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# Phenotypical studies of raw and nanosystem embedded *Eugenia carryophyllata* buds essential oil antibacterial activity on *Pseudomonas aeruginosa* and *Staphylococcus aureus* strains

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## ABSTRACT

Considering the large number of the multiresistant bacterial strains and the increasing need in new antimicrobial formulation, the aim of this study was to evaluate the antipathogenic effect of *E. caryophyllata* essential oil on collection and recently isolated clinical strains of *P. aeruginosa* and *S. aureus*. Experimental assays were carried out by *in vitro* and *in vivo* tests. Qualitative and quantitative assessment of the antimicrobial activity was followed by the analysis of the essential oil influence on enzymatic soluble virulence factors expression. The cytotoxic effect of the vegetal extract was quantified by using HeLa cells. Nanosystem embedded essential oil was used to establish the *in vivo* antipathogenic effect on mice. Qualitative screening results revealed an early microbicidal effect quantified by low minimum inhibitory concentration values. The *in vivo* study showed a stabiliziation of the essential oil biological activities when using nanosystem embedding, that could be used for the design of proper formulations for delivery systems with antimicrobial effect.

**Keywords:** *Eugenia carryophyllata, strains pathogenesis, nanosystem, coated nanoparticles*

## 1. Introduction

*Staphylococcus aureus* and *Pseudomonas aeruginosa* are the most important opportunistic bacterial pathogens in hospitalized patients and immunocompromised hosts. *S. aureus* is a major human pathogen which can cause serious, severe infections such as endocarditis, septicemia, pneumonia and toxic shock syndrome [1]. Due to the continuous emergence and spread of antibiotic resistant strains (e.g. MRSA, VRSA) the treatment options are often severely limited [2,3]. *P. aeruginosa* is an important cause of nosocomial pneumonia, as well as a major pulmonary pathogen in patients with cystic fibrosis and other immunocompromising conditions [4, 5]. Persistent bacterial

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infections involving the opportunistic pathogen *P. aeruginosa* are responsible for much of the morbidity and mortality caused by cystic fibrosis.

The pathogenesis of *P. aeruginosa* and *S. aureus* infections is multifactorial, as suggested by the large number of cell-associated and extracellular virulence determinants possessed by the bacterium. Some of these determinants help colonization, whereas others facilitate bacterial invasion.

Because antibiotic resistances are spreading, current treatment strategies of *S. aureus* infections are becoming more and more ineffective finding a new antimicrobial strategies being of great interest.

Known for their antiseptic, i.e. bactericidal, virucidal and fungicidal, and medicinal properties essential oils are used in embalment, foods preservation and as antimicrobial agents. Essential oils contain a large number of compounds in a complex mixture with multitarget effects on bacterial cells, but many of the compounds are chemically unstable and easily volatile. 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, or for the improvement of the laboratory assay of the biological effects of these compounds [5, 6].

The aim of this study was to evaluate the antipathogenic effect of *E. caryophyllata* essential oil using a stabilizing nanosystem based method.

# 2. Experimental section

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

**2.2. Essential oil GC-MS analysis.** 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). Operation control and the data process were carried out by Agilent Technologies ChemStation software (Santa Clara, CA, USA). The mass spectrometer was calibrated before use with perfluorotributylamine (PFTBA) as a calibration standard. GC-MS parameters was described in a previous study. [7].

**2.3. Bacterial strains.** The antimicrobial activity of the *E. carryophyllata* buds essential oil was tested against bacterial strains recently isolated from clinical specimens as well as reference strains belonging to *S. aureus* and *P. aeruginosa* species (Table 1). The strains were identified by using an automatik Vitec II system. Bacterial suspensions from 15-18 h bacterial cultures developed on solid media were adjusted to 0.5 McFarland density and used for bioassays. The antimicrobial activity was tested on Brain Heart Infusion agar and Chapman media recommended for the tested strains.

**2.4. In vitro tests for the antipathogenic effect of the essential oil**

**2.4.1. Qualitative screening of the antimicrobial effect.** A killing time curve was performed for the tested strains. A stock solution essential oil (EO):dimethyl sulfoxide (DMSO) 1:1 was prepared and added to BHI broth, a concentration of 0.250µL/mL EO being achieved. The qualitative screening of the antimicrobial effect and its dynamic was assessed after 1', 3', 5', 15' and 30' of contact, by performing viable cell counts.

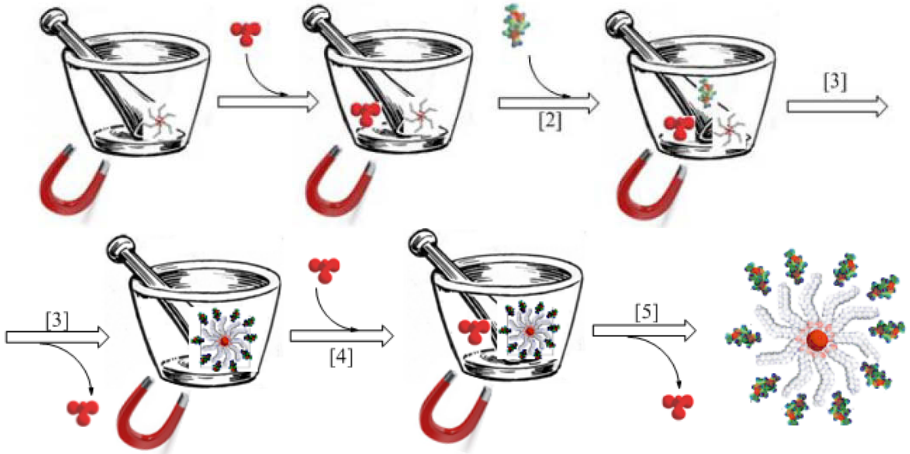
**2.4.2. Quantitative assay of the antimicrobial activity.** MIC (Minimal Inhibitory Concentration) value for the EO was determined by twofold microdilution technique, in 96 multi-well plates, starting from 250µL/mL to 0.125µL/mL, for each tested bacterial strain. Simultaneously, there were achieved serial dilutions for DMSO 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 EO concentration that inhibited the microbial growth.

**Tabel 1:** Clinical origin and species assignment of the strains

| ***Strains*** | ***Code*** | ***Clinical specimen*** | ***Strains*** | ***Code*** | ***Clinical specimen*** |
|---|---|---|---|---|---|
| *S. aureus* | 1 | Sputum | *P. aeruginoasa* | 12 | Sputum |
| *S. aureus* | *2* | Sputum | *P. aeruginoasa* | 13 | Bronchotracheal secretions |
| *S. aureus* | 3 | Would secretion | *P. aeruginoasa* | 14 | Blood cultures |
| *S. aureus* | 4 | Would secretion | *P. aeruginoasa* | 15 | Urine cultures |
| *S. aureus* | 5 | Would secretion | *P. aeruginoasa* | 16 | Sputum |
| *S. aureus* | 6 | Would secretion | *P. aeruginoasa* | 17 | Sputum |
| *S. aureus* | 7 | Would secretion | *P. aeruginoasa* | 18 | Sputum |
| *S. aureus* | 8 | Would secretion | *S. aureus* | 19 | Blood cultures |
| *P. aeruginoasa* | 9 | Blood cultures | *S. aureus* | 20 | Blood cultures |
| *P. aeruginoasa* | 10 | Blood cultures | *S. aureus* | II | ATCC 25923 |
| *P. aeruginoasa* | 11 | Blood cultures | | | |

**2.4.3. Influence of the EO on the expression of soluble enzymatic virulence factors.** The microbial strains were cultivated in nutrient broth with and without addition of subinhibitory concentrations of EO for each strain and the obtained overnight bacterial cultures were used for the performance of the following virulence tests: Plate haemolysis, Protease activity, DNA-se production, Lipase production, Amylase production, Esculin hydrolysis described in a previous study, using methods previously described [8].

**2.5. Nanosystem embedded *Eugenia carryophyllata* buds essential oil.** Magnetite nanoparticles was synthesized by Massart method [9]. Core/shell nanosystem was prepared under microwave condition. Oleic acid was used as surfactant to create the shell. The hybrid nanostructure was prepared according to scheme 1.




*[1] solubilization of core/shell nanosystem in chloroform;[2] essential oil added drop by drop; [3,5] mixing until evaporation the chloroform; [4] chloroform added drop by drop.*

**Scheme 1:** Core/shell/extra-shell hybrid nanostructure

**2.6. In vitro cytotoxic effect of the essential oil.** 5 x $10^5$ HeLa cells were seeded in 25 $cm^2$ flask, in Dulbecco's Modified Essential medium DMEM (Sigma) supplemented with 10% fetal calf serum (Sigma) at 37°C, 5% $CO_2$, in a humid atmosphere. These cells were treated with different dilutions of essential oil stock solution as follows: 1/1000; 1/5000; 1/10000; 1/50000. After 24 h of treatment, cells viability was evaluated using Trypan Blue stain, and the cells were processed for cell cycle analysis. Briefly, the cells were washed in a cold solution of PBS (pH 7.5), then fixed in cold 70% ethanol and stored at –20 $^0$C overnight. The samples were then centrifuged, washed with PBS and then re-suspended in 100 µl PBS, treated with 10 µ1 0.1% RNase A and labelled with 10 µl propidium iodide (10 µg/ml), incubated in the dark at RT for 30 min prior measurement. DNA content of cells was quantified by a FACSCALIBUR flow cytometer and analyzed using FlowJo 8.8.6. software (Ashland, Oregon, USA).

**2.7. In vivo evaluation of the antipathogenic effect of the essential oil**

**2.7.1. Mice.** Generally, mice were 4-5 weeks females or males weighing about 25g. Mice were housed in filtered, clear plastic cages on a 12-h light/dark cycle. Food and water were provided *ad libitum*.

**2.7.2. In vivo tests protocol.** Feeding suspensions were prepared from stock suspensions EO:DMSO 1:1, stabilized EO:DMSO. A 4mL volume of working suspentions was achieved, at 30µL/mL concentration of the EO. Mice were pretreated for 1 week by oral administration (100 µL) with different working suspentions: EO, stabilized EO, ferite nanoparticles, 4 animals/variant. After 1 week half of the mice were injected retroperitoneally with 200µL of a bacterial suspension of 0.5 McFarland density obtained from 16-18h solid culture of *P. aeruginosa* 11. The oral treatment based on EO was carried out for another 2 weeks, following the infection. The mice were evaluated by daily inspection of their behaviour, weight and aspect of the injection site.

# 3. Results section

**3.1. Essential oil GC-MS analysis**. The average yields of essential oil (v/w %, normalized to the part of the plant mass dried weight) extracted from *E. caryophyllata* was 2.25%. The EO from dried buds of *E. caryophyllata* proved to be reach in eugenol and α-cariophylene (92.42% and 5.44% from the total aria) [10]. The qualitative screening for the EO antimicrobial activity proved an early microbicidal effect exhibited on all tested strains (Figure 1). As it could be noticed from Figure 2, the MIC values were generally low especially for *S. aureus* strains (code 1 to 8 and 19 to 21) (≤1µL/mL).

**3.2. Influence of the EO on the expression of soluble enzymatic virulence factors.** The soluble enzymatic virulence factors expression was stimulated by the EO subinhibitory concentrations, particularly in case of lipase production (15 strains, both *S. aureus* and *P. aeruginosa*) followed by DN-ase, haemolysins, gelatinase and lecithinase, in case of *P. aeruginosa* strains) (Table 6).

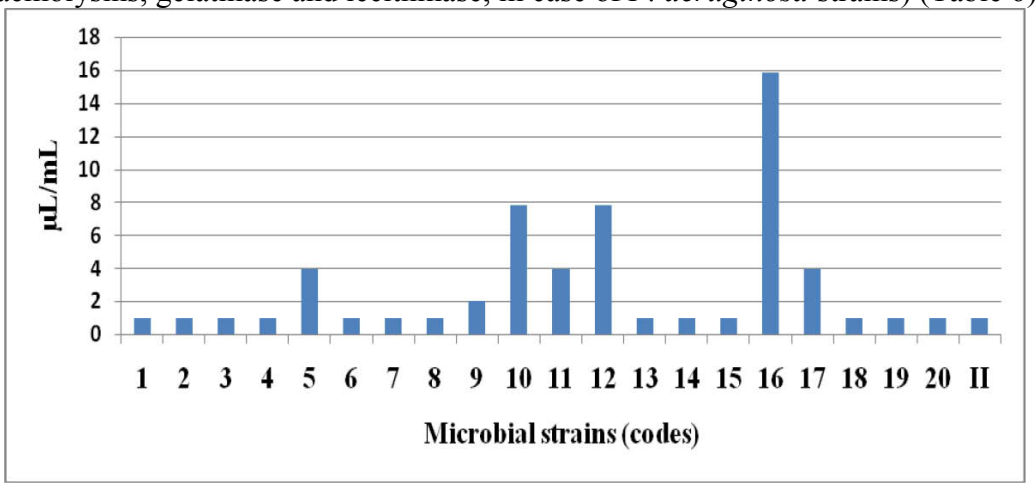



**Figure 2:** The graphic representation of the MIC values obtained for the EO on different bacterial strains

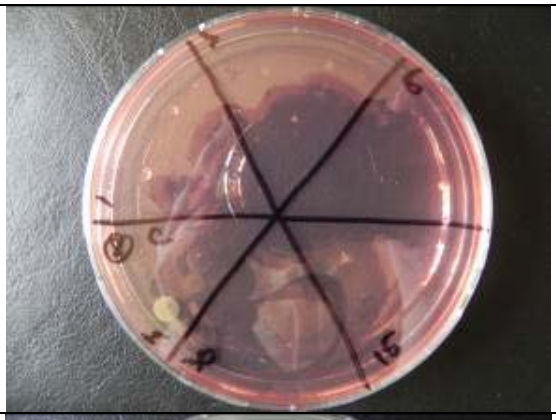
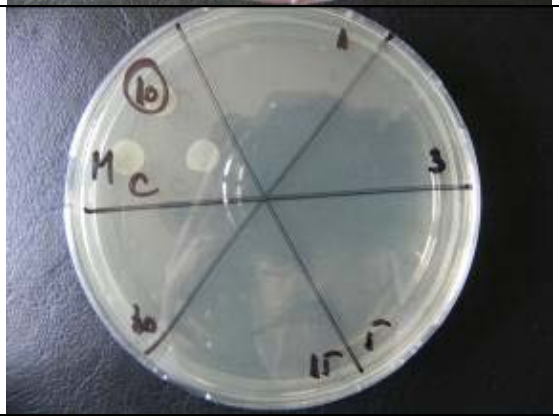
| No. | Tested EO stock solution | Negative control (DMSO) |
|---|---|---|
| 8 |  |  |
| 10 |  |  |

**Figure 1:** Assessment of the antimicrobial activity of the essential oil by viable cell counts

**Table 6.** Influence of the EO on the expression of soluble enzymatic virulence factors

| Virulence factors / Bacterial Strains | DNA-se | | Lecitinase | | Lipase | | Gelatinase | | Esculin hydrolysis | | Hemolysins | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C* | EO** | C | EO | C | EO | C | EO | C | EO | C | EO |
| 1 | - | - | - | - | - | + | - | - | - | - | - | - |
| 2 | - | - | - | - | - | + | - | - | - | - | - | - |
| 3 | - | - | - | - | - | + | - | - | - | - | - | - |
| 4 | - | - | - | - | - | + | - | - | - | - | - | - |
| 5 | - | - | - | - | - | + | - | - | - | - | - | - |
| 6 | - | - | - | - | - | + | - | - | - | - | - | - |
| 7 | - | - | - | - | - | + | - | - | - | - | - | - |
| 8 | - | - | - | - | - | + | - | - | - | - | - | - |
| 9 | - | - | - | - | + | + | + | + | - | - | - | - |
| 10 | - | + | - | + | + | + | + | + | - | - | - | α+ |
| 11 | - | + | - | + | + | + | - | - | - | - | - | - |
| 12 | - | - | - | - | - | + | - | - | - | - | α+ | α+ |
| 13 | +/- | + | - | - | - | +/- | + | + | + | + | β+ | β ++ |
| 14 | +/- | + | - | - | - | +/- | - | +/- | - | - | - | - |
| 15 | - | - | - | - | + | + | + | +/- | +/- | +/- | α+ | α/ β+ |
| 16 | + | + | - | - | - | + | - | - | - | - | β+ | β+ |
| 17 | - | - | - | - | - | + | - | - | - | - | - | β ++ |
| 18 | - | - | - | - | - | + | - | - | - | - | - | α+ |
| 19 | - | + | + | + | + | + | - | - | + | - | β+ | β+ |
| 20 | - | + | - | - | - | + | - | - | + | +/- | - | β+ |
| II | - | - | - | - | + | + | - | - | + | +/- | β+ | β+ |

***Control, **Essential oils***

**3.3. Nanosystem embedded *Eugenia carryophyllata* buds EO.** HR-TEM was used as primary method for the characterization of core/shell nanosystem [9]. The dimension of nanoparticles did not exceed 20 nm.

**Figure 3.** Magnetic field aligns the core/shell/extra-shell nanofluid

**3.4. In vitro cytotoxic effect of the essential oil.** The cell cycle analysis was conducted with the Watson (pragmatic) model (Table 7, Figure 4). Decrease in G0/G1 and increase in synthesis phase was noted in the presence of the essential oil. The cell cycle analysis of HeLa cells treated with increased concentrations of the essential oil (1:5000 dilution) revealed in the left side the appearance of subG0 apoptosis peak, that confirmed the results of Tripan Blue evaluation

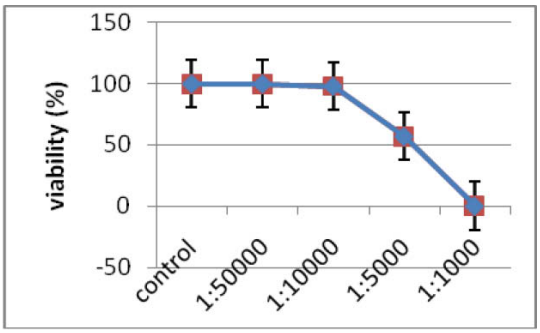



**Figure 4:** Viability evaluation of HeLa cells treated with different essential oil concentration

**Table 7**: The effects of essential oil on HeLa cell cycle progression

| | G0/G1 | S | G2/M |
|---|---|---|---|
| Control | 70.46 | 23.96 | 7.96 |
| Oil dilution 1:50.000 | 67.86 | 24.41 | 5.85 |
| Oil dilution 1:10.000 | 56.04 | 33.99 | 5 |
| Oil dilution 1:5.000 | 49.88 | 33.87 | 7.68 |

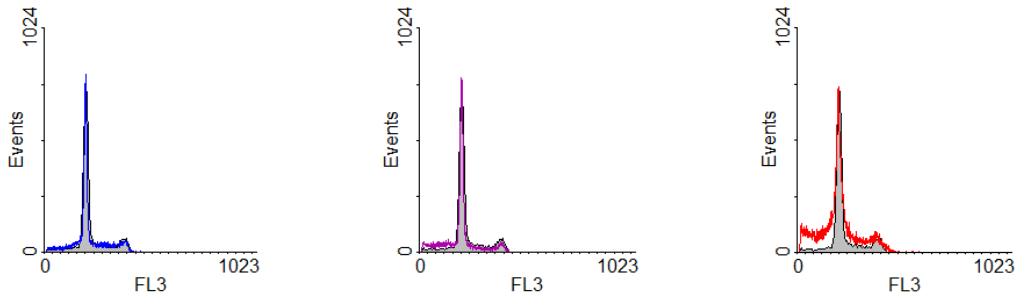
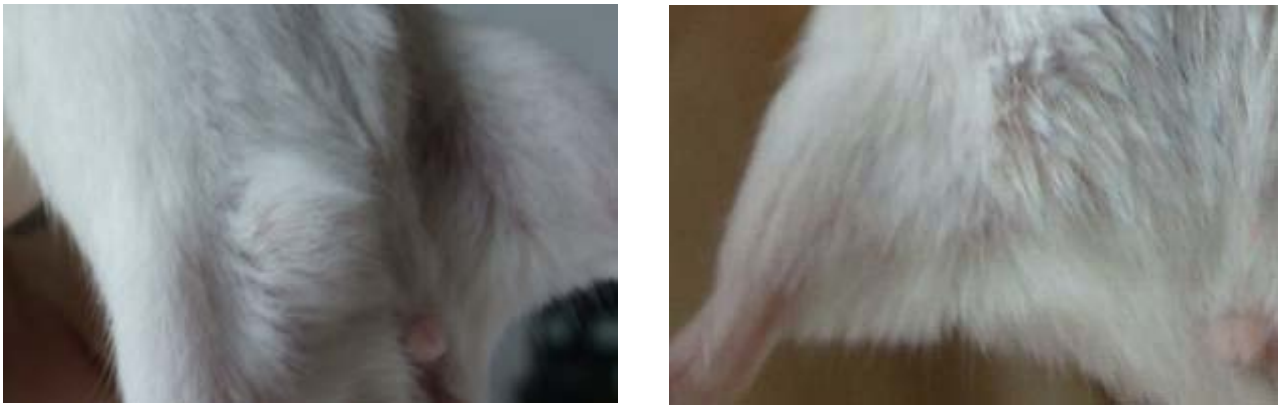



**Figure 5:** The effects of EO on cell cycle progression:
a) dilution 1:50000; b) dilution 1:10000; c) dilution 1:5000.

**3.5. In vivo evaluation of the antipathogenic effect of the essential oil**. After two days of *P. aeruginosa* inoculation by intraperitoneal route, a localized infection appeared in all animals at the injection site, except for the group pretreated with nanosystem embedded stabilized EO (Figure 6). No mortality rate was registered on the entire duration of the experiment.



**a** **b**

**Figure 6:** The effect on *P. aeruginosa* 11 pathogenesis on mice **a.** the occurrence of a localized infection at the injection ste in all working variants, including infection positive control **b.** no localized infection or other sign of infection in mice treated with nanosystem embedded EO.

# 4. Conclusions

The *in vivo* experimental infection on holoxenic mice, revealed the protective effect exhibited by the nanosystem embedded essential oil against the occurrence of localized infections in mice injected with *P. aeruginosa* viable culture. The *Eugenia carryophyllata* essential oil exhibited an early microbicidal effect on all tested *P. aeruginosa* and *S. aureus* stains, with significantly lower MIC values for *S. aureus* strains. The essential oil modulated the expression of soluble virulence factors, by increasing the production of lipase in both species, and of DN-ase, gelatinase, haemolysins and lecithinase, particulary in *P. aeruginosa* strains. The anti-apoptotic effect obtained on HeLa cells suggests a possible anti-tumoral effect of the essential oil. Our *in vitro* and *in vivo* studies for establishing different biological activities of *the E. caryophilata* essential oil suggest that they could be improved and stabilized by embedding it in a nanosystem that could act as a delivery system formulation.

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