

CULTURE METHODS VERSUS FLOW CYTOMETRY FOR THE COMPARATIVE ASSESSMENT OF THE ANTIFUNGAL ACTIVITY OF *EUGENIA CARYOPHYLLATA* THUNB. (*MYRTACEAE*) ESSENTIAL OIL

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Abstract

Opportunistic yeasts especially belonging to *Candida* sp. have been recognized lately as major fungal pathogens. Treatment of fungal infections, particularly nosocomial ones, has lagged behind bacterial chemotherapy and there are substantially fewer antifungal than antibacterial drugs due to the toxic side effects (fungi being eukaryotic cells similar in structure with the host cells) and to limited solutions for the development of new classes of antifungal agents. On the other hand, the lack of standardization and laborious available techniques led to an increasing interest for developing new rapid techniques for assessing the antifungal activity of different compounds. Our aim was to optimize a flow cytometry based method to test the antifungal susceptibility to essential oils, counting as a rapid method able to indications about presumptive action mechanism of essential oil from *Eugenia caryophyllata* dried buds (cloves).

Rezumat

Speciile de levuri aparținând în special genului *Candida* sunt recunoscute ca patogeni oportuniști importanți. În contextul soluțiilor terapeutice limitate pentru tratamentul infecțiilor fungice și al absenței standardizării metodelor de testare a sensibilității la substanțe antimicrobiene, scopul studiului de față a fost de a optimiza un protocol rapid și reproductibil pentru determinarea activității antifungice a unor uleiuri volatile, utilizând tehnica citometriei în flux comparativ cu metodele microbiologice clasice. Rezultatele obținute au demonstrat ca citometria în flux este o metodă rapidă pentru testarea sensibilității uleiurilor volatile la fungi și poate oferi indicații cu privire la mecanismul de acțiune prezumtiv al uleiului volatil din muguri uscați de *Eugenia caryophyllata*.

Keywords: flow cytometry, *Eugenia caryophyllata*, *Candida albicans*, antifungal activity.

Introduction

It is estimated that the human organism has approximately 10^3 cells, but carries approximately 10^{14} microbial cells (comprising the endogenous microbiota) and a series of relationships and interactions have been established between host and its microbiota, as well as between different microbial species, either beneficial or detrimental, from symbiotic relationships to activation of virulence genes in pathogenic bacteria and fungi[1]. However, fungi are opportunistic microorganisms, and from approximately 10^5 species, only 50 (75 or 100 among other authors) have been recognized as human pathogens and identified in clinical specimens.

Candida species are ubiquitous yeasts, that cause a wide spectrum of diseases, including hospital-acquired and device-associated infections (reduced biocid sensibility)[2, 3]. It is also known that the extensive use of the antimicrobial substances led to the emergence of multiresistant strains, increasing the number of nosocomial infections and complicating their clinical picture[4]. In addition, there are far fewer classes of antifungal agents than antibacterial drugs, limiting therapeutic options [5]. The resistance genes occurred in the microbial strains isolated from the hospital environment are originating in the external medium, evolving as non-specific defense mechanisms against the toxic compounds existing in the environment, such as plant metabolites and soil microbiota[3]. Still, it is estimated that are 10^{62} - 10^{63} bioactive compounds in organisms belonging to the three domains of living world and the antimicrobial compounds from the plant material samples are widely studied [6].

The recent interest for medium brought the green chemistry in a strategic position in research field. The antimicrobial assay methods are classified into three main groups, i.e. diffusion, dilution and bioautographic methods. It should be emphasized that many research groups have modified these methods for specific samples such as essential oils and non-polar extracts and this small modifications make it almost impossible to directly compare the results. Validation and selection of primarily screening assays are pivotal to guarantee sound selection of extracts or molecules with relevant pharmacological action and worthy following-up. Primary assay are generally designed for rapid screening [7].

Flow cytometry is a real-time method often used as differential diagnostic tool in oncology-haematology, but in microbiological research too. Essential oils antimicrobial activity testing raises problems related to their volatility and poor solubility in aqueous media and the use of a rapid bioassay is a must. In this context, our aim was to optimize a rapid,

reproducible method based on flow cytometry in order to test the fungal susceptibility to essential oils, exhibiting the advantage of a rapid method that could also provide indications about the presumptive mechanism of action of the essential oil extracted from *Eugenia caryophyllata* dried buds.

Materials and Methods

Plant material and extraction

E. caryophyllata dried buds were purchased from a local supplier and subjected to essential oil extraction. A Neo Clevenger type apparatus according to European Pharmacopoeia 6 was used for performing two microwave assisted extractions from 225g plant material [8].

GC-MS analysis of essential oil

Chemical composition was settled by GC-MS analysis. Gas chromatographic analysis was performed using an Agilent 6890 Series GS System gas chromatograph Detection was carried out with a 5973 mass-selective single quadrupole detector (Agilent technologies). The mass spectrometer was calibrated before use with perfluorotributylamine (PFTBA) as a calibration standard. GC-MS parameters were described in a previous study[9].The essential oil proved to be rich in eugenol and α -caryophyllene (92.42% and 5.44% from the total area).

Fungal strains

The antifungal activity of the *E. caryophyllata* buds essential oil was tested against fungal strains recently isolated from clinical specimens, i.e. *Candida albicans* 173 and *Candida albicans* 172 (urinary tract infection), , *Candida albicans* 2026, *Candida albicans* 131 and *Candida albicans* 177 (bronchotracheal secretions), (Bronchotracheal 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 [10], 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, as well as the remanence of the antimicrobial activity.

Quantitative assay of the antimicrobial activity

Minimum inhibitory concentrations (MIC) assay was performed by twofold micro-dilution technique in 96 multiwell plates [11] for each tested fungal strain. Simultaneously, there were achieved serial dilutions for

DMSO (dimethylsulfoxide) in the same volume, in order to obtain the negative control. An amount of 20 μL of bacterial suspension with the standard density of 0.5 Mc Farland was added in each well. The plates were incubated for 24 h at 37°C, and MICs were read as the lowest essential oils concentration that inhibited the microbial growth.

Assay of the antifungal activity of the essential oil by flow cytometry

Two fold macro-dilution in YPG medium were obtained using a stock solution of the essential oil in DMSO 1:1, working concentrations ranging from 0.05 to $3.125 \times 10^{-3} \mu\text{L/mL}$. Three incubation time working variants for each sample were achieved: 5, 30 min and 24h. The dead cells stained with propidium iodide (50 $\mu\text{g/mL}$) were assessed by flow cytometry. Staining procedure was applied at room temperature 10 min before data acquisition with a FACSCalibur flow cytometer. CellQuest Pro software was used for statistical analysis.

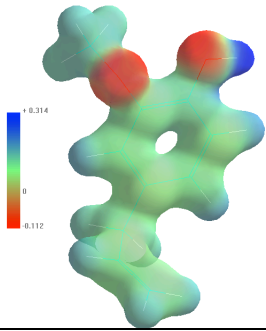
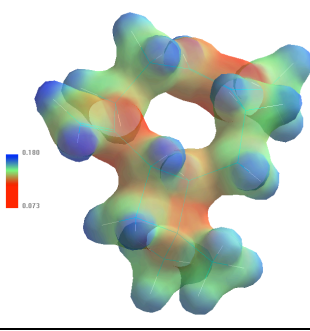
Results and Discussion

Essential oils composition

The average yields of the essential oil (v/w %, normalized to the part of the plant mass dried weight) extracted from *E. caryophyllata* was 2.25%. The essential oil from dried buds of *E. caryophyllata* proved to be rich in eugenol and α -caryophyllene (92.42% and 5.44% from the total area) are presented in Table I as projection structures and perspective representations, with a color gradient charge distribution and the value of the partial charge displayed at each atom. These results were obtained using the MINDO method [12] and optimization by the conjugate gradients method.

Table I

The main compounds and their percentage in *E. caryophyllata* essential oil

	
Eugenol	α -caryophyllene

Antimicrobial activity of essential oil from E. carriophylata buds
Qualitative assay

The kill time assay revealed an early microbicidal and vapor phase effect for the essential oil stock solution (Figure 1).

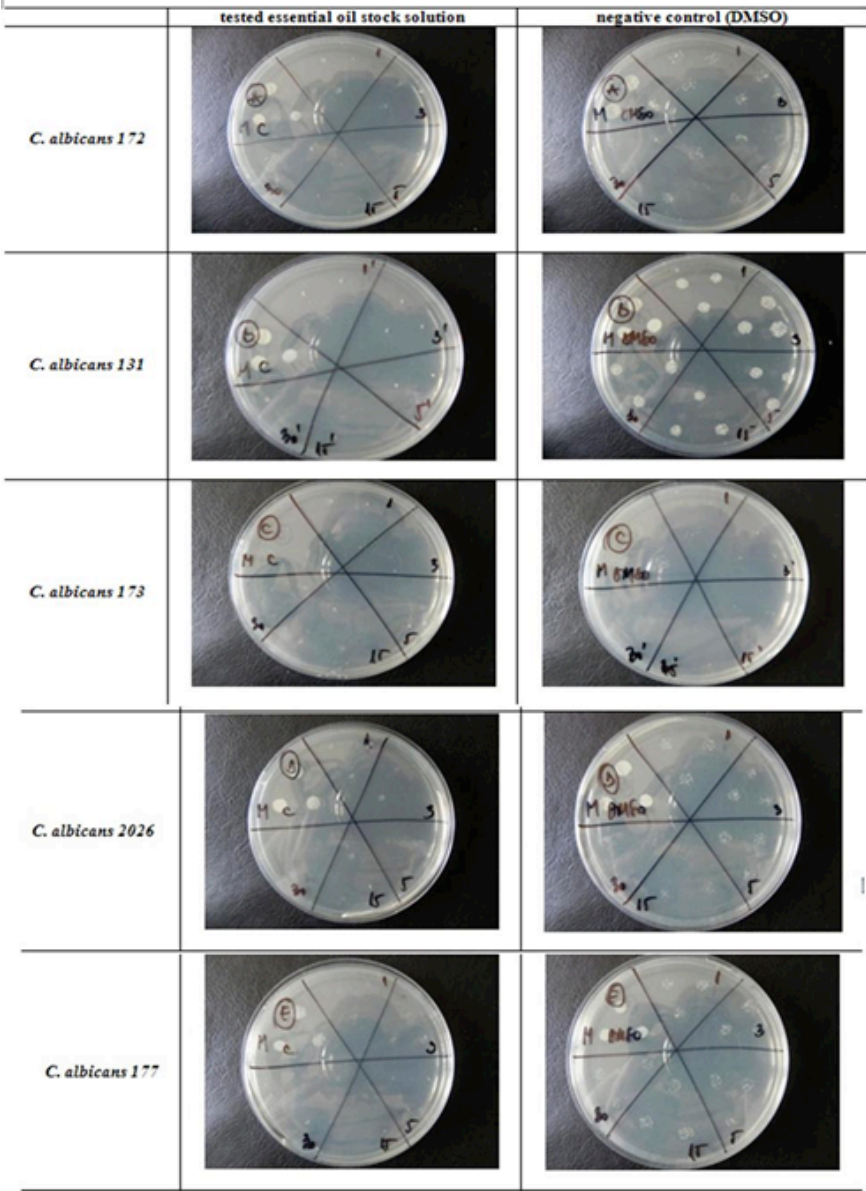


Figure 1.
Assessment of the antifungal activity of the essential oil
by the kill time qualitative assay

Quantitative assay

The microdilution technique proved to be ineffective for MIC assay, probably due to the vapor phase effect which determined a constant and microbicidal effect of volatile compounds in all wells, independently to the achieved concentration. The flow cytometry assay revealed the same early microbicidal effect of the essential oil as already proven by the kill time curve, but having the major advantage of avoiding the vapour phase effect interference and thus making possible the MIC assay. All tested strains exhibited the same MIC value, i.e. $6.250 \times 10^{-3} \mu\text{L/mL}$ after 30min of contact with the essential oil. A late microbicidal effect was noticed after 24 h of contact for *C. albicans* 2026 and *C. albicans* 177 strains, exhibited at a lower concentration of the essential oil ($3.125 \times 10^{-3} \mu\text{L/mL}$).

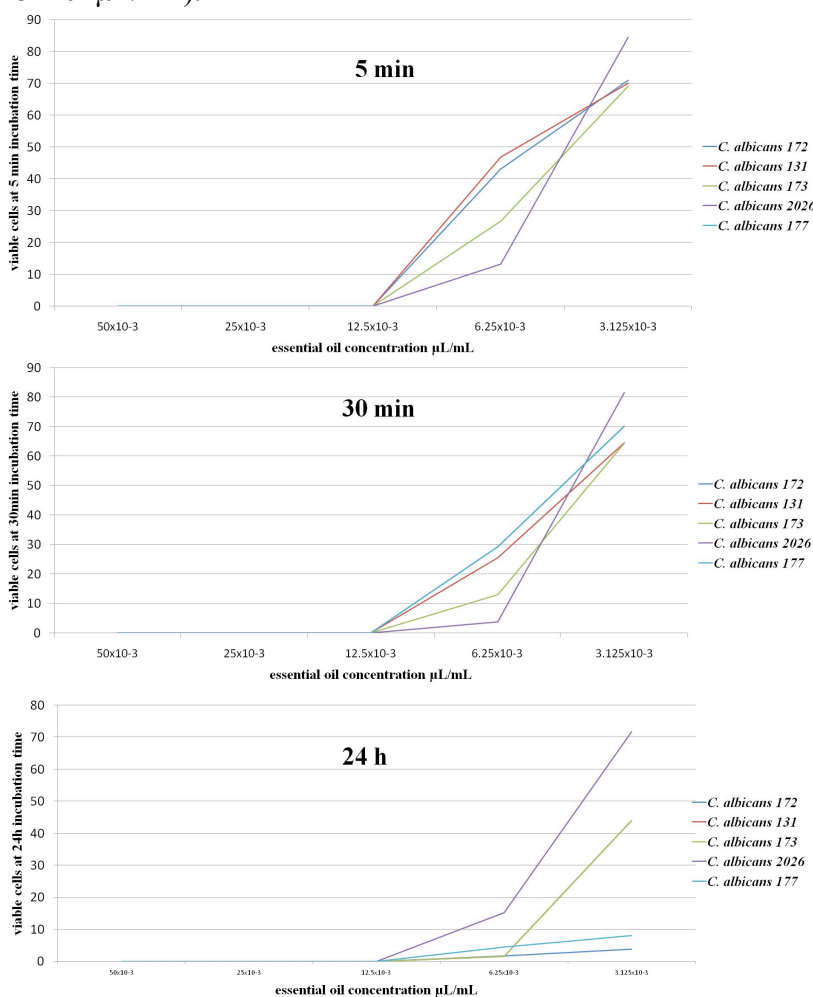
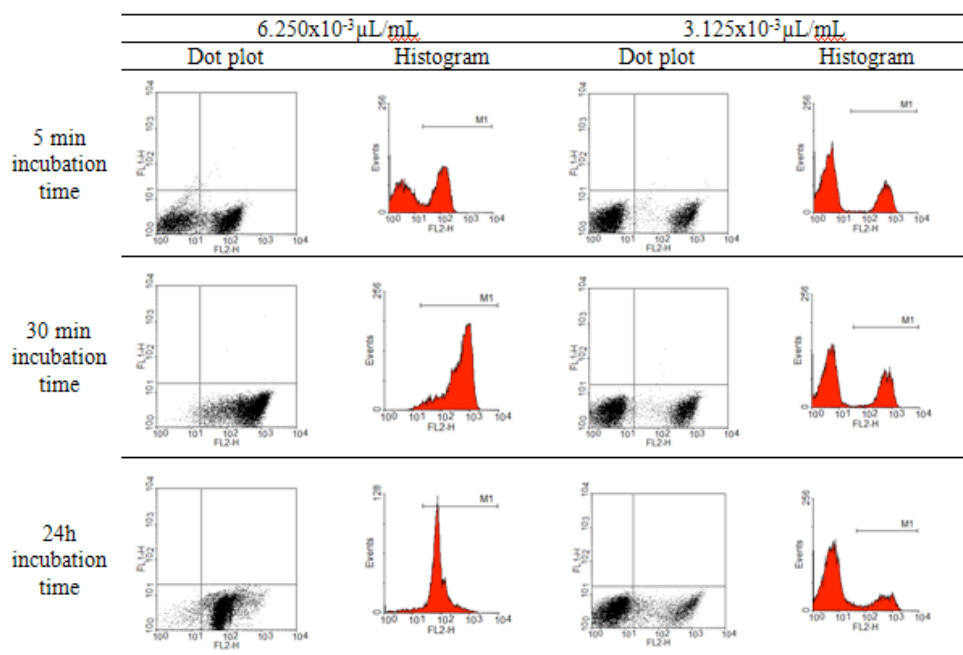


Figure 2

Assessment of MIC value of the essential oil by flow cytometry

**Figure 3.**

Separation of viable/killed fungal cells population by flow cytometry after propidium iodide staining

Conclusions

Although some guidelines for performing antifungal susceptibility testing are presently available, the proposed methods are labor intensive, requiring 48-72h for the incubation time.

Essential oils complex composition and the volatility of their constituents raise specific problems in susceptibility testing, due to their poor solubility, unknown diffusion pattern in solid media and vapor phase effect.

The microdilution technique proved to be ineffective for MIC assay, *versus* flow cytometry which revealed the same early microbicidal effect of the essential oil as the kill time qualitative assay, having the major advantage of avoiding the vapour phase effect interference and thus making possible the MIC determination.

In this context flow cytometry technique could be considered a reliable tool for evaluating the antifungal activity of this type of complex mixtures.

Acknowledgements

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References

1. Alina Maria Holban, Veronica Lazăr: Inter-kingdom cross-talk: the example of prokaryotes - eukaryotes communication, *Biointerface Res. Appl. Chem.*, 1, 3, 2011, 095-110
2. Otilia Banu, Coralia Bleotu, Mariana Carmen Chifiriuc, Bogdan Savu, George Stanciu, Cristina Antal, Magdolna Alexandrescu, Veronica Lazăr: Virulence factors of *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains involved in the etiology of cardiovascular infections, *Biointerface Res. Appl. Chem.*, 1, 2, 2011, 72-77
3. Govindsamy VEDIYAPPAN, Tristan ROSSIGNOL, and Christophe d'Enfert, Interaction of *Candida albicans* Biofilms with Antifungals: Transcriptional Response and Binding of Antifungals to Beta-Glucans *Antimicrobial Agents And Chemotherapy*, 54, 5, 2010, 2096-2111
4. Emilia Panus, Carmen-Mariana Chifiriuc, Otilia Banu, Magda Mitache, Coralia Bleotu, Natalia Rosoiu, Veronica Lazar: Comparative study of resistance and virulence markers in *Escherichia coli* strains isolated from hospital surfaces, clinical specimens and drinking/ marine waters, *Biointerface Res. Appl. Chem.*, 1, 1, 2011, 24-30
5. Richard D. Cannon, Erwin Lamping, Ann R. Holmes, Kyoko Niimi, Philippe V. Baret, Mikhail V. Keniya, Koichi Tanabe, Masakazu Niimi, Andre Goffeau, and Brian C. Monk, Efflux-Mediated Antifungal Drug Resistance, *Clinical Microbiology Reviews*, 22, 2, 2009, 291-321
6. Bala Yauri Muhammad and Ahmed Awaisu, The Need for Enhancement of Research, Development, and Commercialization of Natural Medicinal Products in Nigeria: Lessons from the Malaysian Experience, *Afr J Tradit Complement Altern Med.* 2008; 5(2): 120-130
7. Cos P., Vlietnick A., Vanden Berghe D., Maes L., Antiinfective potential of natural products: How to develop a stronger in vitro „proof of concept”, *Journal of Ethno-Pharmacology*, 106, 2006, 290
8. Crina Saviuc, Alexandru Mihai Grumezescu, Alina Holban, Coralia Bleotu, Carmen Chifiriuc¹, Paul Balaure, Veronica Lazar: Phenotypical studies of raw and nanosystem embedded *Eugenia carryophyllata* buds essential oil antibacterial activity on *Pseudomonas aeruginosa* and *Staphylococcus aureus* strains, *Biointerface Res. Appl. Chem.*, 1, 3, 2011, 111-118, 2011
9. Crina Saviuc, Alexandru Mihai Grumezescu, Alina Holban, Carmen Chifiriuc, Dan Mihaiescu, Veronica Lazar: Hybrid nanostructured material for biomedical applications, *Biointerface Res. Appl. Chem.*, 1, 2, 2011, 064-071
10. Crina Saviuc, Alexandru Mihai Grumezescu, Mariana Carmen Chifiriuc, Coralia Bleotu, George Stanciu, Radu Hristu, Dan Mihaiescu, Veronica Lazăr: *In vitro* methods for the study of artificial fungal biofilms, *Biointerface Res. Appl. Chem.*, 1, 1, 2011, 031-04
11. Crina Saviuc, Alexandru Mihai Grumezescu, Eliza Oprea, Valeria Radulescu, Luminita Dascalu, Mariana Carmen Chifiriuc, Marcela Bucur, Otilia Banu, Veronica Lazar: Antifungal activity of some vegetal extracts on *Candida* biofilms developed on inert substratum, *Biointerface Res. Appl. Chem.*, 1, 1, 2011, 015-023
12. D.E. Mihaiescu, A.M. Grumezescu, D.E. Mogosanu, V. Traistaru, P.C. Balaure, A.S. Buteica, Hybrid inorganic/organic nanomaterial for controlled cephalosporins release, *Biointerface Res. Appl. Chem.*, 1, 2, 2011, 041-047