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HYBRID NANOSYSTEM FOR STABILIZING ESSENTIAL OILS IN BIOMEDICAL APPLICATIONS

C. SAVIUC[a], A.M. GRUMEZESCU[b*], C.M. CHIFIRIUC[a], D.E. MIHAIESCU[b], R. HRISTU[c], G. STANCIU[c], E. OPREA[d], V. RADULESCU[e], V. LAZAR[a]
[a]*University of Bucharest, Faculty of Biology, Microbiology Department*
[b]*University Politechnica of Bucharest, Faculty of Applied Chemistry and Materials Science, Organic Chemistry Department*
[c]*University Politechnica of Bucharest, Faculty of Applied Science, Center for Microscopy - Microanalysis and Information Processing*
[d]*University of Bucharest, Faculty of Chemistry, Department of Organic Chemistry, Biochemistry and Catalysis, Bucharest, Romania*
[e]*University of Medicine and Pharmacy "Carol Davila", Faculty of Pharmacy, Department of Organic Chemistry, Bucharest, Romania*

The microfungi and especially the opportunistic yeasts have emerged as a major cause of human diseases, particularly among immunocompromised individuals and hospitalized patients. Nowadays, medical devices became an essential component in the supportive care of many patients and the association of microbial biofilm related infections with indwelling devices are of great interest. The treatment of fungal infections has lagged behind antibacterial chemotherapy, reason for which the development of alternative or complementary therapeutic strategies become an emergency. The essential oils are proven to exhibit a strong antimicrobial effect on biofilm grown cells, but because of the unstable compounds existing in the complex matrix of the essential oils, the stabilization processes is an important matter. The aim of the present study was to investigate the utility of a new hybrid nanosystem covering treatment for the stabilization of the essential oils and for the further improvement of their antifungal properties. Nanoparticles obtained by a modified Massart method were used in order to obtain a core/shell/coated-shell for the covering treatment of inert substrata, subsequently used for the *in vitro* study of the fungal biofilm development. The biofilm architecture was assessed by confocal laser scanning microscopy. The results are recommending the nanosystem for the stabilization of *Anethum graveolens* essential oils, which proved a significant antibiofilm activity, highlighting the opportunity of using them for the developing of efficient antimicrobial strategies for microbial biofilms related infections.

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1. Introduction

Yeasts represent at present the fourth leading cause of septicemia in the United States, Europe, and Australia, particularly in intensive care units [1]. The incidence of severe fungal infections has steadily increased during the last five decades with the advent of broad spectrum antibacterial agents and of more aggressive chemotherapies. While medical devices became an essential component in the supportive care of many patients, the association of fungal biofilm related infections with indwelling devices has gain a significant impact in human pathology. The treatment of fungal infections, particularly nosocomial ones, has lagged behind bacterial

* Corresponding author: grumezescu@yahoo.com

chemotherapy and substantially fewer antifungals are available due to their toxic side effects (explained by the great level of resemblances between the fungal and host cells, as eukaryotic cells). Despite the major progress made in the indentification of new possible microbial targets, there are still limited solutions for the development of new classes of antibiotics [2]. In this context, the use of the essential oils for the prevention and treatment of fungal infections has been gaining popularity and an increased number of studies, especially over the past decade. It seems that essential oils have a stronger antimicrobial effect on biofilm grown cells by comparison with other vegetal compounds, due to their better diffusion rates and easy contact with the target structures, facilitated by the saturation of incubation atmosphere with their low density fraction [3, 4, 5].

The *Anethum graveolens* L. (Dill plant) species is cultivated extensively in many countries of Europe, Asia and USA, for its use as an aromatic herb and medical applications [6].

The composition of essential oils obtained from dill fruits was described in numerous papers [6, 7, 8]. The fruits of *Anethum graveolens* have been used in traditional medicine as a therapeutical remedy of some digestive problems [9] and to reduce triacylglyceride levels after oral administration [10]. The terpenoid and phenolic compounds are probably responsible for the antimicrobial activity of dill essential oils against Gram-positive and Gram-negative bacteria and fungi [11-16].

It is well known that essential oils contain unstable compounds which could be transformed in some physico-chemical conditions, as follows: oxygen (oxidizing agent for alcohols and aldehydes), light radiation (transforming unsaturated monoterpenic and sesquiterpenic hydrocarbons) and water (involved in ethers and esters hydrolysis). A large variety of methods can be applied to lower the volatility of essential oils compounds in order to use them for the design of a final product, such as: production of microcapsules and microparticles, melt extrusion, melt injection, complex formation, liposomes, micelles, covalent bonding to a matrix, combination of nanocapsules into larger microcapsules, with the largest application in the field of drug delivery. Lowering volatility is also important for the improvement of the laboratory assay of the biological effects of these compounds. [3, 17]. The revolutionary era of nanotechnology is rapidly unfolding nowadays. Nanotechnology, an umbrella term that describes a rapidly evolving interdisciplinary field of technology based on manipulation of matter at a sub-micron scale, embraces objects, mechanisms, assemblies, and various drug delivery systems based on size scales between 1 nm and 100 nm [18-20].

The objectives of the present study were: i) the extraction and characterization of the essential oil from fruits of *A. graveolens* ii) the improvement of its antimicrobial activity by using a nanosystem for covering treatment in order to stabilize it and iii) study of the stabilized essential oil effect on the fungal adhesion on inert substratum, quantified by confocal laser scaning microscopy (CLSM).

2. Experimental

2.1. Microbial strains. The fungal strains were isolated from different clinical specimens and were identified by Vitek II automatic system, as *Candida albicans* 2026, *Candida tropicalis* 4694, *Candida famata* 198, *Candida glabrata* 1957, *Candida krusei* Y5 and *Saccharomyces cerevisia*e 200. The susceptibility tests to different classes of antifungal drugs as well as to essential oils (extracted from *Anethum graveolens*) were previously performed. All tested strains were susceptible to Fluconazole, Itraconazole, Voriconazole, Flucytosin, Amphotericin B as well as to essential oils obtained from *Anethum graveolens* fruits [4].

2.2. Essential oil extraction. *Anethum graveolens* fruits (50g) were hydro-distilled in a Clevenger-type apparatus for 4 h [21].

GC-MS analysis. *Anethum graveolens* fruits (50g) were hydro-distilled in a Clevenger-type apparatus for 4 h [21]. GC–MS analyses were carried out with a Fisons Instruments GC 8000, equipped with an electron impact quadrupole, MD 800 mass spectrometer detector. The electron ionization energy was 70eV, ion-source temperature 200°C and the interface temperature 280°C. A split–splitless injection (split ratio 1:30) at 280°C was employed. A fused silica capillary column 5% phenyl-poly-dimethyl-siloxane (DB-5MS 30 m x 0.32 mm i.d. and 0.25 mm film

thickness, J&W Scientific) was used. The column temperature was programmed as follows: from 40°C (3 min hold) raised at 4°C/min to 250°C and finally hold at 250°C for 10 min. The carrier gas (helium) flow rate was 2mL/min. The identification of compounds was performed using mass spectra library and the Kovats retention indices compared with reported in the literature [22-24].

2.3. Experimental model for the *in vitro* study of biofilm development on the inert substratum represented by core/shell/coated-shell hybrid nanosystem. The Massart method, adapted for microwave conditions has been used previously for core and core/shell synthesis[25]. High resolution transmission electron microscopy was used as a primary characterization method for Fe_3O_4/oleic acid – core/shell nanostructure [20, 26, 27]. The inert substrata for microbial biofilms development assay were obtained by coating the glass coverslips with suspended core/shell nanoparticles (Fe_3O_4/oleic acid:$CHCl_3$ 0,33%(w/v)). In order to achieve core/shell/coated-shell type samples, the extra-shell ($CHCl_3$ diluted essential oil – 160µL/mL) was applied by adsorption in a secondary covering treatment (fig. 1). The layer of functionalized nanoparticles on coverslips was achieved by applying a magnetic field on nanofluid. Subsequently, the coverslips were soft dried and sterilized by ultraviolet irradiation. The experimental model for yeast adherence and biofilm formation on inert substrata, was carried out in 6 multiwell plates for 48h. Coverslips were placed in plastic wells filled with 3mL liquid medium (Glucose broth). Each well was inoculated with 300µL microbial suspension prepared from 24h cultures grown on solid medium (Sabouraud agar) and adjusted to a density corresponding to 0.5 McFarland standard. After every 24h of incubation time, the culture medium was removed, and cover slips were washed three times in phosphate buffered saline (PBS) in order to remove the non-adherent cells and fresh Glucose broth was added [28-31].

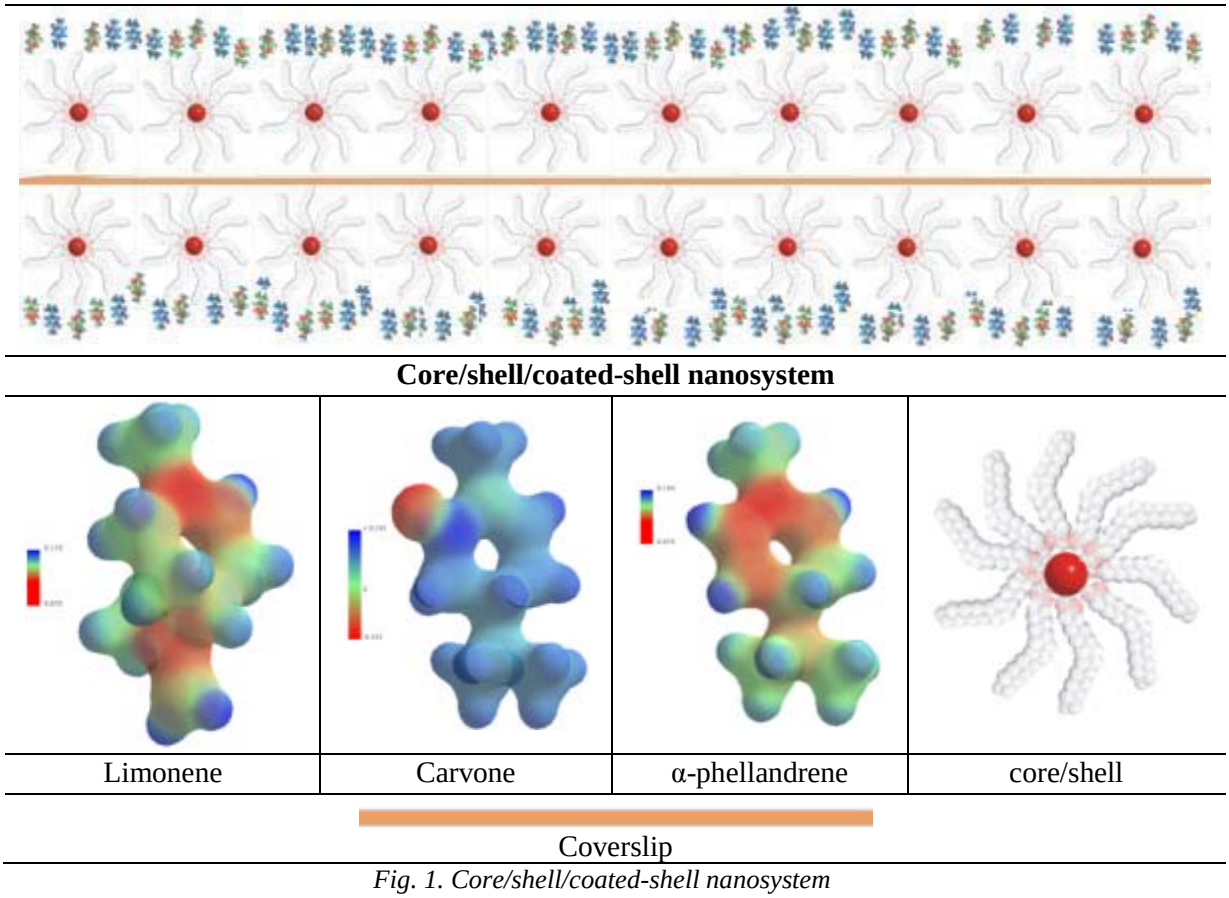

Fig. 1. Core/shell/coated-shell nanosystem

2.4. Direct microscopic examination of microbial adherence and biofilm architecture by CLSM. The samples (coated and uncoated cover slips colonized with fungal biofilms), were removed from plastic wells after 24, 48, 72h of incubation at 25°C, washed three times with PBS,

briefly fixed with cold methanol and dried before microscopic examination. Samples were visualized in reflection mode by using a Leica TCS-SP confocal microscope, equipped with a PL FLUOTAR 40X/0.75 NA objective and a He-Ne laser working on 633nm. A lateral resolution of about 500 nm was achieved. A special Leica software was used for surface topography and statistical analysis.

3. Results

The average yields of essential oil (v/w %, normalized to the part of the plant mass dried weight) extracted from *Anethum graveolen*s fruits were 3.77%. Eleven compounds were identified, having the total area 99.88 % (Table 1). The essential oil proved to be rich in limonene 56.53%, carvone 39.56% and α-phellandrene 1.11%. As for the total area, monoterpenic hydrocarbons accounted for 58.01%, while ethers fraction (dill ether, myristicine and dillapiole) for 0.09%.

Table 1. GC-MS analysis of Anethum graveolens (seeds) essential oil.

No	Compounds	t_R (min)	K.I.	Area (%)
1	α-Tujene	6.11	926	Tr
2	α-Pinene**	6.64	934	0.03
3	β-Myrcene	8.56	990	0.10
4	α-Phellandrene	9.01	1004	1.11
5	*p*-Cymene**	9.64	1024	0.24
6	Limonene**	9.86	1031	56.53
7	Dill Ether	14.81	1184	0.09
8	*cis*-Dihydrocarvone**	15.15	1195	0.09
9	*trans*-Dihydrocarvone**	15.34	1201	2.13
10	Carvone**	16.63	1245	39.56
11	γ-Muurolene	23.07	1477	Tr
TOTAL				99.88

** compounds related with antimicrobial activity
KI - Kovats Index.

The CLSM images revealed the antiadherence effect of the nanosystem coating treatment permiting, as well, the direct examination of uncoated coverslip developed biofilms architecture. The degree of biofilm development on uncoated cover slips was dependent on the tested strain, the ability to form biofilms decreasing in the following order: *C. tropicalis*, *C. albicans*, *C. krusei*, *C. glabrata*, *C. famata* and *S. cerevisiae*. The biofilm development pattern was similar for all tested strains, with maximum architectural complexity in terms of internal canalicular structures and the presence of pseudohyphae at 48h of incubation. The nanosystem coating treatment strongly inhibited the fungal adherence for all tested strains, the adherent cells being virtually absent after 24h of incubation with the coated substrate (fig. 2).

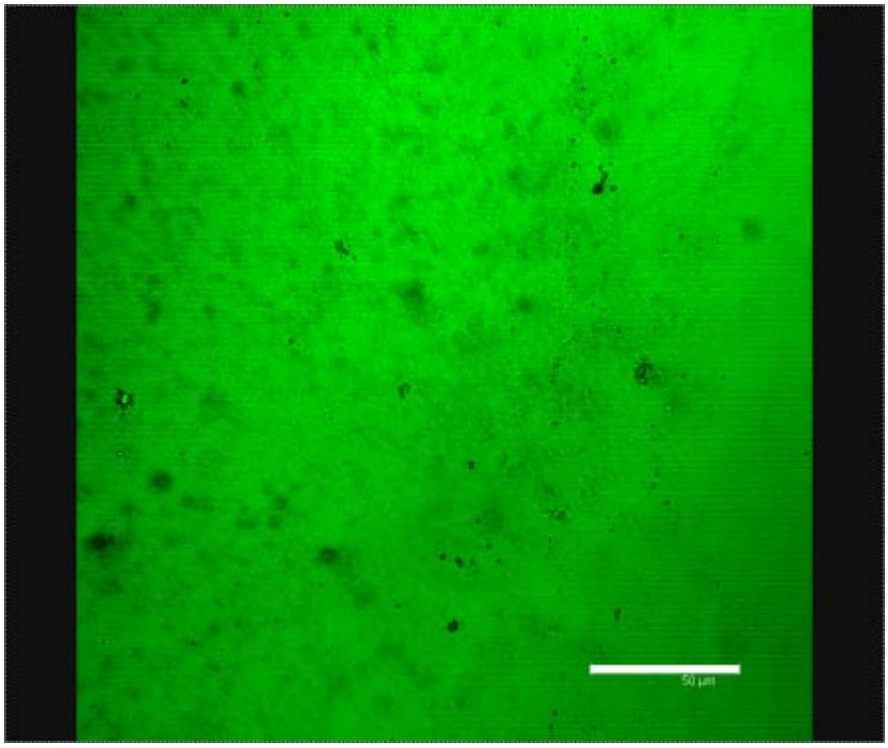

Fig.2.CLSM image of the coated coverslip, colonized with the C. krusei Y5 strain, after 24h of incubation, showing the absence of fungal biofilm.

The dynamic of fungal biofilms development on the glass cover slips and respectively on essential oil based nanosystem revealed that, at 48 h of incubation, the fungal biofilms development on coated coverslips was much more reduced and exhibited a simplified architecture (fig. 3-8). CLSM images of the uncoated coverslips, inoculated with *C. tropicalis* 4694 strain (fig. 3), showed the mature and compact biofilm with pseudohyphae presence and pluristratified zones (a) and rare adherent cells to the coated coverslip (b).

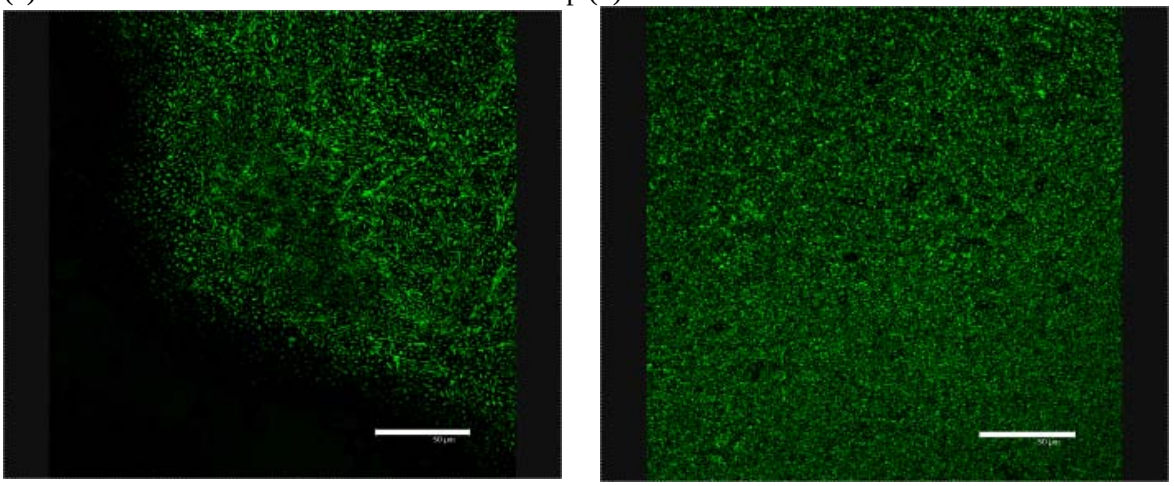

Fig. 3. CLSM images of the uncoated (a) and coated (b) coverslips inoculated with C. tropicalis 4694, after 48h of incubation showing the mature and compact biofilm presence (a) and the significantly reduced adherence profile in case of nanosystem coated substrate (b).

A similar adherence pattern was observed for *C. albicans* 2026, wich instead exhibited a non-homogenously distribution on the glass substrate (fig. 4 (a)).

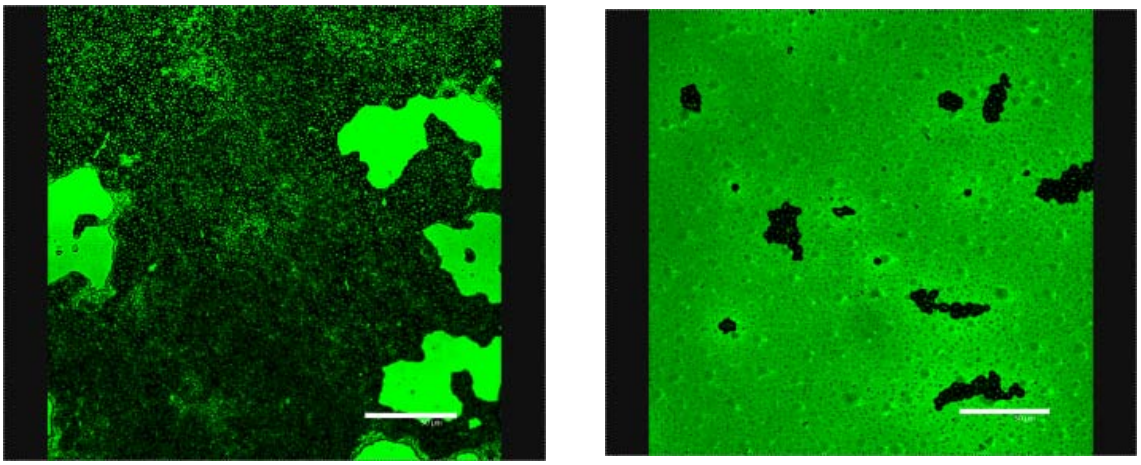

Fig. 4. CLSM images of the C. albicans 2026 inoculated coverlisps after 48h of incubation showing the mature, pluristratified biofilm, non-homogenously distributed on the uncoated coverslip (a) and the rare microcolonies adhered to the coated coverslip (b)

C. krussei Y5 strain developed a thin biofilm, with an internal canalicular structure, on uncoated coupon (fig. 5 (a)) and the rare adherent yeast cells on the coated one (fig. 5 (b)).

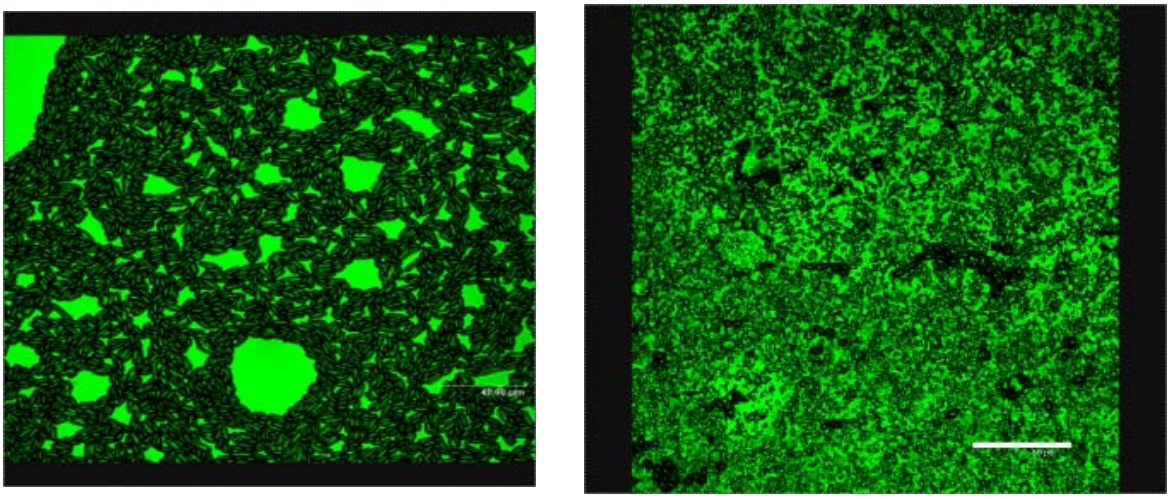

Fig. 5 CLSM images of the C. krusei Y5 inoculated samples after 48h of incubation highlighting the unistratified biofilm formed on uncoated coverslip, with internal canalicular structures of different sizes (a) and rare adherent cells to the coated coverslip (b)

The *C. glabrata* 1957 inoculated coupons showed a monostratified biofilm homogenously distributed on the uncoated coverslip surface (fig. 6 (a)) as well as on the coated one, in wich a central large microbial colony exhibited a midpoint disruption (fig 6 (b)).

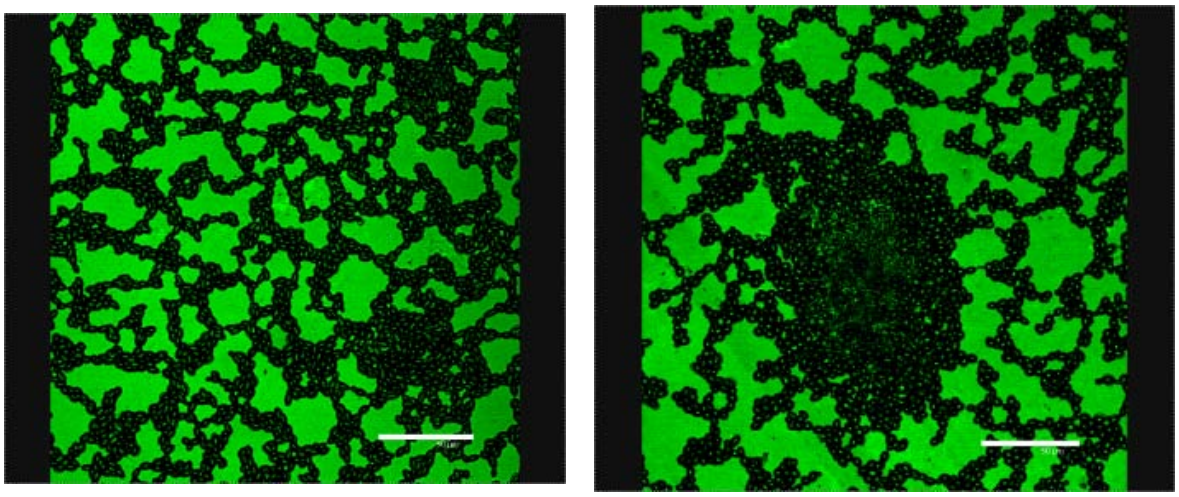

Fig. 6. CLSM images of C. glabrata 1957 inoculated coupons after 48h of incubation emphasize the unistratified biofilm on uncoated (a) as well as on uncoated coverslip (b)

C. famata 198 showed an adherence pattern “in patches” for the macrocolonies formed by adherent fungal cells on uncoated coverslip (fig 7 (a)) and rare isolated yeast cells adhered to nanosystem coated surface (fig. 7 (b)).

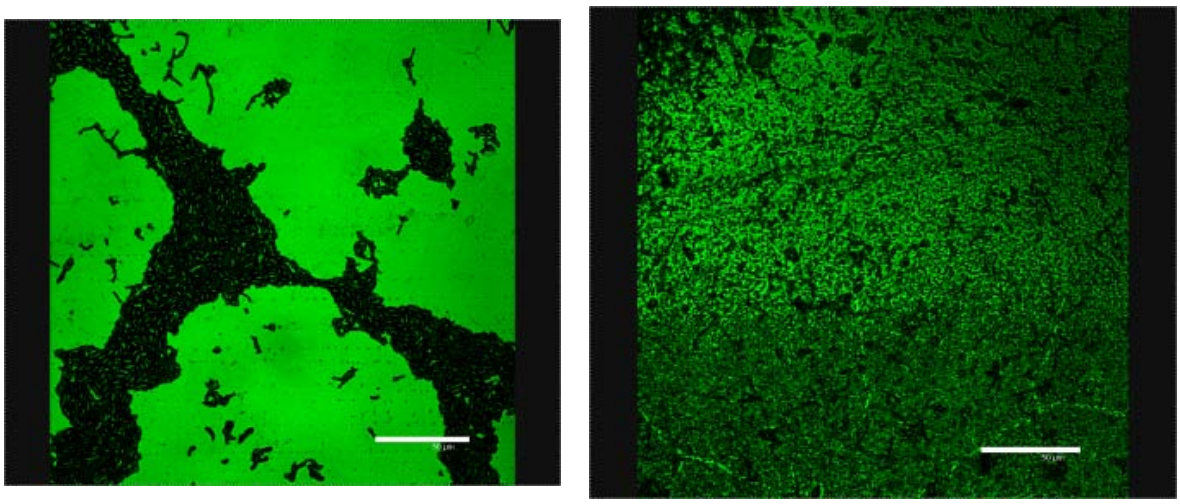

Fig. 7 CLSM image of C. famata 198 inoculated samples after 48 h of incubation showing the adherence pattern “in patches” of uncoated coverslip (a) and the rare cells adhered to the coated coverslip (b)

A well developed, pluristratified, but non-homogenously distributed biofilm was revealed for the *S. cerevisiae* 200 strain inoculated glass coverslip. The nanosystem coated coupons showed instead a less confluent biofilm homogenously distributed on coverslip surface.

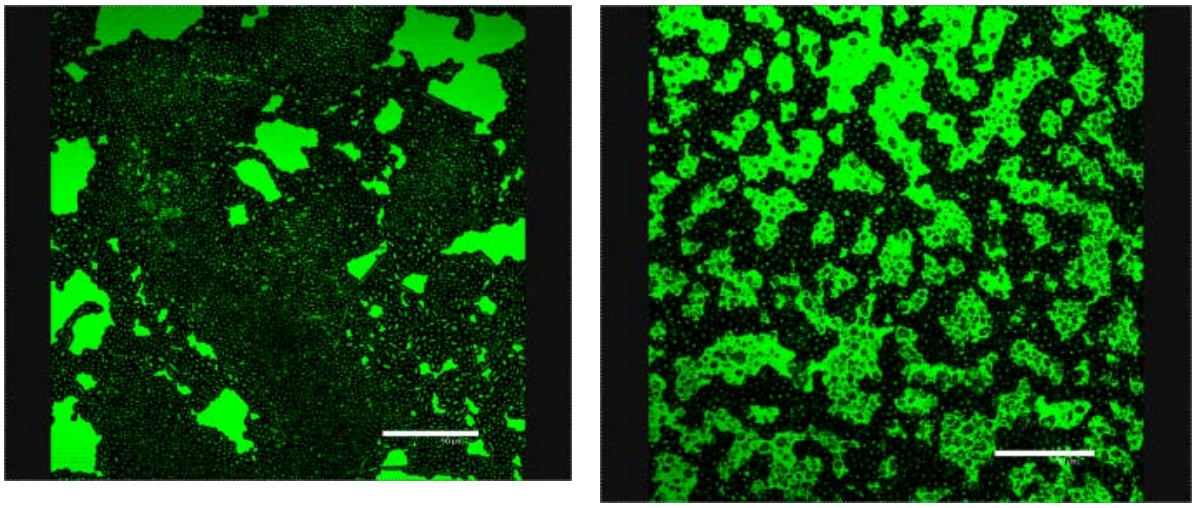

Fig. 8. CLSM image of S. cerevisiae 200 inoculated coverslips, after 48 h of incubation, showing a pluristratified biofilm, homogenously distributed on the glass coverslip (a) and a less confluent biofilm homogenously distributed on the coated coverslip surface (b)

4. Discussions

The analyzed fungal strains have been isolated from biofilms associated human infections, especially related to the respiratory tract. Different species have been chosen, in order to appreciate comparatively the implication of biofilm development capacity in the pathogenicity and virulence of these strains. It is to be noticed that *S. cerevisiae* strain isolated from a pulmonary infection occurred in one immunocompromised patient proved a good ability to form biofilms, this explaining its pathogenic potential [32].

A. graveolens essential oil was previously tested for its anti-pathogenic activity, proving an inhibitory effect on fungal growth, fungal adherence to the plastic substratum and the production of some soluble virulence factors [4]. During the present study, the tested oils proved to have a strong inhibitory effect on the biofilm development ability on the inert substratum, probably due to his balanced composition in terpenes and terpenoides. Moreover, hybrid nanosystem based essential oils proved to be a promising solution for obtaining modified materials for biomedical applications due to the stabilizing interaction between essential oil compounds and nanoparticles support especially by lowering the volatility [33]. The main components identified in the essential oils obtained from *Anethum graveolens* were similar with previous studies of Faber (1997) and Ortan (2008). The antimicrobial activity of some compounds was already cited in the literature: p-cymene, limonene, cis-dihydrocarvone, trans-dihydrocarvone, carvone [34-38]. The development of new strategies for controlling biofilms related microbial infections is strongly dependent on the development of new examination tools based on noninvasive microscopic techniques. In this context, CLSM proved to be an efficient tool, concerning the qualitative appreciation of biofilm architecture complexity, the coverage degree on the substratum surface and the disruption of biofilm in the presence of inhibitory substances. The outer yeast wall components are involved in the adherence process, with an important role in the physical interaction of the pathogenic microbial cells and the host organism and respectively, the inanimate surfaces. According with previous studies, the *A. graveolens* essential oil exhibited antimicrobial effect proven by the killing time curve assay, demonstrating the specific interaction between the microbial cells wall and the plant essential oil chemical constituents. In the present study the tested essential oil adsorbed on the substratum completely abolished the fungal adherence after 24h of incubation and after 48h the fungal adherence was much more reduced as compared with the untreated substratum.

5. Conclusions

The obtained results recommend the nanosystems for the stabilization of *Anethum graveolens* and other essential oils, that proved a more efficient antibiofilm activity, highlighting the opportunity of using them for the developing of efficient antimicrobial strategies for fighting microbial biofilms related infections.

Aknowledgements

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