

**UNIVERSITY OF PÉCS**  
**Doctoral School of Chemistry**

**Investigation of antimycotic susceptibility and antifungal  
activity of Mannich ketones by means of  
chip-based methods**

**PhD Thesis**

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## 1. Introduction

The increasing incidence of the life-threatening, invasive fungal infections is a world-wide problem. The *Candida albicans* is the most prevalent pathogen but the incidence of the non-albicans *Candida* infections and the number of *Aspergillus sp.* caused diseases is increasing, too. The palette of available antifungal agents contains classic, widely used drugs, as well as agents introduced in the last decade. However, the increasing number of fungal infections, the spread of resistance and the demand for the agents with better pharmacokinetic and side-effect profile keeps the research of the antifungal agents a current field of investigations. The same reasons make important the development of fast, time and labor-work saving diagnostic and antifungal-susceptibility tests compared to the traditional ones.

The flow cytometry has been used for microbiology tests, in the research of bacteria, fungi and antimicrobial susceptibility tests. The use of the lab-on-a-chip systems is a peculiar trend in our days. These systems allow a chip platform to realize various analytical challenges in micro dimensions with little demand of sample. However the data available about chip-based microfluidical flow cytometry tests are poor.

In my current work I preformed the antifungal susceptibility tests on *Candida sp.* with classic and microfluidic flow cytometry systems. Beside the traditional antimycotics I examined the anti-*Candida* effect of the potential cytotoxic, antibacterial and antifungal agents Mannich-ketones by chip-based flow cytometry and chip-based capillary gel electrophoresis.

## 2. Aims

The overall aim of this work was the investigation of *Candida* strains by analytical, separation methods. In this quite broad field my concrete aims were:

- the examinations of *Candida* cells by flow cytometry, mainly the determination of antifungal susceptibility (based on literature data).
- The examination of different antifungal agents, on both *Candida albicans* and *non-albicans* strains.
- Based on these results the investigation of an antifungal-susceptibility test by a flow cytometry based chip-platform method, because I did not find such application of this system.
- Beside the traditionally used antimycotics the examination of some different type Mannich ketones with antifungal effect.
- Detection of the qualitative and quantitative changes in the protein profile of *Candida albicans* caused by the Mannich ketones by means of chip-based electrophoresis.
- Study of different type Mannich ketones, the analysis of the relationship between the structure of the molecules and their effect on the protein-profile.

### 3. Materials and Methods

#### 3.1. Materials

In this study international standard **Candida strains** were used, briefly: *Candida albicans* ATCC 90028, *Candida tropicalis* ATCC 90874, *Candida glabrata* 90030, *Candida krusei* 30068.

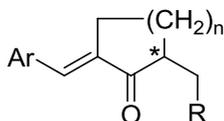
Different **fluorescent dyes** were applied in the flow cytometry based investigations. The propidium iodide (extinction/emission max. 535/617 nm) and the Sytox Green (504/523 nm) are non-permeable nucleic acid dyes capable for the labeling of dead cells. The Syto 60 (652/678) stains both living and dead cells.

Two **antifungal agents** with different mechanisms of action were examined in the susceptibility tests. The **amphotericin B** is a polyene macrolide antimycotic. In my studies it was utilized in the form of Fungizone powder for injection (this contains sodium deoxycholate as adjuvant increasing solubility). The amphotericin B is a fungicide agent, it interacts with the ergosterol components of the fungal cell-wall by forming pores causes the death of the cells. The **fluconazole**, a triazole with fungistatic effect, was utilized in the form of Mycosyst solution for infusion. It inhibits a few steps of the ergosterol biosynthesis and thereby the reproduction of the fungal cells.

#### 3.2. Mannich-ketones

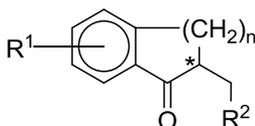
Mannich-bases are widely used in the synthesis of drug molecules for example to increase water-solubility or as intermediates in the preparation of reactive  $\alpha$ -methylene-ketones. The Mannich bases have various biological effects, I would like to emphasize the cytotoxic, antibacterial and antifungal effect of Mannich compounds. The **Mannich-ketones tested in this study**, has been synthesized by my supervisor, Tamás Lóránd in the Department of Biochemistry and Medical Chemistry, University of Pécs, Medical School. The classical Mannich-reaction was performed in ethanol, in the first step 2-arylidene-cycloalkanones intermediates formed in an aldol condensation. The Mannich ketones were isolated as hydrochlorides from the reaction of the corresponding 2-arylidene-cycloalkanone, secondary amine and paraformaldehyde, where the secondary amine was used in excess to prevent competitive aldol-condensation.

The unsaturated and fused Mannich ketones applied in this study are presented in *Table 1* and *2*.



Comp.	n	Ar	R	<i>C. albicans</i> MIC ( $\mu\text{g/ml}$ )
1	1	4'-OCH <sub>3</sub> -C <sub>6</sub> H <sub>4</sub>	4-morpholyl	3.125
2	1	2'-OCH <sub>3</sub> -C <sub>6</sub> H <sub>4</sub>	1-piperidyl	6.25
3	3	phenyl	1-piperidyl	50

*Table 1* – Structure of unsaturated Mannich ketones.



Comp.	n	R <sup>1</sup>	R <sup>2</sup>	<i>C. albicans</i> MIC ( $\mu\text{g/ml}$ )
4	1	H	1-piperidyl	3.125
5	1	H	1-pyrrolidinyl	0.8
6	1	5-OCH <sub>3</sub>	1-piperidyl	3.125
7	2	H	1-piperidyl	12.5
8	2	H	1-pyrrolidinyl	12.5
9	2	H	4-morpholyl	3.125
10	2	H	2-(1,2,3,4-tetrahydro)- izoquinolyl	6.25
11	2	5-OCH <sub>3</sub>	1-piperidyl	12.5
12	2	6-OCH <sub>3</sub>	1-pyrrolidinyl	100
13	2	6-OCH <sub>3</sub>	4-morpholyl	25
14	2	6-OC <sub>3</sub> H <sub>7</sub>	4-morpholyl	25
15	2	7-OCH <sub>3</sub>	1-piperidyl	6.25
16	2	7-OCH <sub>3</sub>	4-morpholyl	12.5
17	3	H	1-piperidyl	6.25
18	3	H	4-morpholyl	12.5

*Table 2.* –Structure of fused Mannich ketones.

### 3.3. Methods

**Flow cytometry** is a method or measuring technique, which is suitable for the examinations of independent cells in a fluid flow. The samples, containing cells in the form of suspensions, exposed to hydrodynamic focusing, then irradiated with a laser source and the optical informations collected, briefly:

- forward light scatter (FSC): inform about the size of the cell
- side light scatter (SSC): proportional to cell granularity or integral complexity
- fluorescence: inform about special feature of the cell, the detected emitted fluorescent intensity is proportional to the amount of fluorophore bonded to the cell

In my study a Becton Dickinson FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) was utilized. It was equipped with a 15 mW (488nm) argon laser and a red diode laser (~635nm). Data collection was in log/log mode, fluorescence intensity, forward and side scatter were collected.

**Microfluidical flow cytometry (cell-chip):** this method based on the principles of flow cytometry but performed on a chip-platform. In this study the flow cytometric part of the Agilent 2100 Bioanalyzer was applied. The measurements were carried out in a pressure-driven system, in the 25x75  $\mu\text{m}$  capillaries of the chip. The apparatus was equipped with a blue LED (470/525 nm) and a red laser (638/680 nm), so it is capable of a two range fluorescence detection. The cells were suspended in a buffer, before measurements the capillary system of the chip was filled with an aqueous solution, while one capillary was filled with a fluorescent dye, which was the reference for the optical system during the measurements. The system is appropriate to sample concentration as far as  $2 \times 10^6$  cell/ml.

**Chip-based electrophoresis:** it is a method based on the principles of capillary gelelectrophoresis. I used the Agilent lab-on-a-chip system for these measurements too, but the electrophoresis block was utilized with the Protein 80 and 230 separation protocols. The capillary system of the chip was filled with a linear non-crosslinked polymer; the separation was based on the size of the molecules. Fluorescent detection was used, the fluorescent dye connected to the proteins through SDS on the chip.

## 4. Results and discussion

### 4.1. Flow cytometric and cell-chip results: detection of living and dead *Candida* cells, differentiation between *Candida* strains.

Flow cytometry is well known for the study of *Candida* strains. However there are only a few studies concerned with cell-chip measurements of fungi. In this study chip measurements was based on the results obtained by conventional flow cytometry so I show my results in that way, too.

Propidium iodide (PI), Sytox Green and Syto 60 nucleic acid dyes were used to differentiate between living and dead fungal cells. In the **flow cytometry** measurements the living and dead *Candida albicans* cells after PI and Sytox Green labeling could be unambiguously separated based on the fluorescence intensity detected in the appropriate area.

The **cell-chip** system collects only the fluorescent signals, therefore only previously dyed cells can be examined. The Syto 60 dye labeled both the living and dead cells, it was good for the determination of the whole cell number of the samples. I achieved the differentiation of living and dead cells with Sytox Green labeling (PI is not compatible with the instrument).

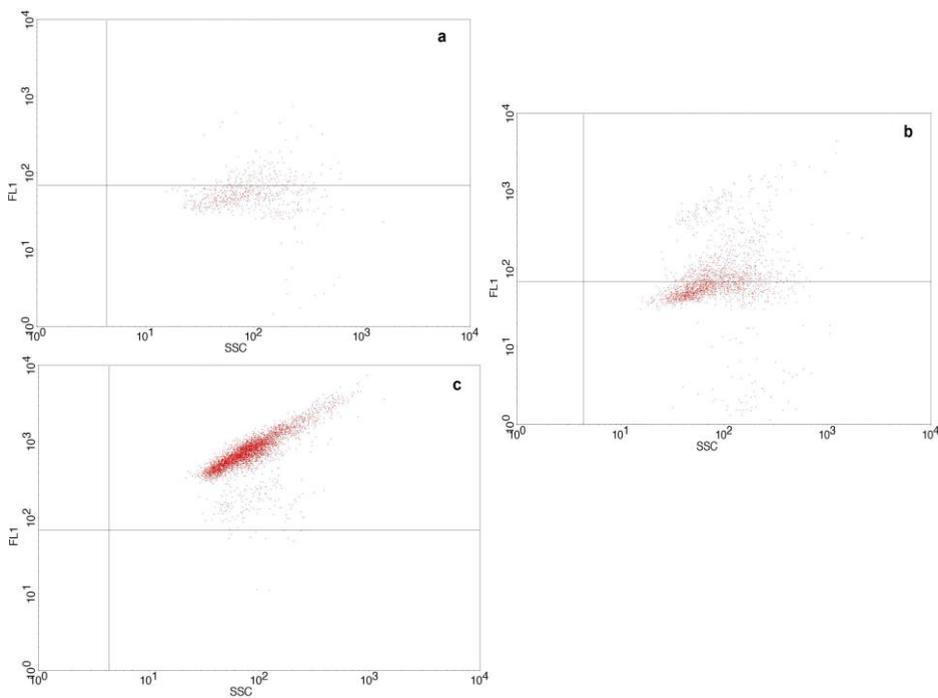
**Different *Candida* strains** were examined. The living and dead cells could be unequivocally separated in case of all *Candida* strains. The different strains could not be separated by this method.

### 4.2. Antifungal susceptibility-tests

The determination of antifungal susceptibility was based on the differentiation between living and dead cells. **Minimal inhibitory concentration (MIC)** was used as the characterization of antimycotic susceptibility; it means the lowest concentration of the antifungal agent which

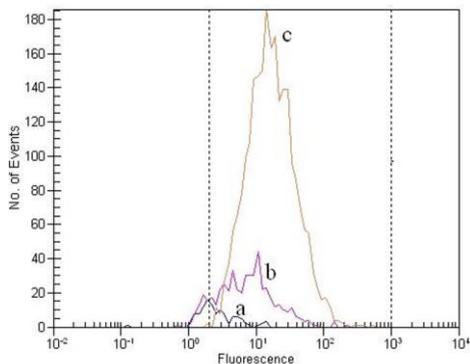
can inhibit the growth of the fungi. The flow cytometry and cell-chip measurements were compared to the standard macrodilution method.

Twofold dilution series were made from each antimycotics and 10-10  $\mu$ l of the prepared *Candida* stock suspensions were added to each tube. After a 24 hour incubation period one half of each sample were stained, the other half were heat-treated before staining, then all samples were measured. **Figure 1** shows these results: *C. albicans* samples were treated with different concentrations of amphotericin B measured by **flow cytometry**.

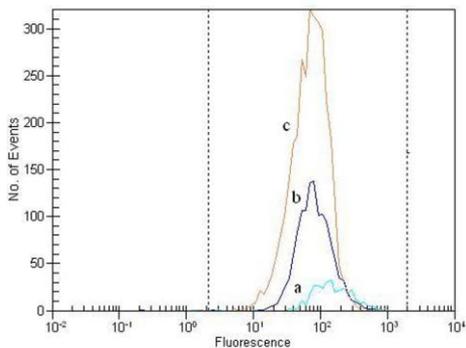


**Figure 1** – Flow cytometry results of *C. albicans* samples treated with amphotericin B, after Sytox Green labeling; heat-treated samples, the fluorescence showed compared to the SSC. The concentration of amphotericin B is 0.625  $\mu$ g/ml on figure a, 0.312  $\mu$ g/ml on b, and 0.156  $\mu$ g/ml on c, respectively.

Based on the results of the flow cytometry measurements the technique was adapted to the **microfluidic cell-chip system**. **Figure 2** and **3** show the cell-chip results of amphotericin B and fluconazole susceptibility tests on *C. albicans*.



**Figure 2** – **Cell-chip** results of **amphotericin B** susceptibility test on *C. albicans*, heat-treated, Sytox Green labeled samples. The concentration of **amphotericin B** is 0.625  $\mu\text{g/ml}$  on figure **a**, 0.312  $\mu\text{g/ml}$  on **b**, and 0.156  $\mu\text{g/ml}$  on **c**, **respectively**.



**Figure 3** - **Cell-chip** results of **fluconazole** susceptibility test on *C. albicans*, heat-treated, Sytox Green labeled samples. The concentration of **fluconazole** is 1  $\mu\text{g/ml}$  on figure **a**, 0.5  $\mu\text{g/ml}$  on **b**, and 0.25  $\mu\text{g/ml}$  on **c**, **respectively**.

At the higher concentration of the antifungal agent the growth of fungi is restricted, the results show only a few, mostly dead cells. In case of lower concentration than MIC the growth of fungi are not inhibited, the results shows lots of mostly living cells, which are visible after heat-treating with Sytox Green labeling.

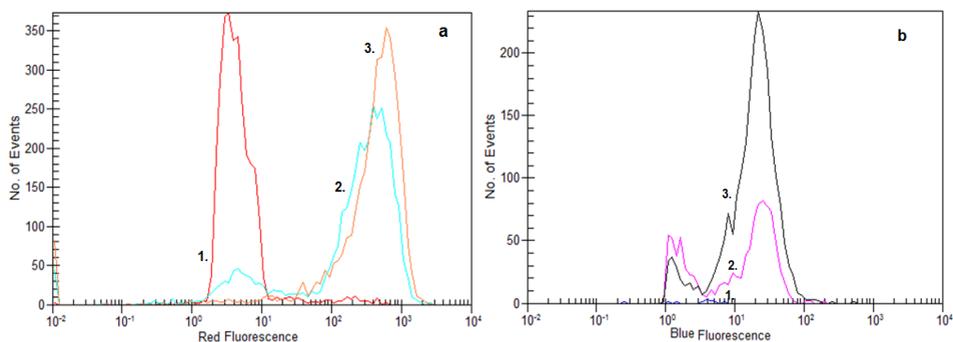
The **MIC value** was defined as the lowest concentration, where the amount of the living cells significantly (min. 50%) decreased compared to the growth control. In case of the amphotericin B this shift was sharper than in case of fluconazole. The difference probably can be explained by the different (fungicid/fungistatic) effect of the antifungal agents.

The **amphotericin B and fluconazole susceptibility** values of the **four Candida strains** obtained by the **three different methods** are shown in **Table 3**.

	Amphotericin B			Fluconazole		
	Macrodil.	FC	Cell-chip	Macrodil.	FC	Cell-chip
<i>C. albicans</i> ATCC 90028	0.312	0.312- 0.625	0.312- 0.625	1	1	0.5
<i>C. glabrata</i> ATCC 90030	0.625	0.625	0.625	>64	>64	>32
<i>C. krusei</i> ATCC 30068	0.312	0.312	0.156	>64	>64	>64
<i>C. tropicalis</i> ATCC 90874	0.156	0.156	0.156	>64	>64	>64

**Table 3.** – MIC values of *Candida* strains obtained by macrodilution method, flow cytometry and cell-chip method ( $\mu\text{g/ml}$ ).

A modified version of this chip-based antifungal susceptibility test was adapted to the MIC determination of unsaturated Mannich ketones on *C. albicans* by Syto 60 and Sytox Green fluorescent labeling. The results show good correlation with the standard methods. **Figure 4** demonstrate the results of compound **1**.



**Figure 4** – determination of MIC values of compound **1** on *C. albicans* by cell-chip method with **Syto 60** (Fig. a) and **Sytox Green** (b) labeling. The concentrations of compound **1** are: **1.** 3.125  $\mu\text{g/ml}$ , **2.** 1,56  $\mu\text{g/ml}$ , **3.** 0.78  $\mu\text{g/ml}$ , respectively.

### 4.3. The effect of Mannich-ketones on the protein composition of *C. albicans*

The effect of **15 fused Mannich ketones** was studied on the **protein-profile of *C. albicans***. Protein profile changes were defined as the appearance of new protein peak on the electropherogram or as the significant increase/decrease in the amount of an existing protein (min. 20% compared to the control).

For this studies the *Candida* cells were grown in a larger amount of broth (400ml), the Mannich ketones were utilized in 2 different concentrations with 1 hour long treatment, simultaneously Mannich-free control sample were made for each compound separately. The proteins were extracted from the fungal cells with a special extraction buffer containing 0.37 M Tris-HCl (pH=7,5), 1mM EDTA, 5mM 2-mercapto-ethanole, 1% Triton X-100 and 1mM phenylmethanesulfonyl fluoride.

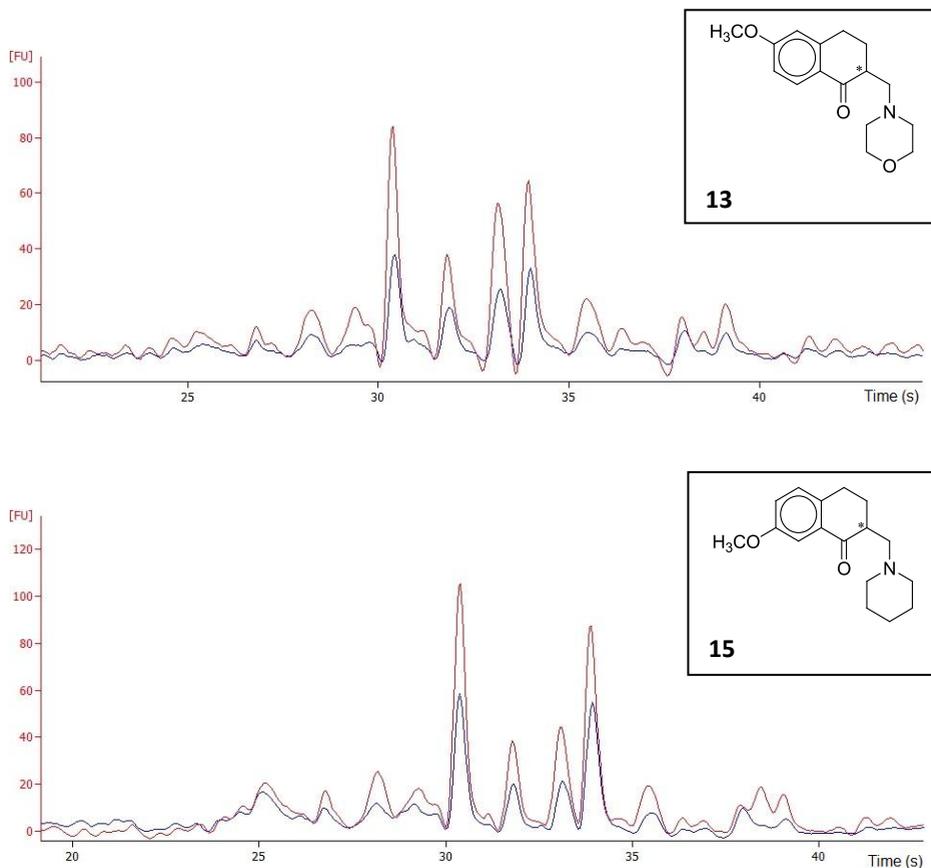
The protein samples were measured by the **capillary gel electrophoresis-based chip system** beside the common polyacrilamide gel electrophoresis. In the chip-based system the separation depends on the size of the protein molecules, due to the utilized reducing agent applied and the SDS, the electropherogram demonstrates the fluorescent intensity compared to the migration time. The method is capable for qualitative and semi quantitative analysis with the aid of internal and external standards.

Based on my studies some of the fused Mannich compounds have significant effect on the proteins of *C. albicans*.

Four compounds out of the fifteen examined, briefly: the **5, 11, 13, 15** compounds caused significant changes. The appearance of new protein could be detected in none of the samples, the effect appears as an increase of the amount of particular proteins, especially in the area of the 30-70 kDa proteins.

**Figure 5** demonstrates the effect of **13** and **15** Mannich ketones on the protein-composition of *C. albicans* in a magnified part of the electropherogram, which shows fluorescence intensity compared to the time. In the electropherogram obtained by chip-based electrophoresis the control samples are shown in blue while the samples

treated with 2xMIC concentrations of the adequate Mannich compound appeared in red. MS studies have been started for the identification of proteins, while samples of one- and two-dimensional electrophoresis gels were tested. Nor the correct proteins identified, neither the appearance of new proteins can be justified until now, further studies are required.



**Figure 5** – enlarged part of the **electropherogram** of *C. albicans* protein samples treated with the **Mannich ketone 13** or **15** obtained by chip-based electrophoresis. The **control samples** are shown in **blue** while the **samples treated with 2xMIC concentrations of Mannich ketones** are shown in **red**.

Based on the results of **15 fused Mannich ketone** studied structure - activity on the protein profile of *C. albicans* relationships cannot be concluded. However a QSAR study carried out by Professor László Prókai ((Department of Pharmacology & Neuroscience, University of North Texas, Health Science Center at Fort Worth, USA) based on my results and the logP values of Mannich ketones. It shows that the ideal logP required for the effect on protein profile is between 1.8-2.5, while the too lipophilic (logP > 2.5) compounds are not effective on the protein profile [L. Prókai, unpublished data].

## 5. Conclusions

In my study **I carried out the antifungal-susceptibility testing of *Candida* cells by flow cytometry**. Two, therapeutically used antimycotics were tested, one with fungicide and one with fungistatic effect. The susceptibility tests were carried out on both *Candida albicans* and *non-albicans* strains.

Based on these results the method was adapted to a microfluidic flow cytometry system which had not been used for similar studies. **Therefore I was able to develop a fast, easy to use chip-based flow cytometric, diagnostic method for antifungal-susceptibility tests.**

Then I concentrate on the study of an interesting, potential antifungal active group of compounds, the Mannich ketones. The **susceptibility (MIC values) of some unsaturated Mannich ketones on a standard *C. albicans* strain was determined** with a modified version of the developed assay.

The cytotoxic and antimicrobial effect of **Mannich ketones** due to several mode of actions. The alkylations of thiol-groups in the target cell is the primary target, however other factors, as the inhibition of protein-synthesis and in case of fungi the inhibition of ergosterol- and chitin-synthesis probably are also important. I chose to study the changes in protein-composition from these modes of action.

A modern, **chip-based gelelectrophoresis** technique was utilized monitoring these changes. The microchip electrophoresis was suitable for the separation of the proteins of *Candida albicans*.

One of my goals was to detect qualitative and quantitative changes in the protein profile of *C. albicans* due to Mannich ketone treatment; in addition to explore the structure-activity relationships. **I studied a group (15 compounds) of fused Mannich ketones.** According to my results some compounds of the fused Mannich ketones with one hour long treatment cause significant changes in the protein profile, the changes mean the increase of the amount of several proteins. However, these results were not sufficient to conclude structure-activity relationships yet.

In conclusions, microfluidical flow cytometry and electrophoresis systems are suitable for the research of several yeasts.

## 6. Publications, papers

### Publications related to the thesis

**O Bouquet**, B Kocsis, F Kilár, T Lóránd, I Kustos.: Amphotericin B and fluconazole susceptibility of *Candida* species determined by cell-chip technology.

Mycoses 2012, 55 (3): e90-96.

IF: 1,278

**O Bouquet**, B Kocsis, F Kilár, I Kustos.: Application of chip-based flow cytometry for amphotericin B and fluconazole susceptibility testing on *Candida* strains.

Methods in Molecular Biology 2013, 968: 149-154.

T Rossignol, B Kocsis, **O Bouquet**, I Kustos, F Kilár, A Nyul, PB Jakus, K Rajbhandari, L Prókai, C d'Enfert, T Lóránd.: Antifungal activity of fused Mannich ketones triggers an oxidative stress response and is Cap1-dependent in *Candida albicans*.

PLoS One 2013, 8(4): e62142.

IF: 3,73

## Posters and presentations related to the thesis

**O Bouquet**, I Kustos, B Kocsis, T Lóránd, F Kilár.: Rapid susceptibility testing of *Candida albicans* by flow cytometry and cell-chip method. 7th International Symposium and Summer School on Bioanalysis, CEEPUS project. 10-15 June, 2007, Pécs, Hungary. Abstract Book, p.16.

**O Bouquet**, I Kustos, T Lóránd, F Kilár, B Kocsis.: Determination of amphotericin B susceptibility of *Candida albicans* by cell-chip method. ISAAC XXIV International Congress. 17-21 May, 2008. Budapest, Hungary. Abstract Book p.253.

**O Bouquet**, I Kustos, T Lóránd, F Kilár, B Kocsis.: Application of cell-chip method to rapid susceptibility testing. 9th International Symposium on Instrumental Analysis. 29 June – 2 July, 2008, Pécs, Hungary. Abstract Book p.55.

**O Bouquet**, I Kustos, B Kocsis, F Kilár, T Lóránd.: Effect of fused Mannich ketones on the protein profile of *Candida albicans*. The Fungal Cell, Annual Scientific Meeting of the British Mycological Society, 1-4 September, 2009, Dundee, UK. Abstract Book p.25.

**O Bouquet**, I Kustos, B Kocsis, F Kilár, T Lóránd.: Examination of the protein profile of *Candida albicans* - effect of fused Mannich ketones. 6<sup>th</sup> International Interdisciplinary Meeting on Bioanalysis. 5-8 November, 2009, Pécs, Hungary. Abstract Book p.41.

**O Bouquet**.: Különböző antifungális vegyületek hatása *Candida albicans* fehérjeprofiljára. Biológus Doktoranduszok Konferenciája, Pécsi Akadémiai Bizottság Biológiai Tudományok Szakbizottságának rendezvénye, Pécs, 2009. november 12-13. presentation