

PROTECTIVE EFFECT OF MAGNETITE NANOPARTICLE/SALVIA OFFICINALIS ESSENTIAL OIL HYBRID NANOBIOSYSTEM AGAINST FUNGAL COLONIZATION ON THE PROVOX® VOICE SECTION PROSTHESIS

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This study is aimed to evaluate a novel nanobiosystem based on magnetic nanofluid and *Salvia officinalis* essential oil for coating the Provox voice section prostheses surfaces with desired antibiofilm properties. The essential oil microwave assisted extraction was performed in a Neo-Clevenger type apparatus and its chemical composition was settled by GC-MS analysis. Fe₃O₄/C₁₈ solubilized in chloroform and oriented in magnetic field was used for coating of the catheter sections of 1 cm. The extra-shell (essential oil) was applied by adsorption in a secondary covering treatment. The fungal biofilm architecture was assessed by confocal laser scanning microscopy (CLSM). The new Fe₃O₄/C₁₈/essential oil nanobiosystem exhibited a fungicidal effect and inhibited the fungal adherence, representing thus, an interesting option for the medical field, opening new directions for the design of modified surfaces with desired and controlled antibiofilm properties.

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1. Introduction

Patients who underwent laryngectomy due to larynx cancer have a possibility to regain ability to speak with the use of fluoroplastic voice prosthesis [1] (figure 1). However, the lifetime of the device is limited, and last for approximately 3–6 months, mainly due to bacterial and fungal biofilm formation, that subsequently causes deterioration of the prosthesis and malfunction of the valve mechanisms. Moreover, the biofilm can be reservoir for chronic and systemic infections [2].

Magnetite nanoparticles have attracted researchers in various fields such as medicine [3], biology [4] and materials science [5-8] due to their multifunctional properties [9], excellent biocompatibility and magnetic properties [10-12], magnetic nanoparticles are the focus of rapidly growing research efforts in controlled drug release [13,14], drug targeting [15], inhibition of microbial biofilm growth [16], resonance magnetic imaging [17,18], bone cancer treatment [3] or antimicrobial therapy [19].

Biofilms are heterogeneous communities of microorganisms entrapped in an extracellular matrix, which limits the penetration of antimicrobial drugs and antibodies [20-22]. Biofilms are characteristically composed of genetically and phenotypically diverse microbial populations [23-26] inhabiting surfaces of tissues, catheters or surgically implanted prosthetic devices: although

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intensively investigated the clinical relevance of fungal biofilms is often uncertain [27]. *Candida albicans* biofilms consist of a dense network of yeasts, hyphae and pseudohyphae encased in a self-produced matrix of extracellular polymeric material that shows extensive spatial heterogeneity and contributes to invasiveness [28,29]. The tendency of *C. albicans* to develop biofilms is clinically relevant because biofilm-associated fungal cells are much more resistant to the traditional antifungal agents that act against their planktonic counterparts and also resist host immune factors [30,31].

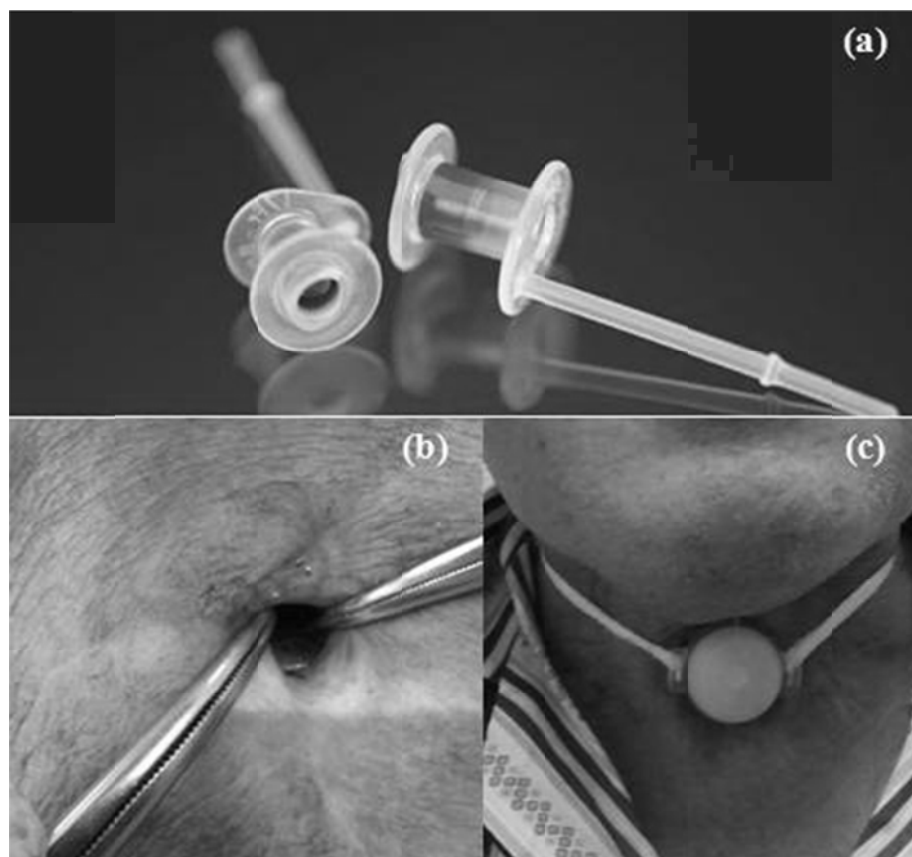


Fig 1. (a) The Provox™ voice prosthesis; (b) introduction of Provox™ voice prosthesis into tracheoesophageal fistula; (c) patient postoperative with Provox Heat and Moisture Exchanger (HME).

It has been observed that essential oils can influence microbial biofilm production [32,33]. *Salvia*, the largest genus of the *Lamiaceae* family, includes about 900 species, spread throughout the world, some of them being economically important since they are used as spices and flavouring agents in perfumery and cosmetics [34,35]. Sage (*Salvia officinalis* L.) is a perennial woody subshrub native to the Mediterranean region that is now extensively cultivated all over the world mainly to obtain dried leaves to be used as raw material in medicine or food industry [36,37]. Volatile oils are complex mixtures of chemical compounds, mainly monoterpenes, sesquiterpenes, and their oxygenated derivatives (alcohols, aldehydes, esters, ethers, ketones, phenols and oxides). The GC-MS analysis of the essential oil composition of several *Salvia* species indicates the 1,8-cineole (eucalyptol) and borneol as its main constituents [38]. The antimicrobial activity of *S. officinalis* was recognized decades ago and was attributed to the presence of 1,8-cineole, thujone and camphor [39]. Several strategies have been proposed to inhibit biofilm formation on medical devices, including the administration of subinhibitory concentrations of some antibiotics [40], use of anti-inflammatory drugs [41], development of new anti-adhesive medical surfaces [42] and coating medical devices with nanoparticles [43,44] or natural products [29]. The use of essential oils as functional antibiofilm agents is increasing. Also, our previous studies proved [29, 30] that core/shell magnetic nanoparticles strongly inhibit the biofilm development onto inert surface.

In this context, this study is focused to evaluate a novel nanobiosystem based on magnetic nanofluid and *S. officinalis* essential oil for coating prosthetic device surfaces with antibiofilm properties.

2. Materials and Methods

Essential oil: extraction and characterization

The essential oil microwave assisted extraction was performed in a Neo-Clevenger type apparatus and its chemical composition was settled by GC-MS analysis. GC-MS analyses were carried out on a gas chromatograph Agilent 6890 Series GC System coupled to a 5973 mass-selective single quadrupole detector (Agilent technologies). A DB-5MS capillary column (30 m x 0.25 mm, 0.25 mm film thickness) was used. The column temperature was programmed to rise from 60 °C to 280 °C at a rate of 5 °C/min. The carrier gas was H₂ with a flow rate of 1.2 ml/min, split ratio 60:1. Operation control and the data process were carried out by Agilent Technologies ChemStation software (Santa Clara, CA, USA).

Hybrid nanobiosystem assembling procedure

Functionalized magnetite is usually prepared by wet chemical precipitation [45, 46]. Magnetic nanoparticles, Fe₃O₄/C₁₈ were prepared according to our previous studies [47] and solubilized in chloroform, oriented in magnetic field and the catheter sections of 1 cm were submerged in nanofluid and extemporaneously dried because of convenient volatility of chloroform (Fe₃O₄/oleic acid:CHCl₃ 0,33%(w/v). In order to achieve core/shell/coated-shell type samples, the extra-shell (CHCl₃ diluted essential oil – 160µL/mL) was applied by adsorption in a secondary covering treatment.

Fungal model

The artificial monospecific biofilms were developed by using *Candida albicans* 110 strain, recently isolated from wound infection and identified by using Vitek II automatic system and previously tested for susceptibility to currently used antifungals (voriconazole, itraconazole, caspofungin, amphotericin B, fluconazole, flucytosin) and some essential oils.

Biofilm development to the Provox voice section prosthesis

The microbial adherence ability was investigated in 6 multiwell plates, in which there have been placed (un)coated prosthetic pieces of 1 cm. Plastic wells were filled with liquid medium, inoculated with 300µL 0.5 McFarland microbial suspension and incubated for 72 hours at 30°C. After each 24 hours the culture medium was removed, the catheters were washed three times in phosphate buffered saline (PBS) in order to remove the non-adherent strains and fresh Glucose broth (GB) was added. Also viable cells counts (VCCs) have been achieved for both working variants (coated and uncoated catheter pieces) at each 24h, in order to assess the biofilm forming ability of the tested strain. In this purpose, the fungal cells were removed from the catheter sections (by vortexing and brief sonication) and the obtained suspension was diluted in PBS, the ten fold serial dilutions being spotted in triplicates on GB and incubated at 37°C for 24 hours. After incubation, the VCCs were performed choosing the optimal dilution that allowed the development of an optimal number of microbial colonies.

Biofilm characterization

For the microbial biofilm architecture assessment, a confocal laser scanning microscopy (CLSM) was used. After 48 and 72h of incubation the samples were removed from the plastic wells, washed three times with PBS, fixed with cold methanol and dried before microscopic examination. Samples were visualized in transmission mode by using a Leica microscope (TCS-SP CSLM model), equipped with PL FLUOTAR (40X NA0.7, electronic zoom 1) and an He-Ne laser tuned on 633 nm wavelength. A lateral resolution of about 600 nm was achieved. The Leica software was used for examining the surface topography.

3. Results and Discussion

Reports on the essential oil composition of this species have been published by several authors [48,49]. Apart from some chemical variations, cis- and trans-thujones are usually the main compounds of *S. officinalis* essential oils [50]. The main constituents identified in the essential oils of *S. officinalis* used in our experiments were cis-thujone and eucalyptol (Table 1), followed by substantial amounts of α,β -pinene and camphene. For the main constituents identified in the essential oil of *S. officinalis*, the obtained concentrations were similar to that obtained by other authors [51,52].

Table 1. Chemical composition of *S. officinalis* essential oil

Peak	r.t.	%	compounds	peak	r.t.	%	compounds
1	4.546	0.445	Tricyclene	17	12.55	0.063	Myrtenol
2	4.882	6.49	α -Pinene	18	13.244	0.038	Carvyl Acetate
3	5.262	7.338	Camphene	19	15.013	0.132	Dihydrocarveol acetate
4	6.035	4.993	β -Pinene	20	15.114	1.701	Bornyl acetate
5	7.468	1.068	m-Cymene	21	15.349	2.068	Myrtenyl acetate
6	7.68	24.706	Eucalyptol	22	16.86	2.446	α -Terpineol acetate
7	8.24	0.06	3-Carene	23	17.487	0.039	Copaene
8	8.486	0.889	τ -Terpinen	24	17.532	0.047	Isobornyl acetate
9	9.36	0.22	α - Terpinolen	25	17.823	0.091	Geraniol
10	9.819	3.089	Linalool acetate	26	18.607	1.945	β -Caryophyllene
11	11.072	29.888	cis-Thujone	27	19.458	0.293	α -Caryophyllene
12	11.364	0.869	Trans-Thujone	28	20.118	0.187	Geranyl propionate
13	11.655	3.847	Camphor	29	21.238	0.082	δ -Cadinene
14	11.99	0.264	4-Terpineol	30	22.48	0.043	Spathulenol
15	12.248	0.045	p-Cymen-8-ol	31	22.603	0.082	Caryophyllene oxide
16	12.394	0.23	α -Terpineol	32	22.805	0.037	β -Guaiene

r.t.= retention time; % = relative content;

The increasing use of indwelling medical devices in conjunction with an ageing/increasingly immunocompromised population has resulted in a surge of hospital acquired *Candida* spp. infections, *C. albicans* ranking high among nosocomial pathogens. *Candida* infections are frequently associated with the formation of biofilms on implantable medical devices [53]. These devices readily support biofilm formation and are responsible for a considerable percentage of clinical candidiasis cases. Several experimental parameters such as the nature of the surface material [54,55] the growth medium [56] and conditions of incubation [57] influence *C. albicans* biofilm formation and structure.

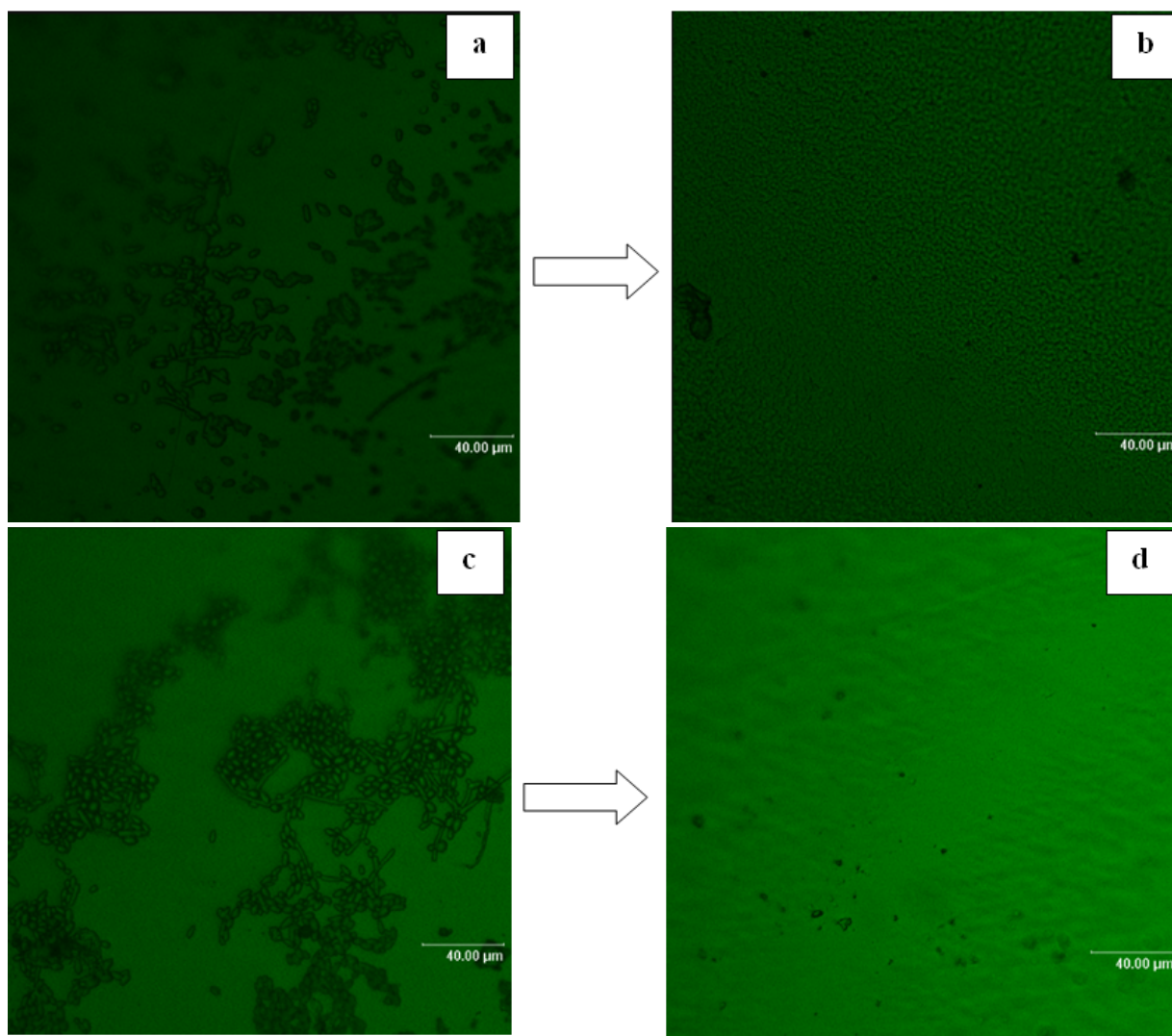


Fig 2. CLSM images of biofilms formed on the Provox voice section prosthesis (coated vs uncoated prosthetic device); a. adherent *C. albicans* 110 biofilm development on the surface of prosthetic device in the absence of the tested nanobiosystem at 48 h; b. the absence of *C. albicans* 110 yeast cells adhered to the surface of prosthetic device coated with the tested nanobiosystem at 48h; c. adherent *C. albicans* 110 biofilm development on the surface of prosthetic device in the absence of the tested nanobiosystem at 72 h; d. the absence of *C. albicans* 110 yeast cells adhered to the surface of prosthetic device with the tested nanobiosystem at 72h

The CLSM examination of the catheter sections colonized with the tested *C. albicans* strains showed that the tested nanobiosystem exhibited an intensive antibiofilm effect, as demonstrated by the absence of yeast cells adhered to the coated surface. In exchange, in the case of control catheter sections with unchanged surface, the fungal cells adhered and formed aggregates, of bigger size at 72 hours, when the morphological conversion from budding yeast to a filamentous form was also more evident (Fig. 2).

The VCCs showed a decrease in the number of colony forming units (CFU) only in case of 48 hours biofilms, demonstrating that the essential oil exhibited an antifungal effect on the tested strain, reducing its multiplication rate and consequently the number of adhered cells. Conversely, at 72 hours the CFU values were quite similar with those of the control, probably due to the “consumption” of the essential oil adsorbed in the nanosystem, the increase in the multiplication rate followed by the fungal adherence to the coated surface (Fig. 3).

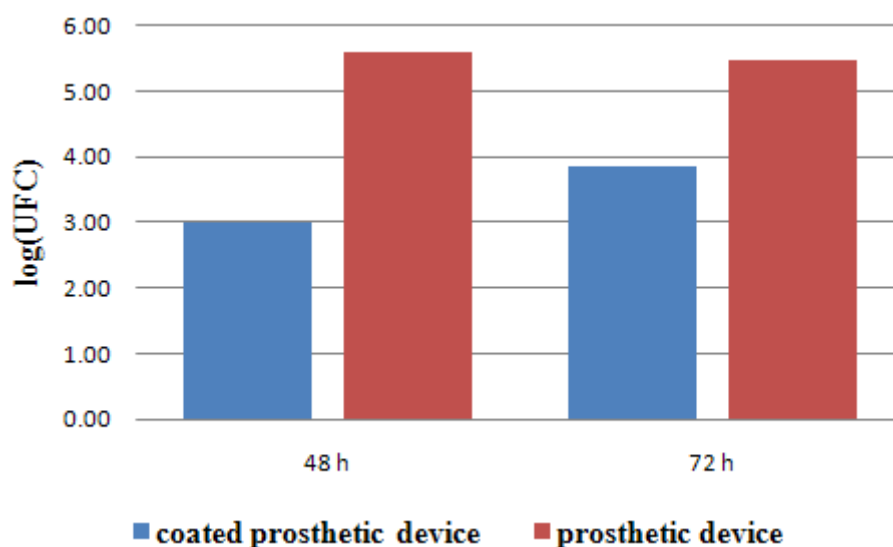


Fig 3. The viable cell counts of fungal cells adhered and embedded in biofilms formed on the Provox voice section prosthesis (coated vs uncoated prosthetic device)

4. Conclusions

The new $\text{Fe}_3\text{O}_4/\text{C}_{18}$ /essential oil nanobiosystem exhibited a fungicidal effect and inhibited the fungal adherence, representing thus, an interesting option for the medical field, opening new directions for the design of modified surfaces with desired and controlled anti-biofilm properties.

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References

- [1] I. Anghel, A.G.Anghel, C.Stefan, R.Matei, *Practica Medicala*, **6**,245 (2011).
- [2] M. Nowak, P. Kurnatowski, *Otolaryngologia Polska*, **64**, 358 (2010).
- [3] E. Andronescu, M. Ficai, G. Voicu, D. Manzu, A. Ficai, *J. Mat. Sci.-Mater. Med.*, **21** , 2237 (2010).
- [4]Tian Xiao-Zhou, Wang Jian-Fang, Qian Si-Wen, Wu Wen-Jian, Gang Xu, *Adv. Nat. Sci.*, **3**, 299 (2010).
- [5] D. Manzu, A. Ficai, G. Voicu B. S. Vasile, C. Guran, E. Andronescu, *Mater.Plast.*, **47**, 24 (2010).
- [6] D. Ficai, A. Ficai, B. S.Vasile, M. Ficai, O. Oprea, C. Guran, E. Andronescu, *Digest J. Nanomat. Biostr.*, **6**, 943 (2011).
- [7] D. Ficai, A. Ficai, M. Alexie, M. Maganu, C. Guran, E. Andronescu, *Rev. Chim.*, **62**, 622 (2011).
- [8] A. M. Grumezescu, E. Andronescu, A. Ficai, C. Saviuc, D. Mihaiescu, M.C. Chifiriuc, *Rom. J. Mat.*, **41**, 383 (2011).

- [9] Gui-Yin Li, Yu-ren Jiang, Ke-long Huang, Ping Ding, Jie Chenb, J. All. Comp., **466**, 451 (2008).
- [10] S.F. Medeiros, A.M. Santos, H. Fessi, A. Elaissari, Int. J.Pharm., **403**, 139 (2011).
- [11] I.H. Labouta, M. Schneider, Int. J.Pharm., **395**, 236 (2010).
- [12] A.M. Grumezescu, E. Andronescu, A. Fikai, D.E. Mihaiescu, B.S. Vasile, C. Bleotu, *Lett. Appl. NanoBioSci.*, **1**, 31 (2012).
- [13] H. Wang, S. Wang, Z. Liao, P. Zhao, W. Su, R. Niu, J. Chang, Int. J.Pharm., **430**, 343 (2011).
- [14] D.E. Mihaiescu, M. Horja, I. Gheorghe, A. Fikai, A.M. Grumezescu, C. Bleotu, M.C. Chifiriuc, *Lett. Appl.NanoBioSci.*, **1**, 45 (2012).
- [15] Y. Zhang, N. Kohler, M. Zhang, *Biomaterials*, **23**, 1553 (2002).
- [16] A.M. Grumezescu, C. Saviuc, A. Holban, R. Hristu, C. Croitoru, G. Stanciu, C. Chifiriuc, D. Mihaiescu, P. Balaure, V. Lazar, *Biointerface Res. Appl. Chem.*, **1**, 160 (2011).
- [17] M.D. Mantle, Int. J.Pharm., **417**, 173 (2011).
- [18] C. Schweiger, C. Pietzonka, J. Heverhagen, T. Kissel, Int. J.Pharm., **408**, 130 (2011).
- [19] G. Tataru, M. Popa, J. Desbrieres, Int. J.Pharm., **404**, 83 (2011).
- [20] C.J. Seneviratne, L. Jin, L.P. Samaranyake, *Oral Dis* **14**, 582 (2008).
- [21] C. Saviuc, A.M. Grumezescu, M. C. Chifiriuc, C. Bleotu, G. Stanciu, R. Hristu, D. Mihaiescu, V. Lazăr, *Biointerface Res. Appl. Chem.*, **1**, 31 (2011).
- [22] V.M. Bubulica, I. Anghel, A.M. Grumezescu, C. Saviuc, G.A. Anghel, M.C. Chifiriuc, I. Gheorghe, V. Lazar, A. Popescu, *Farmacia.*, **60**, 80 (2012)
- [23] E. Panus, M. C. Chifiriuc, O. Banu, M. Mitache, C. Bleotu, N. Rosoiu, V. Lazăr, *Biointerface Res. Appl. Chem.*, **1**, 24 (2011).
- [24] O. Banu, C. Bleotu, M. C. Chifiriuc, B. Savu, G. Stanciu, C. Antal, M. Alexandrescu, V. Lazăr *Biointerface Res. Appl. Chem.*, **1**, 72 (2011).
- [25] I. Anghel, M.C. Chifiriuc, G.A. Anghel, *Farmacia*, **6**, 770 (2011).
- [26] I. Anghel, M.C. Chifiriuc, M. Mitache, L.Marutescu, G.A. Anghel, P. Popa, P. Pelinescu, C. Bleotu, V. Lazar, *Farmacia*. **60**, 21 (2012).
- [27] A. Khodavandi, N.S. Harmal, F. Alizadeh, O. J. Scully, S. M. Sidik, F. Othman, Z. Sekawi, Kee P. Ng, P. P. Chong, *Phytomedicine* **19**, 56 (2011).
- [28] P. C. Braga, M. Culici, M. Alfieri, M. D. Sasso, *International Journal of Antimicrobial Agents* **31**, 472 (2008).
- [29] C. Saviuc, A. M. Grumezescu, E. Oprea, V. Radulescu, L. Dascalu, M. C. Chifiriuc, M. Bucur, O. Banu, V. Lazar, *Biointerface Res. Appl. Chem.*, **1**, 15 (2011).
- [30] A. M. Holban, V. Lazăr, *Biointerface Res. Appl. Chem.*, **1**, 95 (2011).
- [31] S.P. Hawser, L.J. Douglas, *Antimicrob Agents Chemother*, **39**, 2128, (1995).
- [32] C. Saviuc, A. M. Grumezescu, A. Holban, C. Bleotu, C. Chifiriuc, P. Balaure, V. Lazar, *Biointerface Res. Appl. Chem.*, **1**, 111 (2011).
- [33] G. Ramage, S.P. Saville, B.L. Wickes, J.L. Lopez-Ribot, *Appl Environ Microbiol* **68**, 5459 (2004).
- [34] E. A. Hayouni, I. Chraief, M. Abedrabba, M. Bouix, J.Y. Leveau, H. Mohammed, M. Hamdi, *International Journal of Food Microbiology* **125**, 242 (2008).
- [35] A. P. Longaray Delamare, I. T. Moschen-Pistorello, L. Artico, L. Atti-Serafini, S. Echeverrigaray, *Food Chemistry* **100**, 603 (2007).
- [36] J. Bruneton, *Pharmacognosy, Phytochemistry Medicinal Plants*. Lavoisier Intercept, London, UK (1999).
- [37] I. Bettaieb, N. Zakhama, W. Aidi Wannes, M.E. Kchouk, B. Marzouk, *Scientia Horticulturae* **120**, 271 (2009).
- [38] M. Z. Haznedaroglu, N. U. Karabay, U. Zeybek, *Fitoterapia*, **72**, 829 (2001).
- [39] V. Jalsenjak, S. Peljnjak, D. Kustrak, *Pharmazie*, **42**, 419 (1987).
- [40] P. Furneri, A. Garozzo, M. Musumarra, A. Scuderi, A. Russo, G. Bon-figlio, *Int. J. Antimicrob. Agents* **22** 164 (2003).
- [41] C. Arciola, L. Montanaro, R. Caramazza, V. Sassoli, D. Cavedagna, *J. Biomed. Mater. Res.* **42**, 1 (1998).
- [42] N. Cerca, S. Martins, G. B. Pier, R. Oliveira, J. Azeredo, *Research in Microbiology* **156**, 650 (2005).

- [43] C. Saviuc, A.M. Grumezescu, C.M. Chifiriuc, D.E. Mihaiescu, R. Hristu, G. Stanciu, E. Oprea, V. Radulescu, V. Lazar, *Digest J. Nanomat. Biostr.*, **6**, 1657 (2011).
- [44] A. M. Grumezescu, C. Saviuc, M. C. Chifiriuc, R. Hristu, D. E. Mihaiescu, P. Balaure, G. Stanciu, V. Lazar, *T. NanoBioSci.*, **10**, 269, (2011).
- [45] E. Andronescu, A.M. Grumezescu, A. Fikai, I. Gheorghe, C. Chifiriuc, D.E. Mihaiescu, V. Lazar, *Biointerface Res. Appl. Chem.*, **2**(3), 332. (2012).
- [46] A.M. Grumezescu, E. Andronescu, A. Fikai, B.S. Vasile, G. Voicu, D.E. Mihaiescu, C. Bleotu, *Lett. Appl. NanoBioSci.*, **1**, 56 (2012)
- [47] A.S. Buteică, D.E. Mihaiescu, A.M. Grumezescu, B.Ş. Vasile, A. Popescu, O.M. Mihaiescu, R. Cristescu, *Digest J. Nanomat. Biostruc.*, **5**, 927 (2010).
- [48] V. Radulescu, S. Chiliment, E. Oprea, *J. Chromatogr. A* **1027**, 121 (2004).
- [49] D.T. Velickovic, M.S. Ristic, N.V. Randjelovic, A. Smelcerovic, *J. Essent. Oil Res.* **14**, 453 (2002).
- [50] E. Pinto, L. R. Salgueiro, C. Cavaleiro, A. Palmeira, M. J. Goncalves., *Ind. Crops Prod.*, **26**, 135 (2007).
- [51] M. Marino, C. Bersani, G. Comi, *International J. Food Micro.*, **67**, 187 (2001).
- [52] E. Putievsky, D. Ravid, N. Diwan-Rinzler, D. Zohary, *Flav. Frag. J.*, **5**, 121 (1990).
- [53] C.A. Kumamoto, *Curr. Opin. Microbiol.* **5**, 608 (2002).
- [54] S.P. Hawser, L.J. Douglas, *Infect. Immun.* **62**, 915 (1994).
- [55] J Chandra, JD Patel, J Li, et al, *Appl. Environ. Microbiol.* **71**, 8795 (2005).
- [56] B.P. Krom, J.B. Cohen, G.E. McElhaney Feser, R.L. Cihlar, *J. Microbiol. Meth.* **68**, 421 (2007).
- [57] A.M. Gallardo-Moreno, M.L. Gonzalez-Martin, C. Perez-Giraldo, J.M. Bruque, A.C. Gomez-Garcia, *J. Colloid. Interf. Sci.*, **271**, 351 (2004).