showing freshly prepared bead formulation 1, formulation 3, formulation 6, and dried formulation 6.

the slower the drying process. In particular, formulations prepared using GA showed a faster drying rate than calcium alginate beads. These results suggest that for the same cross-linking agent, longer exposure time led to an increased rigidity of the polymer, thereby showing a decreased desorption rate of liquid from the beads.

These data were confirmed by SEM analysis. From photomicrographs of formulations 1 to 3 (Figure 4), it is clearly visible that different exposure times to the cross-linking agent determined a different surface morphology. The beads exposed for a shorter time have a rough irregular surface that becomes smoother as the exposure time increased. The same dependence on exposure times was observed for beads prepared using GA (formulations 4-6), which always showed a very fast drying rate as a consequence of a low degree of cross-linkage.

All freshly prepared beads showed a high encapsulation capacity (Figure 5) as already reported in literature with similar formulations.[23] In particular, formulation 1, prepared using 10% $CaCl_2$, was found to be the formulation with the highest calculated encapsulation capacity (101.0 ± 4.1). In general, for all formulations, the longer the exposure time, the lower the encapsulation capacity. This behavior is probably due to a partitioning of the essential oil components in the water phase: an increase in the exposure time led to an increase in the partition of the essential oil in the water solution.

Table 2. Composition and Size of *Artemisia arborescens* L Essential Oil Loaded Bead Formulations*

Formulations	Cross-linking Agent		Exposure Time (min)	Size Freshly Prepared Beads (mm)	Size Dried Beads (mm)
	$CaCl_2$	GA			
1	10%	—	10	3.32 ± 0.87	2.15 ± 0.48
2	10%	—	20	3.35 ± 0.92	2.32 ± 0.42
3	10%	—	30	3.24 ± 0.71	2.40 ± 0.51
4	—	1%	10	4.56 ± 0.98	3.45 ± 0.23
5	—	1%	20	4.27 ± 1.10	3.51 ± 0.18
6	—	1%	30	4.35 ± 1.21	3.26 ± 0.24

*GA indicates glutaraldehyde.

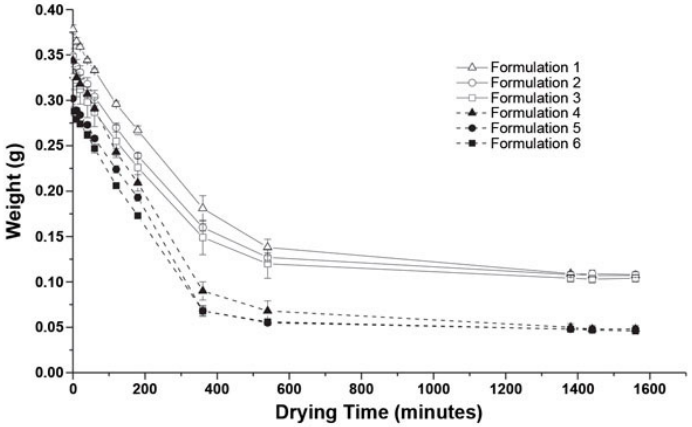

Figure 3. Effect of cross-linking on drying time of beads.

Drying of the beads led to a slight decrease in the encapsulation capacity of up to 12.17% ± 0.7% (formulation 1) when $CaCl_2$ was used. The use of GA determined a greater decrease in the entrapped essential oil content. For formulations 4, 5, and 6, a decrease of 68.11% ± 3.09%, 65.69% ± 2.14%, 68.06% ± 2.17% in essential oil content was found, respectively. This can be explained by considering the cross-linking reaction medium. Kulkarni et al[23] and Yeom and Lee[24] prepared cross-linked alginate beads using an acetone or methanol solution of GA and HCl as a catalyst. In this study, the use of these solvents led to very low encapsulation capacity owing to the extraction of the essential oil during the bead formation (data not shown). Probably, the use of water instead of these solvents is not the optimal reaction medium for the cross-linking reaction between the hydroxyl groups of sodium alginate and the aldehyde groups of GA. Therefore, a weak gel with fewer cross-linkages was obtained, and this gel was not able to retain the essential oil inside the formed beads. Moreover, a simple touch analysis was performed to emphasize the difference in the consistency of GA and $CaCl_2$ gels.

The encapsulation capacity variation (R%) for the 6 dried formulations was studied over a 3-month period. Figure 6 shows only a slight decrease of R% for calcium alginate beads. In particular, at the end of the experiments, formulations 1, 2, and 3 retained ~80%, 69%, and 65% of the total encapsulated oil, respectively. For formulations 4, 5, and 6,

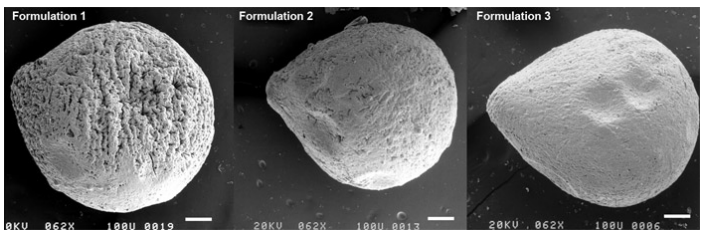

Figure 4. Scanning electron photomicrographs of formulations 1 to 3 (bar = 250 μm) original magnification × 30.

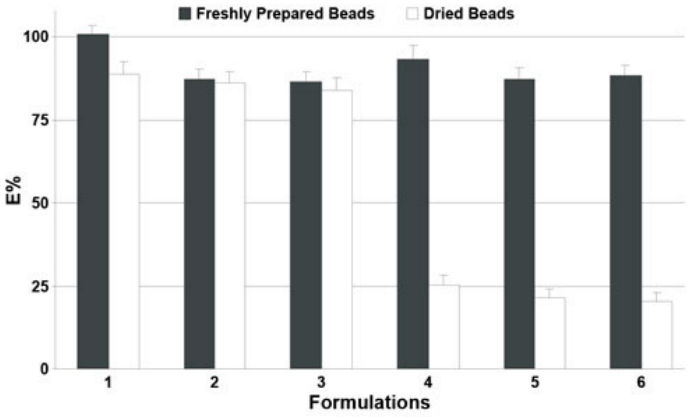

Figure 5. Encapsulation capacity (E%) of freshly prepared and dried bead formulations 1 to 6.

prepared using GA, only ~2%, 3%, and 4%, respectively, of the entrapped essential oil was still inside the beads after 3 months. As already stated, this behavior can be explained as a consequence of the low degree of cross-linkage of alginate when GA is used in an aqueous medium.

The essential oil in vitro release from the beads was studied for 24 hours in order to evaluate the influence of the nature of cross-linking agents ($CaCl_2$ or GA), the time exposure, and the drying process. All in vitro release experiments were performed in 1% Tween 80 PBS in order to avoid water saturation because of the low solubility of *Artemisia arborescens* essential oil in water.

Results of the in vitro release of the freshly prepared and dried formulations 1 to 6 are presented in Figure 7. Because of GA-azulene derivative interactions, in the release study from GA beads only camphor and β-thujon were considered. Release rate of the essential oil from all formulations 1 to 6 seems to be slightly affected by the exposure time to the cross-linking agent. More influencing parameters on the release rate are the type of cross-linking agent ($CaCl_2$ or

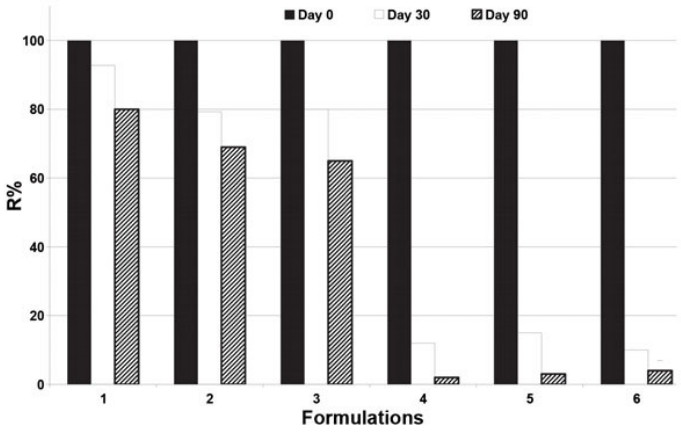

Figure 6. Encapsulation capacity variation (R%) of dried bead formulations during storage at room temperature.

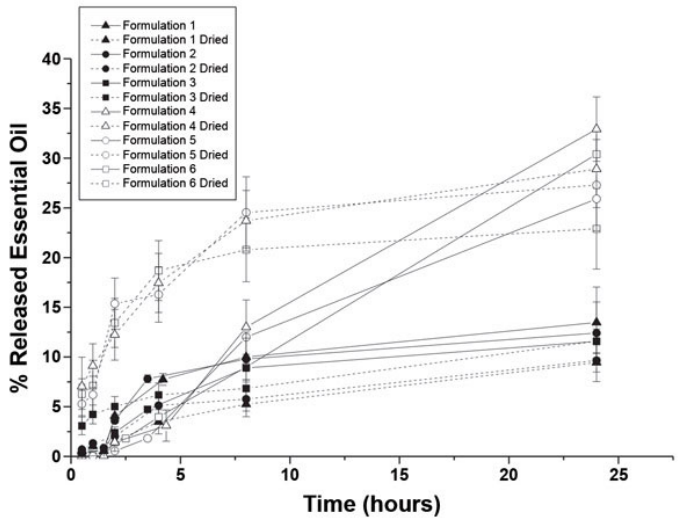

Figure 7. In vitro release of freshly prepared (solid line) and dried (dash line) formulations 1 to 6.

GA) and the drying process. Both fresh and dried $CaCl_2$ formulations 1 to 3 showed a slower release than GA formulations 4 to 6. In fact, as can be seen in Figure 7, freshly prepared calcium alginate beads were able to control the essential oil release if compared with the corresponding fresh GA formulations 4 to 6. After 24 hours, the fresh beads 1 to 3 released from 11.57% ± 3.12% (formulation 1) to 13.47% ± 3.56% (formulation 3) of the encapsulated oil, while the fresh GA beads released a 1.92- to 2.84-fold higher amount of the oil. This different release behavior is related to the results of the drying study described above and further supports the hypothesis that GA beads have a lower cross-linkage degree than $CaCl_2$ beads. Therefore, the GA-containing alginate matrix is not able to retain the essential oil components if compared with the $CaCl_2$ formulations.

The drying process, in the case of $CaCl_2$ formulations, led to a light, not statistically significant ($P > .05$), decrease in the essential oil release rate showing a maximum release ranging from 9.45% ± 0.96% (dried formulation 3) to 11.59% ± 1.22% (dried formulation 1) after 24 hours. In the freshly prepared swelled formulations, alginate chains are hydrated and the diffusion spaces are larger. The release rate of the essential oil components from the $CaCl_2$ beads decreases after the drying process because of the reduction of the alginate chain distance, namely, the diffusion spaces between the polymer chains.

Release experiments from the GA-dried formulations showed a different profile. In fact, the essential oil release was faster than the fresh formulations in the first 6 hours of the experiments, but then the release rate decreased and became almost constant. In particular, formulation 4 showed the fastest release with 32.92% ± 3.24% of the essential oil released after 24 hours.

The highest release rate in the first hours of the experiments obtained from the dried GA-containing beads could be owing to the drying process, which led to a concentration of the oil components in the superficial strata of the beads. Therefore, the activation of the matrix was very fast as shown by the burst release of the oil (ranging from 5.28% ± 1.54% to 7.04% ± 2.94%) in the first hour. On the contrary, the opposite profile obtained from the freshly prepared beads 4 to 6 could be explained as the consequence of a longer activation process of the matrix that, once hydrated, cannot retain the essential oil as a consequence of the low cross-linkage degree.

CONCLUSION

Results obtained during this study have shown that *Artemisia arborescens* L essential oil can be encapsulated in calcium alginate beads with good yields. Calcium alginate beads have also demonstrated their capability to control essential oil release. On the contrary, the use of GA as a cross-linking agent is not appropriate because it led to interactions with some oil components (azulene derivatives) and to a low degree of matrix cross-linkage. Further studies are in progress in order to characterize essential oil release kinetics.

ACKNOWLEDGMENTS

This work was supported by a grant from Ministero dell'Università e della Ricerca (MIUR) and Progetti di Rilevante Interesse Nazionale (PRIN) 2004.

REFERENCES

1. Hammer KA, Carson CF, Riley TV. Antimicrobial activity of essential oils and other plant extracts. *J Appl Microbiol.* 1999;86:985–990.

2. Schnitzler P, Schon K, Reichling J. Antiviral activity of Australian tea tree oil and eucalyptus oil against herpes simplex virus in cell culture. *Pharmazie.* 2001;56:343–347.

3. Bouzouita N, Kachouri F, Hamdi M, Chaabouni MM. Antimicrobial activity of essential oils from Tunisian aromatic plants. *Flavour Frag J.* 2003;18:380–383.

4. Manohar V, Ingram C, Gray J, et al. Antifungal activities of origanum oil against Candida albicans. *Mol Cell Biochem.* 2001;228:111–117.

5. De M, Krishna DEA, Banerjee AB. Antimicrobial screening of some Indian spices. *Phytother Res.* 1999;13:616–618.

6. Valenti D, De Logu A, Loy G, et al. Liposome-incorporated Santolina insularis essential oil: Preparation, characterization and in vitro antiviral activity. *J Liposome Res.* 2001;11:73–90.

7. Sinico C, De Logu A, Lai F, et al. Liposomal incorporation of Artemisia arborescens L essential oil and in vitro antiviral activity. *Eur J Pharm Biopharm.* 2005;59:161–168.

8. Sherif A, Hall RG, el-Amamy M. Drugs, insecticides and other agents from Artemisia. *Med Hypotheses.* 1987;23:187–193.

9. Ballero M, Poli F, Sacchetti G, Loi MC. Ethnobotanical research in the territory of Fluminimaggiore (southwestern Sardinia). *Fitoterapia.* 2001;72:788–801.

10. Becerra M, Baroli B, Fadda AM, Blanco-Mendez J, Gonzalez-Siso MI. Lactose bioconversion by calcium-alginate immobilization of *Kluyveromyces lactis* cells. *Enzyme Microb Tech.* 2001;29:506–512.

11. Sugawara S, Imai T, Otagiri M. The controlled release of prednisolone using alginate gel. *Pharm Res.* 1994;11:272–277.

12. Kikuchi A, Kawabuchi M, Watanabe A, Sugihara M, Sakurai Y, Okano T. Effect of Ca^{+}-alginate gel dissolution on release of dextran with different molecular weights. *J Control Release.* 1999;58:21–28.

13. Cho NH, Seong SY, Chun KH, et al. Novel mucosal immunization with polysaccharide-protein conjugates entrapped alginate microspheres. *J Control Release.* 1998;53:215–224.

14. Gohel MC, Jani GK, Amin AF, Patel KV, Gupta SV. Application of classical experimental design for the development of theophylline microspheres. *J Control Release.* 1997;45:265–271.

15. Albarghouthi M, Fara DA, Saleem M, El-Thaher T, Matalka K, Badwan A. Immobilization of antibodies on alginate-chitosan beads. *Int J Pharm.* 2000;206:23–34.

16. Sezer AD, Akbuga J. Release characteristic of chitosan treated alginate beads. I. Sustained release of macromolecular drug from chitosan alginate beads. *J Microencapsul.* 1999;16:195–203.

17. Sezer AD, Akbuga J. Release characteristic of chitosan treated alginate beads. II. Sustained release of low molecular drug from chitosan alginate beads. *J Microencapsul.* 1999;16:687–696.

18. Aynie I, Vauthier C, Chacun H, Fattal E, Couvreur P. Spongelike alginate nanoparticles as a new potential system for the delivery of antisense oligonucleotides. *Antisense Nucleic Acid Drug Dev.* 1999; 9:301–312.

19. Lambert G, Fattal E, Couvreur P. Nanoparticulate systems for the delivery of antisense oligonucleotides. *Adv Drug Deliv Rev.* 2001;47:99–112.

20. Adams RP. *Identification of the Essential Oil Components by Gas-Chromatography/Mass Spectroscopy.* Carol Stream, IL: Allured Publishing Corp; 1995.

21. National Institute of Standards and Technology. NIST Scientific and Technical Databases [database online]. *The NIST Mass Spectral Search Program for the NIST/EPA/NIM Mass Spectral Library Version 1.7.* 1999. Available at: http://www.nist.gov/srd/index.html. Accessed: November 5, 1999.

22. Sacco T, Frattini C, Bicchi C. Constituents of essential oil of Artemisia arborescens. *Planta Med.* 1983;47:49–51.

23. Kulkarni AR, Soppimatha KS, Aminabhavi TM, Daveb AM, Mehta MH. Glutaraldehyde cross-linked sodium alginate beads containing liquid pesticide for soil application. *J Control Release.* 2000;63:97–105.

24. Yeom CK, Lee KH. Characterization of sodium alginate membrane cross-linked with glutaraldehyde in pervaporation separation. *J Appl Polym Sci.* 1998;67:209–219.