

Hybrid nanostructured material for biomedical applications

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ABSTRACT

The purpose of this study was to assess the antibacterial activity of a novel hybrid organic-inorganic nanostructured material based on Fe₃O₄/PEG₆₀₀/Citrus maxima essential oil. The core/shell material was synthesized by reacting PEG₆₀₀ with Fe₃O₄ nanoparticles, under microwave condition. The core/shell was modified by adsorption of C. maxima essential oil as an extra-shell. The nanostructured material was characterized by HR-TEM and FT-IR. The hybrid nanomaterial was screened for their *in vitro* antimicrobial activity against Gram – positive and Gram-negative bacterial strains. The *in vitro* assay of the influence of the magnetic biomaterial on bacterial adherence to cellular and acellular substrata and biofilm development demonstrated that novel hybrid nanostructured materials could be used for the development of novel antimicrobial materials with anti-adherent properties.

Keywords: C. maxima, core/shell, hybrid nanostructured biomaterial, antibiofilm, antimicrobial activity

1. Introduction

As biomaterials are defined as any matter, surface, or construct interacting with biological systems, they have a great potential for biomedical applications. The biomaterials are used for the design of medical devices that replace parts or substitute functions of living systems and are intimately related with their tissues. To date, biomaterials are used clinically and experimentally in the field of implantology, drug delivery, extracorporeal blood separation columns and so on. The materials meant for assisting in any course of medical treatment are routinely being made up of many different types of surfaces. The medical device surfaces may act as a substrate for microbial attachment, the initial step in colonization and biofilm formation [1].

Despite the beneficial role of biofilms in environmental technologies and bioprocesses, they can be detrimental to both human life and industrial processes by causing infections, food and water contamination, biofouling etc. [1, 2]. Antibiotic recalcitrance/tolerance is the major obstacle faced by the medical community in regard to biofilm related infections, several well-recognized puzzles in microbiology remained unsolved for decades deriving from this, i.e. latent bacterial infections,

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unculturable microorganisms, persister cells and difficult treatment. Approximately 70% of bacterial strains frequently associated with nosocomial infections are resistant to at least one of the drugs commonly used to treat them [3]. For any medical device the infection related complication represents a risk factor which cannot be controlled and affects the performance of the device [4].

The continuous emergence of microbial resistance to existing antibiotics and the decline in the formulation of new one have generated an increasing interest in herbal medicines with anti-infective potential [2]. Essential oils are complex multicomponent mixtures originating from plants, well known for their multiple biological activities, including their antimicrobial properties [5].

The purpose of this study was to quantify the antimicrobial and antibiofilm activity of a novel hybrid inorganic/organic material based on *Citrus maxima* essential oil.

2. Experimental section

2.2. Extraction and characterization of *C. maxima* essential oil

C. maxima epicarp, fresh samples was manually grounded and hydrodistilled in a Neo-Clevenger type apparatus performing a simple microwave assisted extraction. Chemical composition was settled by GC-MS analysis. Gas chromatographic analysis was performed using an Agilent 6890 Series GS System gas chromatograph fitted with a splitless injector for a low background with an injector liner split/splitless under a column head pressure of 12.5 psi and H_2 as carrier gas at a flow rate of 1.2 ml/min. Oven temperature was programmed from 50 to 300 °C at 5 °C/min. Injector and detector temperatures were 250 °C. A capillary column DB5-MS fused-silica J&W Scientific Inc. (30m×0.25mm i.d.; 0.25µm film) was used. 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. Identification of the chromatogram peaks as well as quantification of the compounds was performed following the methodology previously described [6, 7].

2.1. Synthesis and characterization of hybrid nanostructured biomaterial

Nanoparticles of Fe_3O_4 were synthesized by hydrolysis in an aqueous solution containing ferrous and ferric salts with KOH. The dimensions of Fe_3O_4 nanoparticles were in the 5-10 nm range and they were previously characterized by High Resolution Transmission Electron Microscopy [8-11].

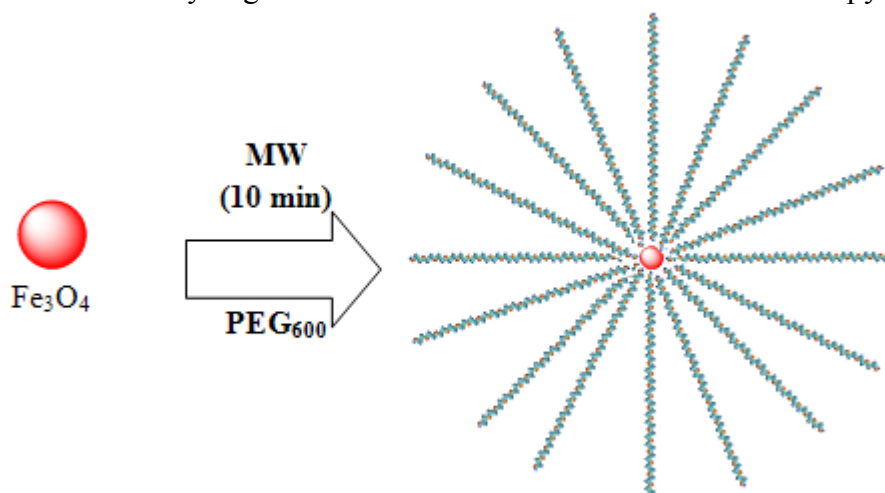


Figure 1: Synthesis of core/shell under microwave condition

Core/shell nanoparticles (fig. 1) have been synthesized under microwave conditions [8-11]. The $\text{Fe}_3\text{O}_4/\text{PEG}_{600}$ hybrid nanomaterial was characterized by FT-IR. IR spectroscopic measurements were performed using a Nicolet 6700 spectrometer provided with a ZnSe crystal based ATR module. The spectra were recorded over the wavenumber range of $650\text{--}4000\text{ cm}^{-1}$ with a resolution of 4 cm^{-1} for Fe_3O_4 as well as $\text{Fe}_3\text{O}_4/\text{PEG}_{600}$. Essential oil of *C. maxima* has been adsorbed on the surface of nanostructured material as an extra-shell (fig. 2). The excess of the essential oil was removed by applying a magnetic field and washing with hexan. The hybrid nanostructured material was dried at 30°C over night.

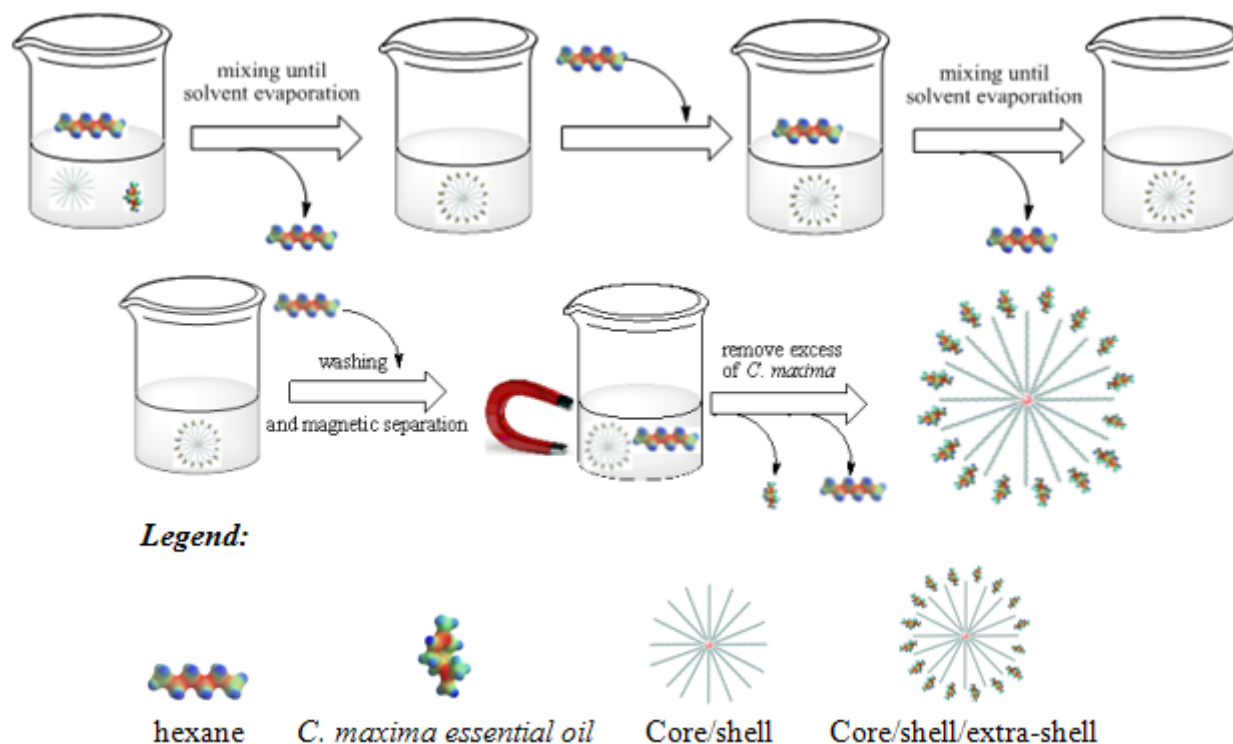


Figure 2: Schematic representation of extra-shell adsorption on hybrid nanostructured material

2.2. Assessment of the antibacterial activity of the essential oil

Collection as well as recently isolated from clinical specimens bacterial strains were used for assessing the antimicrobial effect, belonging to the following species: *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* MRSA 1684, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecalis* 86, *Escherichia coli* ATCC 25922, *Escherichia coli* ESBL 1586, *Klebsiella pneumoniae* 1771.

The antibacterial activity of *C. maxima* essential oil was quantified by a kill-time curve method. Microbial suspensions performed in liquid medium (MH broth) were left in contact with the essential oil diluted with dimethyl sulfoxide (DMSO) (1:1, v/v) up to 30 minutes. The obtained inocula were seeded thereafter onto solid medium (MH) after 1, 3, 5, 15 and 30 minutes of contact, incubated at 37°C for 24 h in order to perform the viable cell counts. The assessment of bacterial strains susceptibility to nanoparticles impregnated with essential oil was performed by following the minimum inhibitory concentration (MIC) protocol using liquid medium twofold microdilutions in 96 multi-well plates.

2.3. Bacterial strains adherence on inert and cellular substratum

CV (violet crystal) staining technique was used for quantifying bacterial strains adherence to the inert substratum represented by the plastic wells. Negative controls for nanoparticles and DMSO were used. After performing the MCI protocol, the wells were subsequently emptied, washed 3 times with PBS (phosphate buffered saline), fixed with cold methanol and stained with CV 1%

solution for 30 min. The biofilm formed onto the plastic wells was resuspended in 30% acetic acid and the intensity of the colored suspension was assayed by measuring the absorbance at 490 nm [10, 12].

For the assessment of bacterial adherence on cellular substratum a modified Cravioto method (Cravioto, 1979) was used [1]. HeLa cells were routinely grown on 6 multi-well plates in MEM enriched with 10% heat-inactivated (30min at 56°C) fetal bovine serum (Gibco BRL), 0.1mM nonessential amino acids (Gibco BRL), and supplemented 0.5 mL of gentamycin (50 µg/mL) (Gibco BRL) and incubated at 37°C for 24 h. HeLa cells monolayers grown in 6 multi-well plastic plates were used at 80% confluency. Essential oil impregnated nanoparticles suspensions in liquid medium (MH) (5mg/mL) were obtained, seeded with 0.5 McFarland bacterial suspensions for each strain and incubated for 5 hours. For the adherence assay, the HeLa cell monolayers were washed 3 times with PBS and 1mL MEM medium and 100 µL from the obtained cultures were added. The inoculated plates were incubated for 2 h at 37°C. After incubation, the monolayers were washed 3 times with PBS, fixed with cold methanol and stained with Giemsa stain solution (1:20). The plates were washed, dried at room temperature overnight, and microscopically examined.

3. Results section

3.1. GC-MS analysis of *C. maxima* essential oil

There were performed two extractions and the average yields of essential oil (v/w %) normalized to total plant mass (fresh weight) were 0.97 % and 1.01% respectively. The main components found in essential oil of *C. maxima* epicarp were: limonene and β -pinene, with 74.878 and 21.267% from the total area.

Table 1: Identification of compounds by GC-MS and Kovats index

Peak	T _R	%	Compound	identification*[6,7]
1	6.696	0.284	α -pinene	RI, MS
2	7.255	21.267	β -pinene	RI, MS
3	8.374	74.878	Limonene	RI, MS
4	8.97	0.203	(E)-ocimene	RI, MS
5	10.545	0.053	Linalool	RI, MS
6	14.747	0.124	Citral	RI, MS
7	18.234	0.056	geranyl acetate	RI, MS
8	19.623	0.074	β -caryophyllene	RI, MS
9	20.66	0.036	Farnesene	RI, MS
10	21.232	0.722	δ -Elemene	RI, MS
11	28.828	1.254	Nootkatone	RI, MS
Total		98.951		

*MS: Identification by NIST MS library, RI: Identification by Kovats index

3.1. Fourier Transform infrared spectroscopy (FT-IR) characterization of hybrid organic/inorganic nanomaterial

In Figure 3 the IR spectrum of Fe₃O₄ and Fe₃O₄/PEG₆₀₀ are presented. The main absorption bands of Fe₃O₄/PEG₆₀₀ are: 845, 944, 1093, 1248, 1348, 1451, 2865 and 3454 cm⁻¹.

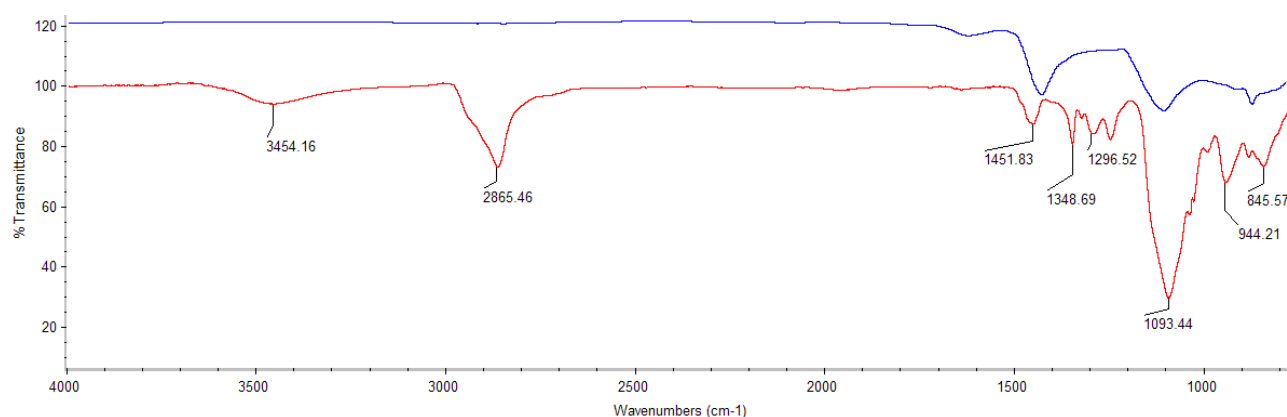


Figure 3: FT-IR spectra of Fe_3O_4 (blue line) and $\text{Fe}_3\text{O}_4/\text{PEG}_{600}$ – core/shell (red line)

3.2. Antibacterial activity of the essential oil.

The essential oil exhibited a low antibacterial activity on the tested strains. The earliest and most evident bactericidal effect was obtained for *E. coli* ATCC 25922. For *S. aureus* and *E. faecalis* strains a growth inhibition effect was noticed (fig. 4).



Figure 4: Early bactericidal effect of the *C. maxima* essential oil on the *E. coli* ATCC strain

3.3. Bacterial adherence on inert substrata.

The quantitative assay of the antimicrobial activity (MIC protocol) revealed that the essential oil impregnated nanoparticles exhibited a low MIC (0.072mg/mL) only against *E. faecalis* 86, for the other strains the microbial growth being slightly stimulated by the tested materials (fig. 5). An inhibition of bacterial adherence to the inert substratum was observed for the *E. coli* ESBL 1586, *E. coli* ATCC and the two *E. faecalis* tested strains, but suprisingly, no dose-effect correlation could be established, the antibiofilm activity being noticed at an intermediary concentration (0.03 mg/mL) (fig. 6). The high concentrations of the nanoparticles slightly stimulated the bacterial adherence to the inert substratum. These results are supported by the microscopic examination data, showing that suspended essential oil impregnated nanoparticles proved to be a preferential support for the bacterial adherence, forming mixed aggregates, that either adhered to the plastic wells or offered an enlarged surface for bacterial growth in suspension phase, the growth rate exceeding the potential

antimicrobial activity of the essential oil, as reflected in the low antimicrobial activity revealed by the MIC assay.

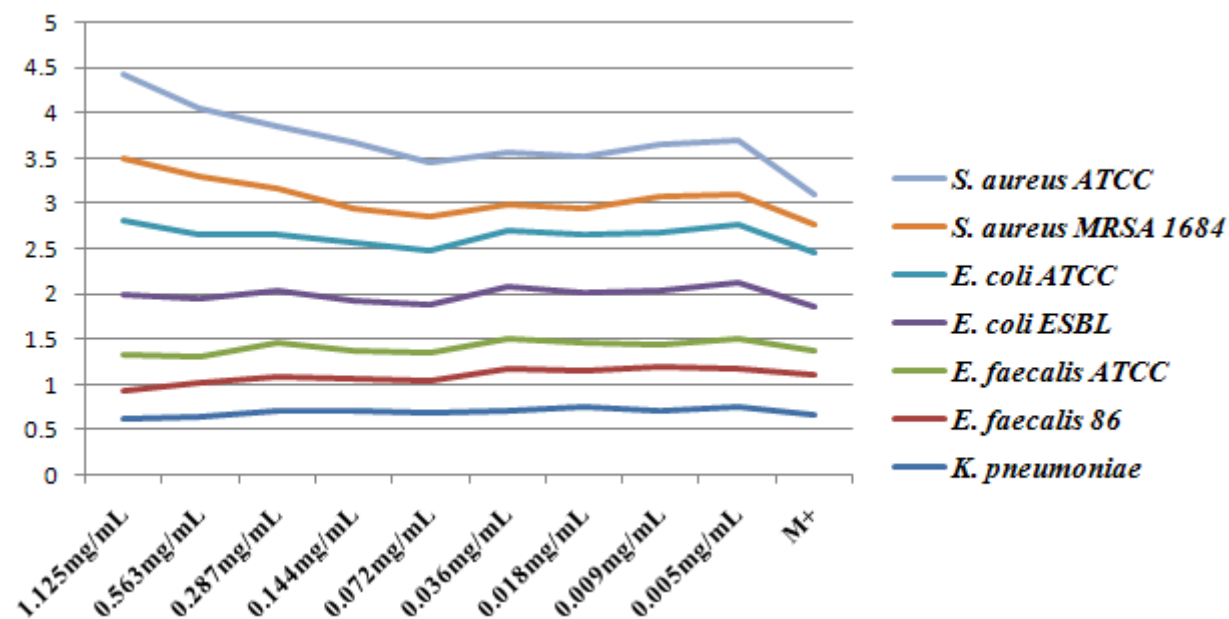


Figure 5: Influence of hybrid nanostructured material on the microbial growth rate of different bacterial strains (M+ - positive control, i.e. liquid medium seeded with bacterial inocula)

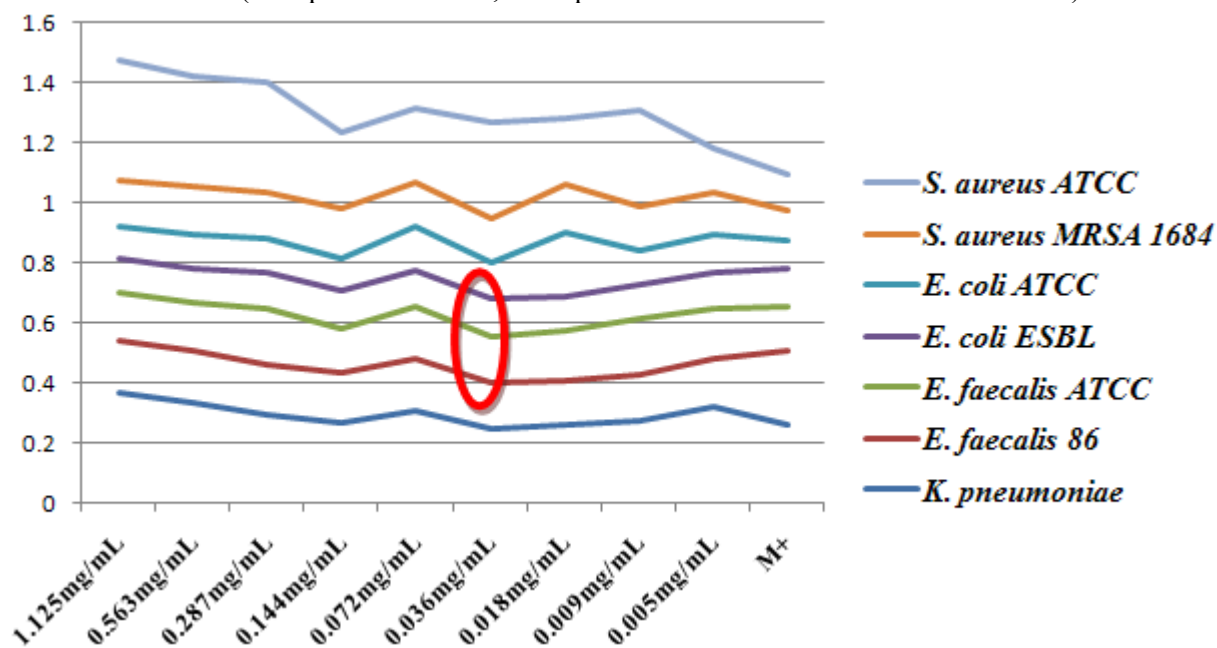


Figure 6: Influence of hybrid nanostructured material on the microbial strains ability to develop biofilms on the inert substratum represented by plastic wells (M+ - positive control, i.e. liquid medium seeded with bacterial inocula)

3.4. Adherence on cellular substrata

The tested bacterial strains formed aggregates with the impregnated nanoparticles, showing a reduced ability to adhere to the cellular substrata. However, when present, a diffuse aggregative adherence pattern to the HeLa cells was observed (fig. 7).

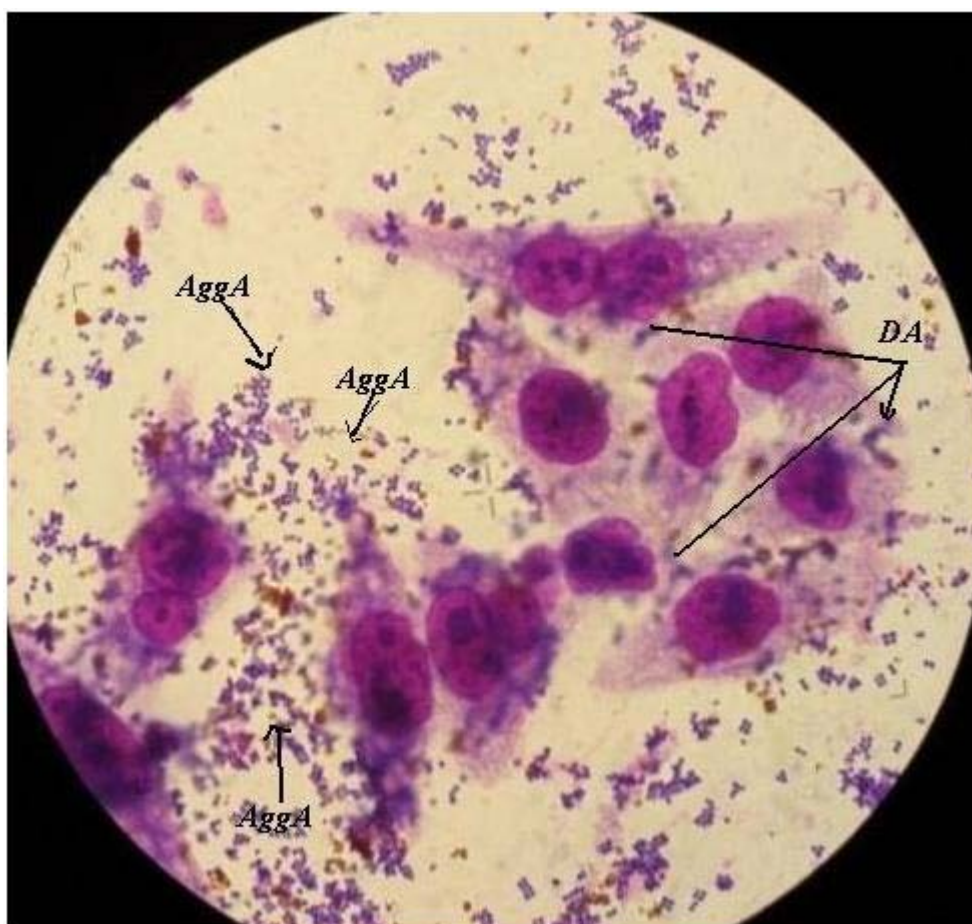


Figure 7: Microscopic image of the HeLa cells monolayer infected with bacterial cultures (*S. aureus* ATCC 25923) obtained in liquid medium containing essential oil impregnated nanoparticles (Giemsa staining, immersion oil, x1000)

4. Conclusions

Microwave-assisted hydrodistillation proved to be a better method for essential oils extraction in terms of yield and time of extraction comparing with previously tested simple extraction technique. The obtained hybrid nanostructured biomaterial exhibited a low antimicrobial activity. The antibiofilm activity is dependent on the suspended nanostructured biomaterial concentration and the tested strain. The microbial adherence to the inert substratum was inhibited by average concentrations of nanoparticles only in case of *E. coli* and *E. faecium* strains, while the adherence to the cellular substratum was inhibited for all tested strains, irrespective to the nanoparticles concentration. Our results recommend the hybrid inorganic/organic nanomaterial with low concentrations of active compounds matrix for obtaining medical devices formulations with antiadherence surfaces.

5. References

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