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The use of fluorescent optical respirometry to study the antimicrobial activity of plant products and evaluation of the pharmaceutical preparations

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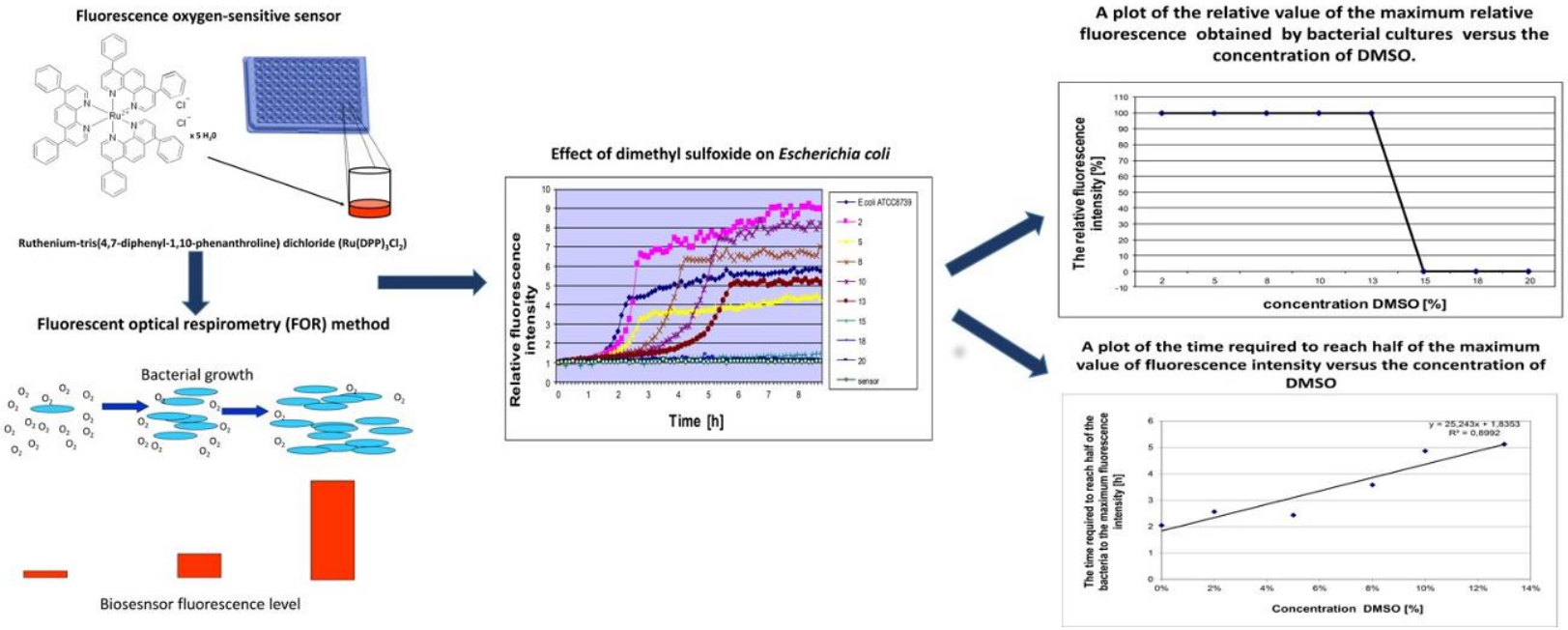
The use of fluorescent optical respirometry to study the antimicrobial activity of plant products and evaluation of the pharmaceutical preparations

Graphical Abstract



Abstract: A fluorescence oxygen-sensitive sensor, ruthenium-tris(4,7-diphenyl-1,10-phenanthroline) dichloride ($Ru(DPP)_3Cl_2$), the fluorescence of which depends on the amount of oxygen in the test sample, was applied in the fluorescent optical respirometry (FOR) method. Molecular oxygen is a fluorescence quencher. Growing microorganisms consume oxygen, thus influencing the intensity of fluorescence in the sample. The fluorescence optical respirometry test method was performed to evaluate the effect of chemical (DMSO) and environmental (temperature, heavy metals: $ZnCl_2$, $NiSO_4x7H_2O$, $CuSO_4x5H_2O$) factors, plant extracts on aerobic bacteria. FOR allows to detect bacteria in sterile and non-sterile pharmaceutical products. It has been shown that MIC values obtained by fluorescence optical respirometry are consistent with the results of the MIC determinations made by serial dilution method. The FRO method allows detection of growing bacteria in pharmaceutical preparations in real time. This method also allows rapid, unequivocal detection and counting of living bacterial cells, and also allows to check the durability of the preservation of pharmaceutical products.

Keywords: fluorescence optical respirometry **;**ruthenium-tris(4,7-diphenyl-1,10-phenanthroline) dichloride ($Ru(DPP)_3Cl_2$); plant extracts; oxygen sensor; pharmaceutical products;

Introduction

The determination of the number of microorganisms is very important in the biotechnology, pharmacy and food industries. Monitoring the quality of pharmaceuticals and food products requires fast, sensitive and selective methods to detect a small number of viable bacterial cells. Isolation of the natural compounds presented in the food with antibacterial properties requires testing of the many samples, against many bacteria in a short time.

Introduction

Counting bacteria on agar plates, membrane filters, and using the “most probable number" are basic methods used to determination of the living bacterial cells. The plate count and membrane filter methods require a long incubation time (1-3 days), colonies may be formed by several related species of bacteria, and full identification takes up to seven days. The "most probable number" method gives the opportunity to determine the approximate number of bacteria in a diluted test sample by measuring turbidity after incubation. The methods requires clear, dispersed samples without inhibitory factors

Introduction

Techniques based on the detection of nucleic acids and fatty acid assays are characterised by short detection time (6 to 18 h) and high sensitivity (about 10 cfu/ml). Unfortunately, cell detection requires several steps, specialised equipment and trained personnel. It is also difficult to distinguish between dead and living cells.

The serial dilution method in broth, used in clinical microbiology and studies of the antimicrobial effect of various chemical compounds, allows for determination of the minimum inhibitory concentration (MIC).

This method can be used to test (examine) a large number of samples on several strains of bacteria; unfortunately, the length of assay time and the impact of the physical properties of the sample affect the results these are some of the few disadvantages of the method. However, it is considered a standard method of determining the chemical toxicity of bacteria.

Introduction

Another method of microbial growth analysis in cultures using so-called fluorescence optical respirometry (FOR). This method is based on analysis of the fluorescence of an oxygen-sensitive sensor, the fluorescence of which is dependent on the amount of oxygen in the tested sample.

Molecular oxygen quenches the fluorescence; microorganisms growing in culture consume the oxygen, thus affecting the fluorescence intensity of the sample. By analysing variations in the intensity of the fluorescence in the cultures it can track the metabolic activity of microorganisms (Figure1).

FOR assays have been successfully applied to monitoring the oxygen respiration of yeast, bacteria and mammalian cells, as well as for measuring the action of some antibiotics and chemicals compounds.

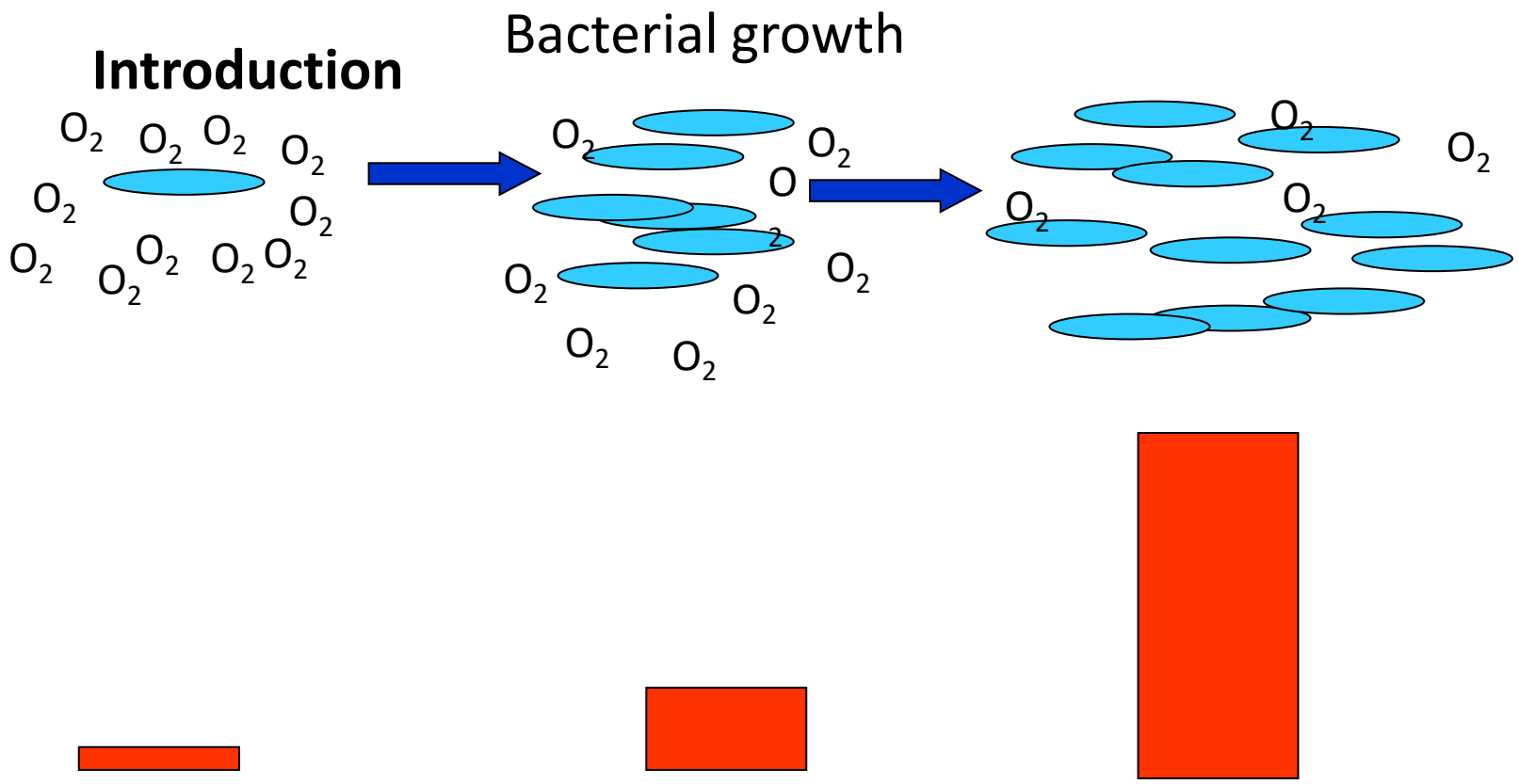
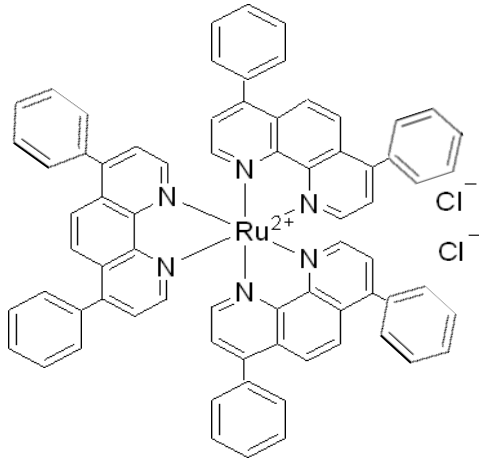


Figure 1. Growth of bacteria in the presence of a fluorescent sensor. Molecular oxygen quenches the fluorescence; microorganisms growing in culture consume the oxygen, thus affecting the fluorescence intensity of the sample.

Introduction

In the present study we used oxygen-sensitive sensor: ruthenium-tris(4,7-diphenyl-1,10-phenanthroline) dichloride ($Ru(DPP)_3Cl_2$) (Figure 2).

A



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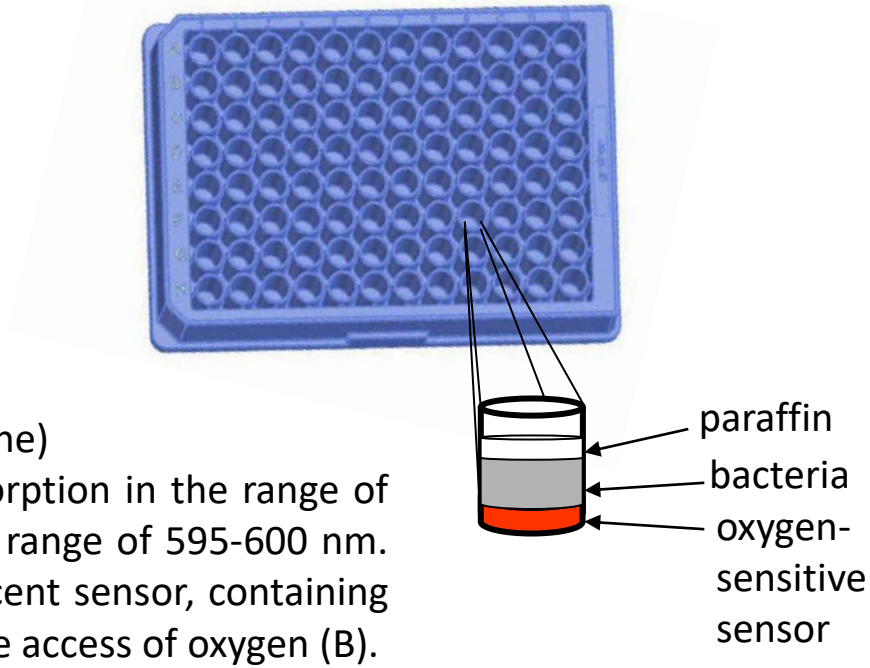

Figure 2. Ruthenium-tris(4,7-diphenyl-1,10-phenanthroline) dichloride ($Ru(DPP)_3Cl_2$) (A). Maximum wavelength absorption in the range of 440-475 nm, maximum emission of fluorescence in the range of 595-600 nm. View of a well in a 96-well plate, coated with a fluorescent sensor, containing the test sample (bacteria) and paraffin, which cuts off the access of oxygen (B).

Results and discussion

Effect of dimethyl sulfoxide on bacteria

The dimethyl sulphoxide (DMSO) is often used as a solvent in studies of antimicrobial properties of chemical compounds, what is necessary in the case water-insoluble compounds. It is known that DMSO at higher concentrations has a toxic effect on microorganisms. There have been reports of the possible use of DMSO as an active substance in medicines.

The results of experiments that analysed the effects of different concentrations of DMSO on bacteria e.g *Escherichia coli,* show the figures on the next slide.

Results and discussion

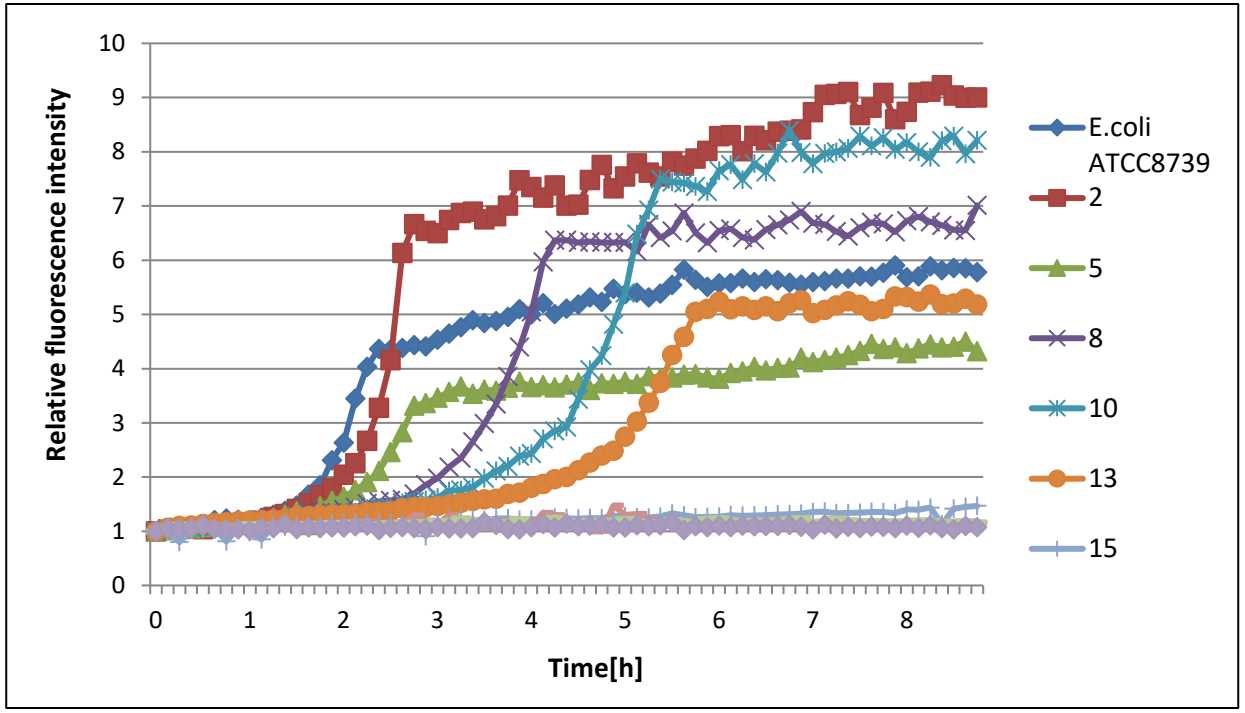

Figure 3. A plot of the relative fluorescence intensity of *Escherichia coli* (ATCC 8739) culture against time for different concentrations of DMSO in culture. Data presented in the legend containing concentrations of DMSO expressed in percentage.

Figure 3 makes it clear that DMSO concentrations, which inhibit the growth of bacterial cultures, are higher than 13% (vol/vol) for *E coli*. The curves of fluorescence intensity obtained for culture in the presence of 2, 5, 8, 10, and 13% DMSO retain the same sigmoidal character, but in the presence of increasing concentration of DMSO they are increasingly shifted in time. The higher the concentration of DMSO, the greater the retardation.

Results and discussion

The dependence between the time, at which the fluorescence intensity reaches half the maximum value and the concentration of DMSO is shown in the figure above for *E.coli* (Figure 4)

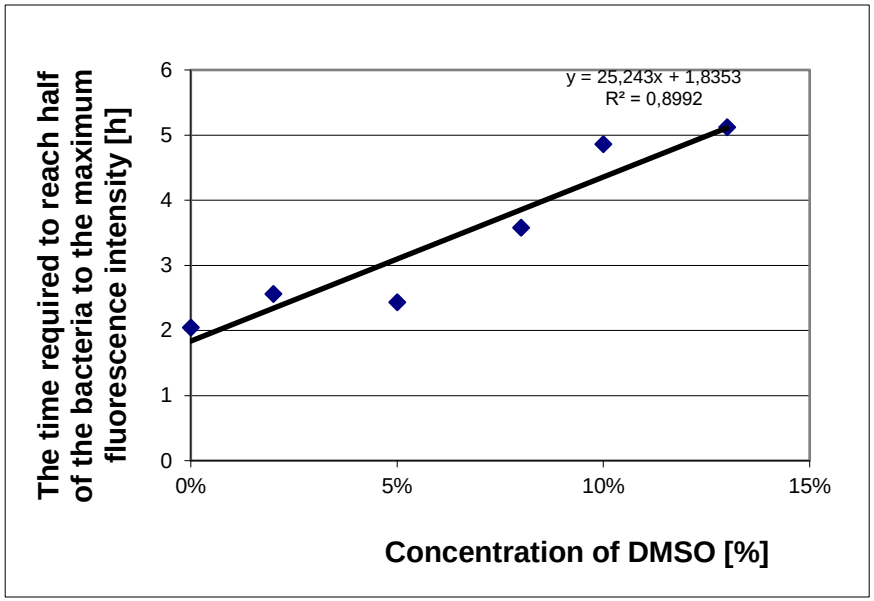

Figure 4. A plot of the time required to reach half of the maximum value of fluorescence intensity versus the concentration of DMSO.

The graph in figure 4 shows the linear dependence of the culture-specific parameter on DMSO concentration.

The graph shows also, that as the concentration of DMSO increases in the sample of bacteria, the time required to reach half of the maximum value of fluorescence intensity is proportionally extended.

Results and discussion

The minimum inhibitory concentration (MIC) determination for DMSO on the basis of optical fluorescence respirometry gives values identical to the MIC values determined by the serial dilution method.

Optical respirometry method provides much more information compared with the traditional method, particularly when this relates to the toxic effect of low concentrations of DMSO on bacteria. In addition, the fluorescence optical respirometry will significantly reduce the time of the experiment. The MIC values can be obtained after four hours. In contrast, by increasing the initial concentration of bacteria in culture, this result can be obtained with a significant reduction in test time to 1-2 hours.

Results and discussion

Measurement of the effect of plants extracts for bacteria

Despite increasing progress in the field of microbiology, infectious diseases are still a significant cause of morbidity and mortality worldwide, where drug-resistant strains of pathogenic bacteria and fungi are becoming more common. The need to develop more effective and safe antimicrobials drives research focused on plant-derived compounds as a source of new antimicrobial drugs. Therefore, preliminary in vitro screening for the antimicrobial activity of plant extracts can serve as a guide to selecting those with significant activity as potential resources for such new drugs and thus as promising candidates for further phytochemical and pharmacological studies

Results and discussion

Examples of the use of FRO to study the effect of *Lonicera caerulea* var. *edulis* 'Wojtek' flowers extracts (Figure 5A) and *Rubus idaeus* shoots extracts (Figure 5B) against bacteria e.g *Staphylococcus aureus* are presented in the figures below.

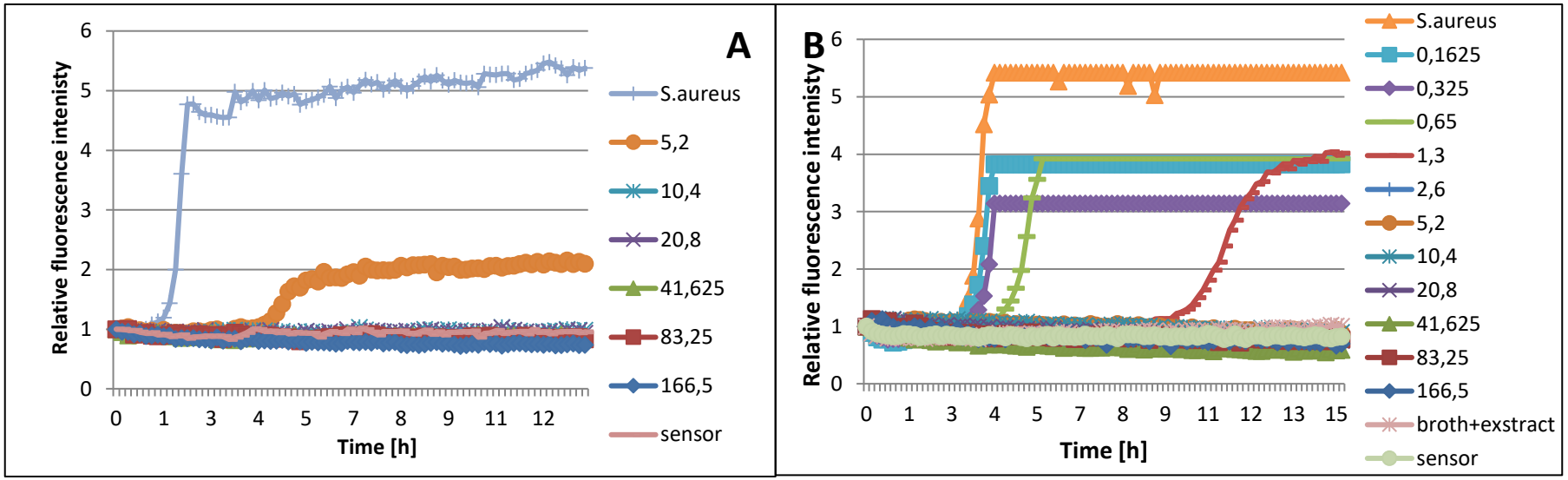

Figure 5. The dependence of the relative fluorescence intensity of bacterial culture of *S. aureus* strain against time for different concentrations of *Lonicera caerulea* var. *edulis* 'Wojtek' flowers extracts (Figure 5A) and *Rubus idaeus* shoots extracts (Figure 5B). In the legend the extracts concentration expressed in mg/mL was given.

Results and discussion

Figure 5A and 5B above makes it clear that extracts concentrations, which inhibit the growth of bacterial cultures, are the identcal as the MIC results obtained by serial dilution method. The curves of fluorescence intensity obtained for culture in the presence of concentrations lower than MIC retain the same sigmoidal character, but in the presence of increasing concentration of extracts they are increasingly shifted in time.

The results obtained with FOR are identical to the traditional method for all bacteria. However, the results obtained are more objective, independent of the physical properties of the sample, in a much shorter time.

Results and discussion

FRO method suitability test for the detection of aerobic bacteria in sterile and non-sterile pharmaceutical products.

FRO method suitability test was performed on the basis of guidelines contained in monographs 2.6.1 and 2.6.12, chapters usefulness of the method, of the European Pharmacopoeia 11.0.

The microorganisms used: *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 663, *Escherichia coli* ATCC 8739, *Salmonella enterica* ATCC 13076, *Pseudomonas aeruginosa* ATCC9027, *Candida albicans* ATCC 10231

Results and discussion

FRO method suitability test for the detection of aerobic bacteria in sterile and non-sterile pharmaceutical products.

The sterility test is applied to substances, preparations or articles which, according to the Pharmacopoeia, are required to be sterile.

To test the presence of aerobic bacteria in pharmaceutical products we transferred the appropriate amount of the preparation directly into the culture medium, so that the volume of the product is not more than 10 percent of the volume of the medium. After the content of the container or containers has been transferred to the culture medium, a small number of viable micro-organisms (not more than 100 CFU) were added to the medium. Suitable strains of micro-organisms are indicated in Table Ph.Eur.

Results and discussion

FRO method suitability test for the detection of aerobic bacteria in sterile and non-sterile pharmaceutical products.

Microbiological examination of non-sterile products: microbial enumeration tests. The tests allow quantitative enumeration of mesophilic bacteria and fungi that may grow under aerobic conditions.

We followed the instructions in Ph.Eur. The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. The method for sample preparation depends upon the physical characteristics of the product to be tested.

We added a sufficient volume of the microorganism suspension to the prepared samples and controls to obtain an inoculum of no more than 100 CFU. The volume of the inoculum suspension is not exceeded 1 percent of the volume of the diluted product.

Results and discussion

Products used for testing: ear drops „Ototalgin” manufactured by Farmina sp.zoo, Kraków; eye drops “Polcrom” manufactured by Polfa Warszawa, Warszawa; cough syrup “Dexa Pico” manufactured by Herbapol-Lublin, Lublin, Injectio Natrii Chlorati Isotonica volume 10mL manufactured by Polpharma, Starogard Gdański. Diet supplement herbal syrup “Gardlox” manufactured by S-lab sp zoo, Mirków. Poland; (Figure 6,7)

Results and discussion

FRO method suitability test for the detection of aerobic bacteria in sterile pharmaceutical products on example Injectio Natrii Chlorati Isotonica (INCI) of *Escherichia coli* (Figure 6)

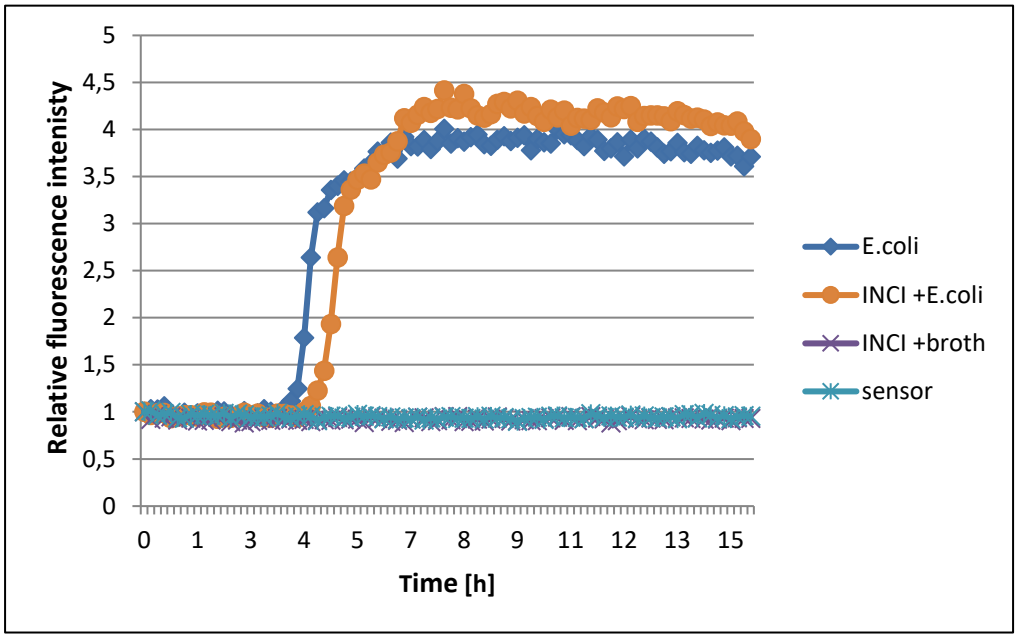

Figure 6. The dependence of the relative fluorescence intensity of bacterial culture of *E.coli* against time for Injectio Natrii Chlorati Isotonica (INCI).

The figure 6 shows the curves of fluorescence intensity obtained for culture in the presence of sterile product. It have the sigmoidal character, characteristic for the bacterial growth. The use FOR is possible to detect aerobic bacteria in sterile pharmaceutical products, even after 4 hours, when the initial suspension is not greater than 10^2 cfu/ml. With a lower inoculum, the detection time will probably be longer, but it will be shorter than that time required for the traditional method (14 days).

Results and discussion

FRO method suitability test for the detection of aerobic bacteria in non-sterile pharmaceutical products cough syrup “Dexa Pico” and diet supplement herbal syrup “Gardlox”; e.g. *Staphylococcus aureus* (Figure7)

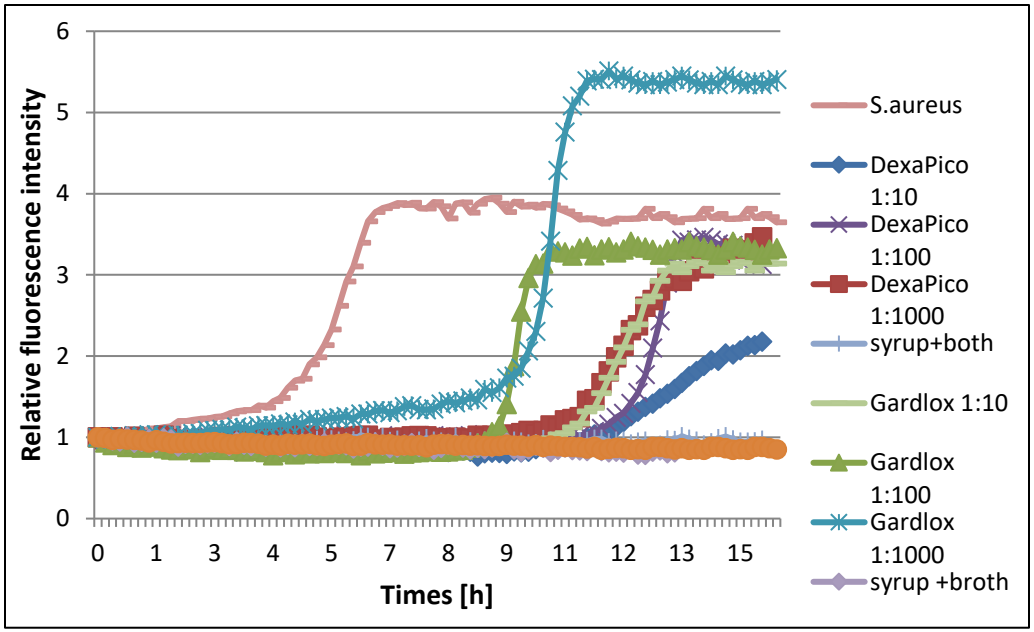

Figure 7 The dependence of the relative fluorescence intensity of bacterial culture of *S.aureus* against time for different dilutions of Dexa Pico and Gardlox.

Using FOR we can detect aerobic bacteria in non-sterile pharmaceutical products (Figure 7). The curves of fluorescence intensity obtained for culture in the presence of different dilutions of non-sterile product have the same sigmoidal character, but in the presence of increasing concentration of products they are increasingly shifted in time. It is important to neutralize the preservatives present in the products. Detection time is shorter than that required for the traditional method (≤ 3 days).

Conclusions

Our researcher demonstrated the usefulness of FOR as a quick and objective screening method for demonstrating the antimicrobial properties of plant extracts.

We have shown that with FOR we can quickly and clearly detect aerobic microorganisms in sterile and non-sterile pharmaceutical products.

We believe FOR can be useful for the pharmaceutical and cosmetic preservation test, which will be our next stage of research