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Short communication

Long-term antifungal activity of volatile essential oil components released from mesoporous silica materials

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ABSTRACT

Antimicrobial volatile substances from plants have become known as a suitable alternative to synthetic pesticides and food preservatives. The study tested the antifungal activity against *Aspergillus niger* of seven volatile essential oil components from plants—allyl isothiocyanate, carvacrol, cinnamaldehyde, diallyl disulfide, eugenol, thymol, and thymoquinone. To provide long-term effects by controlled release and ease of application, these substances were encapsulated into mesoporous silica material MCM-41 and then compared to the effects of pure substances. Significant antifungal activity was verified in five out of the seven tested substances. These results were correlated with the evaporation rate of pure and encapsulated substances. It has been proven that by encapsulating selected volatiles, excluding sulfur compounds, their long-term effectiveness is ensured by controlled release and easy handling, with positive effects for their antifungal activity.

1. Introduction

Essential oil components (EOC) have antimicrobial activity that can be a suitable alternative to synthetic pesticides and the preservatives of food (Antunes and Cavaco, 2010; Bakkali et al., 2008; Nedorostova et al., 2008). However, due to high hydrophobicity and volatility, they can lose this activity before acting as a fungicide (Laird and Phillips, 2012). Encapsulation in microcapsules has been developed for the protection and preservation of the effectiveness of bioactive substances (Kailasapathy, 2009; Prado et al., 2011; Park et al., 2012).

In recent years, as an alternative to polymeric materials, silica mesoporous supports (SMPS) have been used as inorganic scaffolds for the storage and release of drugs and organic molecules (Kresge et al., 1992). SMPS provide unique features such as stability, biocompatibility, large load capacity, and the possibility to include gate-like scaffoldings on the external surface for the design of nanodevices for on-command delivery applications (Munoz et al., 2003; Schmaljohann, 2006; Vallet-Regi et al., 2001, 2007). SMPS can be prepared in different sizes with pores ranging from 2–10 nm. Encapsulation of volatile compounds from plants into mesoporous silica material MCM-41 should significantly facilitate this application and provide a longer term efficacy of controlled release without affecting antimicrobial activity.

This work evaluates the antifungal activity against *Aspergillus niger*, an important pathogen of fruit and vegetables, particularly in the post-harvest period and other stored agricultural products such as seeds, rice, coffee, walnut kernels, sunflower seeds and other substrates (Krijgsheld et al., 2013). Its action on the substrates leads to changes in their organoleptic properties and the nutritional value is decreased. Much more serious, however, is the

Abbreviations: EOC, essential oil components; *A. niger*, *Aspergillus niger*; MID, minimal inhibitory dose; SDA, sabouraud dextrose agar; SMPS, silica mesoporous support; SMPS-AITC, encapsulated allyl isothiocyanate; SMPS-Ca, encapsulated carvacrol; SMPS-Ci, encapsulated cinnamaldehyde; SMPS-DD, encapsulated diallyl disulfide; SMPS-EUG, encapsulated eugenol; SMPS-Thy, encapsulated thymol; SMPS-Tq, encapsulated thymoquinone.

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production of toxic secondary metabolites – mycotoxins. It has been shown that certain strains of *A. niger* produce ochratoxin A (Schuster et al., 2002). From numerous studies it was found that ochratoxin A damages the kidneys, carcinogenic and teratogenic effects (central nervous system disorder in the fetus) and suppresses the immune function (Kuiper-Goodman and Scott, 1989). Moreover, in immunocompromised patients various aspergillosis, e.g., pulmonary infection or ear canal may occur (Schuster et al., 2002). For these reasons *A. niger* was selected for this study. The main objective of this work was to determine and compare the antifungal activity and the evaporation rate of seven volatile compounds when encapsulated in MCM-41 and when pure.

2. Materials and methods

2.1. Encapsulation of SMPS-EOC

Encapsulation was performed by adding 10 mg of the selected compound into 20 mg of MCM-41. Then, the vials were inserted into a vortex and shaken for 24 h in an oven at 40 °C. Using this approach, the final mesoporous materials SMPS-AITC, SMPS-Ca, SMPS-Ci, SMPS-DD, SMPS-Eu, SMPS-Thy and SMPS-Tq, loaded with allyl isothiocyanate, carvacrol, cinnamaldehyde, diallyl disulfide, eugenol, thymol, and thymoquinone, respectively, were prepared.

2.2. Antifungal assays

Testing was performed in vitro in petri dishes of 60 mm diameter, by modified disc diffusion method (Martos et al., 2012). Into each dish 5 ml SDA (Sabouraud Dextrose Agar) was poured, as the nutrient medium for *A. niger* (ATCC 6275, Czech Collection of Microorganisms, Brno, CZ). The antifungal activity of doses of 0.5 mg, 1 mg, 2 mg, and 4 mg of the substances in pure form was compared to the encapsulated materials; meaning that 1.5 mg, 3 mg, 6 mg, and 12 mg of SMPS-EOC. Next, 50 μL of the spore suspension (5×10^6 spores per mL) were vigorously mixed with the encapsulated and pure substances and evenly dispersed onto the agar. Inoculated petri dishes with or without empty MCM-41 were used as a control. All petri dishes were cultivated in a dark box at 25 °C with high humidity. All selected substances of defined doses were tested in triplicate and the fungus growths observed were established from minimum inhibitory doses (MID) of selected compounds. The degree of growth of the fungus was visually evaluated and documented at three, six, and fourteen days. Dishes with concentrations of substances that remained after two weeks without any visible growth of *A. niger* were re-inoculated with the fresh spores of the same density, and then antifungal activity was further evaluated and documented.

2.3. Evaporation test

The evaporation rate was monitored at 25 °C (suitable for fungal growth). In this test, 30 mg of individual SMPS-EOC, which means 10 mg of pure compound, and 10 mg of non-encapsulated compounds in the pure form, were weighed into small watch glasses in triplicate. The changes in weight were monitored after 24, 48, 72, and 144 h. Weight changes expressed as a percentage of evaporated compounds were calculated using the average evaporation rate of individual substances.

Table 1
MID (mg) of selected compounds encapsulated and pure tested against *A. niger*, rated at 3, 6, and 14 days.

MID of pure and encapsulated EOC (mg) against *A. niger*						
	3 days		6 days		14 days	
	Encaps.	Pure	Encaps.	Pure	Encaps.	Pure
Thymol	0.5	0.5	0.5	2	0.5	4
Thymoquinone	1	0.5	1	1	1	N
Eugenol	1	0.5	1	4	2	4
Carvacrol	0.5	0.5	0.5	0.5	0.5	0.5
Cinnamaldehyde	0.5	0.5	1	0.5	1	1
Diallyl disulfide	1	N	N	N	N	N
Allylisothiocyanate	1	N	4	N	N	N

N: *A. niger* was not inhibited by even the highest dose tested.

3. Results and discussion

The antifungal test was developed over two weeks (Table 1). After 14 days, the antifungal activity was confirmed in five of the seven tested compounds (thymol, thymoquinone, eugenol, carvacrol, and cinnamaldehyde), but generally in higher doses, especially in the non-encapsulated forms.

Thymol, thymoquinone, and eugenol are demonstrably more effective in a state of encapsulation than when in the pure state. After 14 days, the MID of encapsulated thymol and encapsulated thymoquinone was 1 mg and encapsulated eugenol was 2 mg. These treatments were therefore re-inoculated (Table 2). Three days after the re-inoculation, the MID of SMPS-Eu was still 2 mg. The MID of SMPS-Thy increased to 2 mg, while SMPS-Tq was not active even at 4 mg. Six days after the re-inoculation, the only active formulations were SMPS-Eu and SMPS-Thy at 4 mg. Fourteen days after the re-inoculation—or 28 days after the treatment—only SMPS-Eu remained active.

Comparing the results from the antifungal assays with desorption assays (Fig. 1), SMPS-Thy showed antifungal activity eight times higher than that of pure thymol and showed that it is vaporized substantially slower than pure thymol. Thymoquinone performed very well against *A. niger* in the encapsulated state, which is correlated with the speed of evaporation: SMPS-Tq was very slowly vaporized. SMPS-Eu, and to a lesser extent also SMPS-Ca and SMPS-Ci, were initially vaporized significantly more than the pure form, this apparent paradox could be explained by the presence of the compound in the surfaces of the MCM-41 pores. The great surface area of the material seemingly works as a wick in these cases, which hastens the initial evaporation rate. The antifungal assays performed in SMPS-Ca and pure carvacrol showed high activity in both cases. However, in the test evaporation rate, after six days all pure carvacrol had evaporated; it can be con-

Table 2
MID (mg) of selected compounds encapsulated and pure tested against *A. niger*, re-inoculated rated at 3, 6, and 14 days.

MID of pure and encapsulated EOC (mg) against *A. niger* re-inoculated						
	3 days (17 complete)		6 days (20 complete)		14 days (28 complete)	
	Encaps.	Pure	Encaps.	Pure	Encaps.	Pure
Thymol	2	N	4	N	N	N
Thymoquinone	N	N	N	N	N	N
Eugenol	2	N	4	N	4	N

N: *A. niger* was not inhibited by even the highest dose tested.

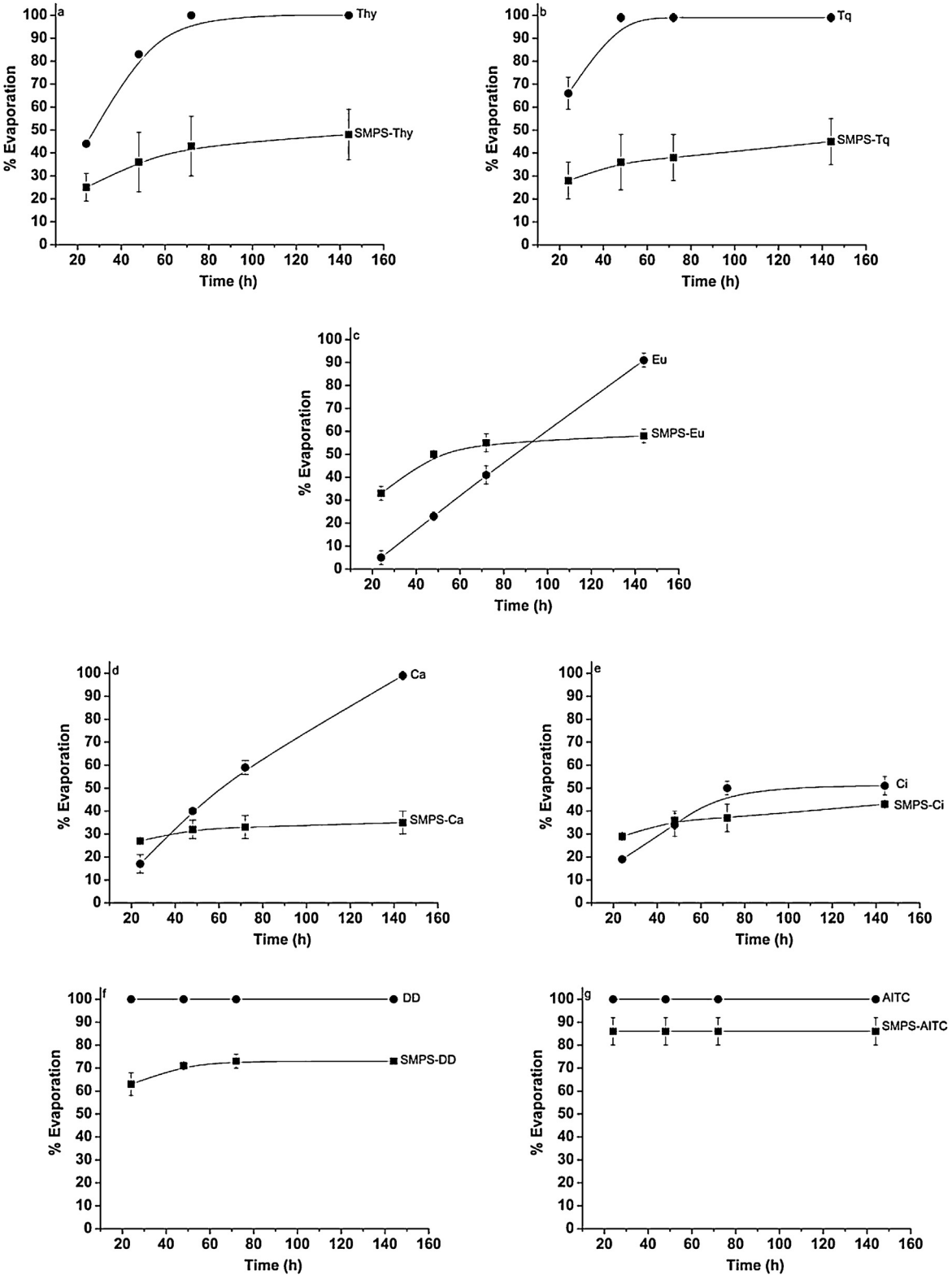

Fig. 1. Comparison during evaporation encapsulated (a) SMPS-Thy, (b) SMPS-Tq, (c) SMPS-Eu, (d) SMPS-Ca, (e) SMPS-Ci, (f) SMPS-DD, and (g) SMPS-AITC with a rate of evaporation of pure compounds at 25 °C.

cluded that carvacrol completely destroyed the *A. niger* spores during the first three days of the inoculated study. Cinnamaldehyde appeared to be very stable during the evaporation rate; it showed only small differences between SMPS-Ci and pure cinnamaldehyde and both showed high antifungal activity. The non-encapsulated sulfur compounds diallyl disulfide and allyl isothiocyanate in our experiments appeared to be almost ineffective, and only slightly effective when encapsulated. In the evaporation rate assay it was confirmed that both compounds had completely evaporated during the first day. In contrast, in the previous studies, these compounds

were rated as highly effective against bacteria (Nedorostova et al., 2009; Park et al., 2012) and even fungi (Kloucek et al., 2012). This difference from previous studies could be explained by the differences in the assay design, because in the tests described there the vapor escape from the petri dishes was limited. In the current test, petri dishes without any restriction of air circulation were used, so the highly volatile sulfur compounds probably escaped before they could show any significant antifungal activity. For the mechanism of action of the tested agents it has been demonstrated that EOC are preferentially absorbed onto the lipophilic surface of mycelia and that the greater the surface area of mycelia the stronger the inhibitory effect. It was hypothesized that EOC irreversibly cross link with components in the fungal cell membrane causing the leakage of intracellular components (Cavanagh, 2007). Regarding individual EOC used in this study, particularly essential oils containing cinnamaldehyde, eugenol, thymol and carvacrol showed the highest inhibitory activity against *A. niger* (Kloucek et al., 2012). When testing the fungi-static activity of essential oils in comparison to isolated components, it appears that this activity is directly related to the chemical composition of the essential oil. Phenols (eugenol, carvacrol, thymol) are specifically more antifungal, even though acids (cinnamic and hydrocinnamic acids) also exhibit remarkable fungi-static properties (Saad et al., 2013). Generally, the most active essential oils can be sorted in decreasing order of antimicrobial activities: oregano (carvacrol) > clove (eugenol) > cinnamon (cinnamldehyde) > thyme (thymol) > mustard (allyl isothiociynate) (Shaabana et al., 2012).

As stated above, this work aimed to develop delivery systems using mesoporous scaffolds for the sustained antifungal activity of EOC. Compiling the results from the seven EOC, carvacrol, cinnamaldehyde, thymol, thymoquinone, and eugenol showed in some concentration fungicidal activity inhibiting the fungus growth for 14 days. Meanwhile, diallyl disulfide and allyl isothiocyanate showed only fungistatic activity; they were able to retard the growth for only the first 24 h as had been also demonstrated by Park et al. (2011), where the lethal effect of AITC between 12 h and 24 h were detectable. In the literature, some examples of EOC encapsulation (Kailasapathy, 2009; Prado et al., 2011) demonstrated EOC volatility reduction inside micro-capsules, but in those studies no biological assay were performed. Lai et al. (2006) were formulating a new delivery system for ecological pesticides by the incorporation of *Artemisia arborescens* essential oil into solid lipid nanoparticles. In their study, those nanoparticles were able to reduce the rapid evaporation of essential oil. Chen et al. (2011) developed a new nanophase material loaded with the biological pesticide Pyoluteorin. In this study, a bioactivity experiment showed prolonged antifungal effects compared with pure pesticide, but only for four days. In our work, in almost all the cases, EOC inside the MCM-41 mesoporous showed better effect against *A. niger* than the pure EOC during 14 days, because EOC inside the mesoporous were releasing in a controlled manner. The diffusion of the EOC from the pores of the MCM-41 kept the effective concentration for longer time, diminished by their high volatility in the pure form, which is the reason why the SMPS-EOC that had slow evaporation showed better antifungal effects.

4. Conclusions

Antifungal activity was verified by five out of seven of the tested substances, which were able for more than 14 days to inhibit the growth of *A. niger*. The encapsulated substances showed significantly higher antifungal activity after 14 days than the same substance in a pure state in the majority of the cases tested, which is correlated with the speed of evaporation. Their encapsulation within the MCM-41 may significantly facilitate the use of these compounds in medicine, the food industry, and in agriculture. Natural compounds, such as thymol, thymoquinone, and eugenol encapsulated in MCM-41 may find applications, such as in integrated pest management or organic agriculture, those are currently in high demand. Encapsulation of these substances is able to provide controlled release according to the needs of vegetation and, at the same time, thus contributes to the reduction of environmental pollution.

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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.2015.01.019.

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