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TESTING ANTIFUNGAL ACTIVITY OF SOME ESSENTIAL OILS USING FLOW CYTOMETRY

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Abstract

The use of natural antifungal compounds has become a viable alternative for fighting fungal infections since high rates of resistance to synthetic antifungal compounds has emerged. Classical techniques aimed to routinely investigate fungal susceptibility are often limited when using natural essential oils, because of their instability and great volatility that may lead to false results. In this study, we report the results obtained by classical antimicrobial susceptibility testing techniques and flow cytometry regarding the effect of some volatile oils on different Candida clinical isolates. The obtained results revealed that flow cytometry is a very useful and precise technique in investigating the influence of essential oils on the fungal cells, surpassing the disadvantage of their volatility and thus reducing false results often obtained by using the classical methods.

Keywords *Flow cytometry, antifungal activity, candida spp., essential oils.*

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Introduction

One of the most promising antifungal strategies is based on the identification of vulnerable virulence pathways that can be targeted with novel antifungal therapies [1,2]. An important and modern trend in contemporaneous microbiology world is the identification and use of natural antimicrobial compounds that have the advantage of being less detrimental for the host and possibly less susceptible for development of microbial resistance. Few studies are available regarding the application of some alternative and traditional medicine on treatment and prevention of *Candida* infections [3,4]. The use of plant extract or plant-derived chemicals to treat diseases by topical, subcutaneous and systemic application has stood the test of time. In recent years, there has been a gradual revival of interest [5-9] in the use of medicinal plants in developing countries because herbal medicines have been reported safe and without any adverse side effect especially when compared with synthetic drugs. Also, there is little or no report of any form of microbial resistance emerged during the use and administration of herbal medicines [10]. Our previous studies revealed that some essential oil extracts may have significant antimicrobial effect, affecting key virulence mechanisms as adherence and biofilm formation [6-9]. We could also demonstrate that essential oils may be successfully coupled with some nanostructured materials, which could be further developed as trading vectors for natural compounds for an enhanced efficiency [11-13]. Since many plant extracts have usually reported to exhibit a high antimicrobial activity, even when used in very low

amounts, they are the perfect contenders in developing new antimicrobial strategies and therapies. One of major limitation of the classical techniques that involve volatile vegetal compounds for assessing antimicrobial activity is precisely their high volatility and instability, aspects that may conduct to false results. The purpose of this study was to characterize and compare some recent different *Candida* clinical isolates, and to investigate the antifungal effect of some freshly extracted essential oils by using the flow cytometry technique in order to minimize the volatility due effect.

Experiment Details

Strains and growth conditions. 15 fungal strains belonging to *Candida albicans* (*C. albicans*) species were isolated from different clinical specimens (urinary tract isolates, wound secretions, upper airway secretions, and blood cultures) and identified using Vitek II automatic system. Clinical samples were obtained from patients hospitalized in the National Institute for Cardiovascular Diseases, Prof. C.C. Iliescu of Bucharest, during October 2011-March 2012. The identified *Candida* strains and their clinical origin are indicated in table 1. After identification the fungal strains were preserved as permanent stocks in the microbial collection of the Microbiology Laboratory of the Faculty of Biology, University of Bucharest. For further experiments *Candida* strains were plated on Sabouraud agar and inoculated in YPG broth.

Table 1: *Isolation sources of the tested strains*

Strain	Isolation sources
C.albicans 903	wound secretions
C. albicans 110	wound secretions
C. albicans 3	blood culture
C. albicans 774	tracheo- bronchial secretions
C. albicans 279	tracheo- bronchial secretions
C. albicans 983	uroculture
C.albicans 948	tracheo- bronchial secretions
C.albicans 103	tracheo- bronchial secretions
C. alibcans 1137	uroculture
C. albicans 772	tracheo- bronchial secretions
C. albicans 771	wound secretions
C.albicans 980	uroculture
C. albicans 648	tracheo- bronchial secretions
C. albicans 918	tracheo- bronchial secretions
C.albicans 957	wound secretions

Qualitative screening of the antimicrobial activity. The qualitative screening of the susceptibility spectra of different Candida strains to the essential oils was performed by the kill-time curve, i.e. the microbial strains were kept in contact with the essential oil for 1', 3', 5,' 15' and 30', viable cell counts being thereafter performed in order to appreciate the fungistatic or fungicidal effect.

Essential oils extraction. *Anethum graveolens*, *Rosmarinus officinalis*, *Mentha piperita*, dried plant material, were hydro-distilled in a Clevenger-type apparatus according to our recently published papers [14,15]. Standardized eucalyptol was purchased from Sigma-Aldrich.

GC-MS analysis. The obtained essential oils were subsequently dried on Na_2SO_4 anhidrous and stored in dark botles at 4°C untill further analysis. For GC-MS phytochemical assessment the essential oils were diluted 1:1000 with CH_2Cl_2 and injected. Gas chromatographic analysis was performed using an Agilent 6890 Series GC System. Detection was carried out with a 5973 mass-selective single quadrupole detector (Agilent technologies). Operation control and data process were carried out by Agilent Technologies ChemStation software (Santa Clara, CA, USA). The mass spectrometer was calibrated before use with perfluorotributylamine as a calibration standard. The working conditions were: H2-carrier gas, flow: 1.2 ml/min, temperature program 50/300^{o}C with a ramp rate of 5^{o}C/min; the temperature of the injector and of the detector was 250^{o}C, and a DB5-MS (30m; 0.25 mm id; 0.25 µm) column.

Flow cytometry assay. Twofold macro-dilution in YPG medium was obtained using a stock solution of essential oil in DMSO at ratio 1:1, concentrations ranging from 3.125 to 50µL/mL. Two different incubation times 15min and 24h were used for each sample. The live/dead staining was performed using propidium iodide (5µg/mL) and the samples were analyzed using a FACSCalibur(BD) flow cytometer. All the steps were carried out at room temperature, staining being achieved 20min before data acquisition. CellQuest™ Pro software was used for statistical analysis.

Results and Discussions

The *Anethum graveolens* essential oil proved to be rich in limonene, carvone and α-phellandrene [11]. *Mentha piperita* essential oil proved to be rich in β-pinene, limonene, menthone, isomenthol, menthol [15]. *Rosmarinus officinalis* essential oil proved to be rich in α-pinene, β-pinene, eucalyptol, camphor and caryophyllene [12] (figure 1). All these results are in concordance with reported literature [16-18].

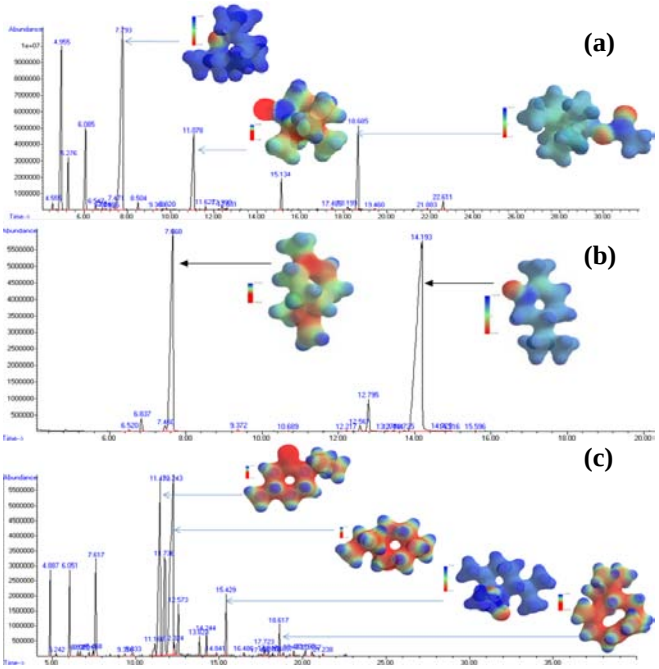

Figure 1: *GC-MS analyses of the essential oils: Rosmarinus officinalis (a), Anethum graveolens(b), Mentha piperita(c)*

The major limitation of classical methods used for investigation of antimicrobial activity of essential oils is that volatility of the compound could not be controlled. This aspect is critical because the evaporated amount of certain compounds may accumulate in the test microtiter plate environment will be sufficient to kill all microorganisms from the plate, feature that make impossible the screening of many conditions in the same time. Our results demonstrate that due to their great volatility even very low amounts of oil seem enough to kill *C.albicans* used strains, leading to false results and making difficult the estimation of a minimum inhibitory concentration (MIC) of the oil mixture (figure 2).

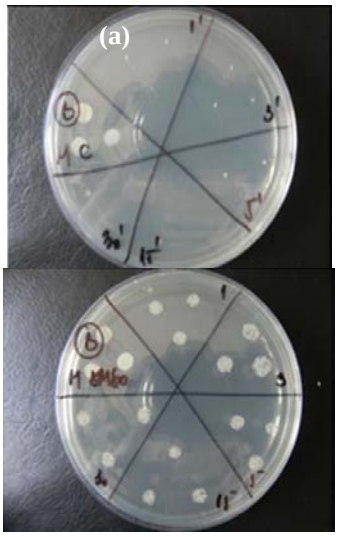

Figure 2: *Kill time approach for qualitative screening of antifungal activity for A. graveolens essential oil: (a) viable count analysts of C.albicans 774 strain grown in the presence of a very low amount of Mentha piperita oil in a microtiter plate; (b) control plate-DMSO treated samples*

Bearing in mind the volatility of the oils and the great number and diversity of clinical isolates our next task was to find a suitable method for the rapid screening of the antifungal activity of some natural essential oils. We compared the results achieved by flow cytometry with the data obtained when a microtiter plate killing time assay was used. Even though an early microbicidal effect was observed by both killing time assay and flow cytometry at different concentrations (figure 3), flow cytometry technique allowed us to set a MIC value for each tested essential oil tested (figure 4).

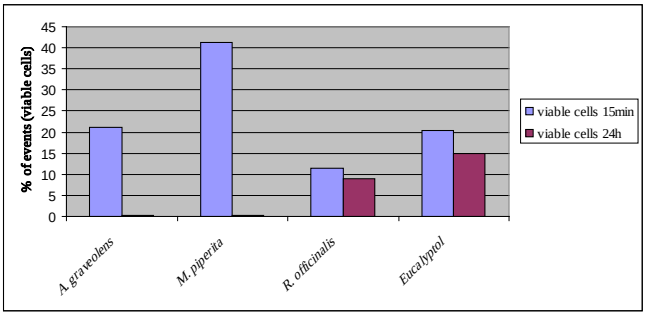

Figure 3: *Viable cells at established MIC for C. albicans 980, after 15 min of contact with essential oils solutions*

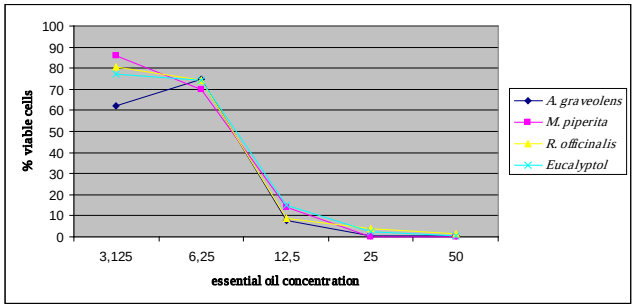

Figure 4: *Minimum inhibitory concentration for C. albicans 980 after 24 h of incubation time with the essential oils and eucalyptol analytical standard.*

Anethum graveolens and *Mentha piperita* showed the same MIC against tested fungal strains, and *Rosmarinus officinalis* which had an antimicrobial activity comparable to that of the eucaliptol standard. MIC were ranged between 25 and 12.5µL/mL. Flow citometry technique revealed a strain specific effect of the essential oils and an essential oil specific profile (figure 5) when MIC essential oil concentrations are tested after 15 min of contact time. This proves the early microbicidal effect in order *M. piperita*, *A. graveolens*, *R. officinalis* and eucalyptol probably correlated with rapid permeability effect of cell envelopes. Furthermore, flow cytometry technique revealed the fact that different *Candida* strains may respond slightly different to the same oil concentration, showing the usefulness of this tool in investigating the antimicrobial activity of volatile essential oils.

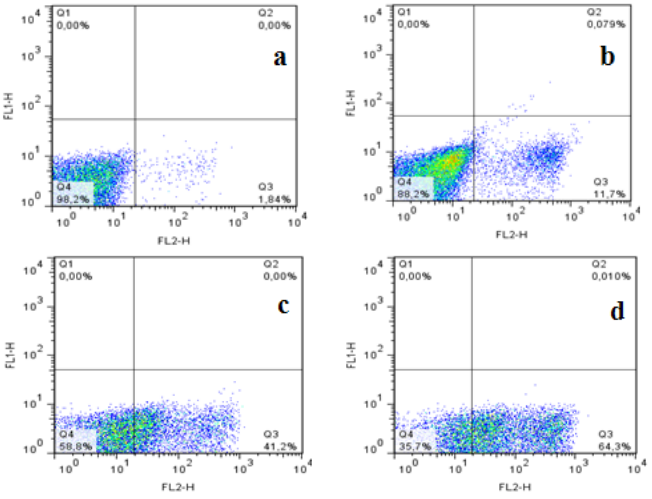

Figure 5: *Flow citometry dot plot analysis for 24h incubation time (a) viable cells control (b, c, d) binary serial concentration of essential oil from Rozmarinus officinalis against C. albicans 131 strain.*

Conclusions

Due to the emergence of synthetic antibiotic and antifungal resistant strains, novel natural compounds with antimicrobial activities are urgently needed. Besides, appropriate methods to cover the needs imposed by the usage of those complex mixtures are required. Our results demonstrate that natural essential oils are very effective antifungal mixtures, revealing a great killing activity against some of the most encountered human pathogenic *Candida* species. Essential oils complex composition and their volatility raise specific problems in susceptibility testing, due to their poor solubility, unknown diffusion pattern in solid media and vapor phase effect. The data demonstrate that due to their great volatility even very low amounts of oil seem enough to kill *C. albicans* used strains, leading to false results and making impossible the estimation of the minimum inhibitory concentration (MIC) of the oil mixture. In this context flow cytometry technique has proved to be a very useful tool in investigating the antifungal activity of the essential volatile oils, excluding volatility effect of those complex mixtures that often leads to false results.

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