VYTAUTAS MAGNUS UNIVERSITY

FACULTY OF NATURAL SCIENCES

DEPARTMENT OF BIOLOGY

Sophia Badieva Vedi

MULTIDRUG RESISTANCE IN *CANDIDA ALBICANS*

Bachelor thesis

Biology and genetics study program, State Code 612C10003,

Biological Sciences

Supervisor: Prof. Habil. Dr. Rimantas Daugelavičius _____ _____

(signature) (date)

Defense: Prof. Dr. Saulius Mickevičius_____ _____

(signature) (date)

Kaunas, 2020

Experimental work was done: During autumn semester 2019- spring semester 2020, Department of Biochemistry, Faculty of Natural Sciences, Vytautas Magnus University.

Reviewer of the Bachelor's thesis: Erika Juškaitytė

Defence of the Bachelor's thesis: Defence of the thesis will be held on Thursday, June 18th, 2020, at 9:00 at a remote public meeting of the Bachelor's Thesis Defense Commission, Vytautas Magnus University, Department of Biology.

Address of the Department: Department of Biology, Faculty of Natural Sciences, Vytautas Magnus University, Vileikos street 8, LT-44404, Kaunas, Republic of Lithuania.

Bachelors's thesis is defended by: Sophia Badieva Vedi

(Signature)

Supervisor: Prof. Habil. Dr. Rimantas Daugelavičius

(Signature)

Head of the Department: Prof. Dr. habil. Algimantas Paulauskas

(Signature)

TABLE OF CONTENTS

SUMMARY

Author of diploma paper:	Sophia Badieva Vedi
Full title of diploma paper:	Multidrug resistance in *Candida albicans*
Diploma paper advisor:	Prof. Habil. Dr. Rimantas Daugelavičius
Presented at:	Vytautas Magnus University, Faculty of Natural Science, Kaunas, 2020
Number of pages:	37
Number of tables:	2
Number of pictures:	20

The development of multidrug resistance in pathogenic yeast species is a growing cause for concern in modern medicine. The natural process of developing resistance is exacerbated by incorrect or untimely use of antifungal drugs leading to reduced efficiency of previously successful treatments. Research into underlying causes and mechanisms of MDR and testing new compounds that could have antifungal properties is crucial in developing new effective treatments and combating MDR in pathogens. This research project focusses on explaining the biochemical processes governing MDR such as the action of protein pumps in fungal cell membranes, conducting experiments with well-studied antifungals and using new compounds with antifungal properties to establish minimum inhibitory concentration (MIC) values, testing combinations of these antifungal compounds and observing differences in MIC values between wild type strains and clinical isolates of *Candida albicans*. The effectiveness of the compounds is reflected in MIC values obtained through graphing the results after incubating microplates with yeast cells and compounds and measuring the absorbency through a microplate reader.

SANTRAUKA

Diplominio darbo autorius: Sophia Badieva Vedi

Visas diplomo darbo pavadinimas: *Candida albicans* dauginis atsprumas vaistams

Diplominio darbo vadovas: Prof. Habil. Dr. Rimantas Daugelavičius

Pateikta adresu: Vytauto Didžiojo universitetas, Gamtos mokslų fakultetas, Kaunas, 2020 m

Puslapių skaičius: 37

Lentelių skaičius: 2

Nuotraukų skaičius: 20

Šiuolaikinėje medicinoje didėja susirūpinimas dėl patogeninių mielių atsparumo vaistams. Natūralų atsparumo vystymosi procesą pagreitina neteisingas ar nesavalaikis priešgrybelinių vaistų vartojimas, dėl kurio sumažėja anksčiau sėkmingai naudotų vaistų veiksmingumas. Kuriant naujus veiksmingus gydymo būdus, būtini MDR priežasčių ir mechanizmų tyrimai bei naujų junginių, kurie galėtų pasižymėti priešgrybelinėmis savybėmis, sintezė. Šiame tiriamajame darbe siekiama išsiaiškinti su MDR susijusius biocheminius procesus, pavyzdžiui, baltyminių pompų veikimo patogeninių mielių ląstelių membranose ypatumus, atlikti eksperimentus su gerai ištirtais priešgrybeliniais preparatais ir naudoti naujus junginius, turinčius priešgrybelinių savybių. Buvo siekiama nustatyti minimalias slopinančiąsias koncentracijas (MIC), išbandyti šiuos priešgrybelinius junginius ir nustatyti MIC vertės skirtumus tarp laukinio tipo padermių ir *Candida albicans* klinikinių izoliatų. Junginių efektyvumas atsispindi MIC vertėse, gautose grafiškai pateikiant rezultatus, gautus inkubuojant mikroplokštes su mielių ląstelėmis ir junginiais ir išmatuojant mielių suspensijų drumstumą mikroplokštelių skaitytuvu.

LIST OF ABBREVIATIONS

ABC - ATP-binding cassette

CDR - *Candida* drug resistance

MDR - Multidrug Resistance

MFS - Major facilitator superfamily

NAC - Non-albican *Candida*

NBD - Nucleotide-binding domain

OD - Optical density

PDR - Pleiotropic drug resistance

TET- Tetrandrine

TMD - Transmembrane domain

INTRODUCTION

MDR is defined as insensitivity or resistance of a microorganism to the administered antimicrobial medicines (which are structurally unrelated and have different molecular targets) despite earlier sensitivity to it. These resistant microorganisms, including bacteria, fungi, viruses and parasites are able to combat attack by antimicrobial drugs, which leads to ineffective treatment resulting in persistence and spreading of infections. Due to an exponential increase in new resistance mechanisms and decrease in efficiency of treating common infectious diseases, it results in failure of microbial response to standard medical treatment, leading to prolonged illness, higher expenditures for health care, and an immense risk of death. This phenomenon has lead to the widely-known occurrence of the 'Antibiotic-resistance crisis' and the term 'superbugs' referring to the microbes causing it. Although the development of MDR is a natural phenomenon, the inappropriate use of antimicrobial drugs, inadequate sanitary conditions, inappropriate food-handling, and poor infection prevention and control practices contribute to emergence of and encourage the further spread of MDR. The development of MDR in yeast is due to a number of mechanisms. The most documented mechanism is enhanced extrusion of drugs mediated by efflux pump proteins belonging to either the ABC (ATP-binding cassette) superfamily or MFS (major facilitator superfamily). These drug-efflux pump proteins are localized on the plasma membrane, and their content affects their proper functioning. Several studies demonstrate that fluctuations in membrane lipid composition affect the localization and proper functioning of the MDR efflux pump proteins. (Panwar et al., 2008).

This paper examines effects of antifungal compounds from various families on the growth of pathogenic yeast, and how these compounds interact with the MDR efflux pump machinery in the yeast's cell membranes.

Subject of research: pathogenic yeast *Candida albicans*; Wild Type strain ATCC10231, clinical isolate 110717. Brewer's yeast *Saccharomyces cerevisiae* as model organism; Wild Type, pdr5 strains.

Significance of research: *C. albicans* is a very well-known and ubiquitous commensal organism that is commonly found as part of a healthy human gut flora. However, under certain conditions, this organism can become pathogenic and cause candidiasis in humans. In light of growing concerns of MDR developing in pathogenic yeast, it is essential to examine and re-evaluate the action of traditional antifungals against *Candida*, as well as test new compounds that could

supplement the efficiency of first-line antifungals, or demonstrate antimicrobial activity of their own.

Objectives:

1. To determine the active concentrations of the most popular antifungal drugs - azoles and polyenes, on cells of *C. albicans* strains.
2. To determine interaction of naturally-occuring efflux pump inhibitors - tetrandrine and octanoic acid with the studied drugs.
3. To compare growth response of pathogenic *C. albicans* and model yeast *S. cerevisiae* to the studied drugs and the efflux inhibitors.

I. LITERATURE REVIEW

1.1 Antifungal Compounds

1.1.1 Azoles

Azole antifungals are the most frequent class of drugs used to treat *Candida* infections. Azole antifungals such as fluconazole are often preferred treatment for many *Candida* infections as they as they are inexpensive, exhibit limited toxicity, and are available for oral administration. Azoles are synthetic compounds that include 2 groups, imidazoles and triazoles. Triazoles have 3 atoms of nitrogen in the azole ring, imidazoles have two. The primary mechanism of action is inhibition of lanosterol 14-alpha-demethylase biosynthesis in the endoplasmic reticulum, an enzyme required for the synthesis of ergosterol, the main component of fungal cell membranes, resulting in the accumulation of the toxin 14-α-methyl-3, 6-diol. As the concentration of ergosterol is reduced, the cell membrane structure is altered, leading to the inhibition of fungal growth (Sanguinetti *et al*., 2015). Imidazole agents include miconazole, ketoconazole and clotrimazole. Triazole compounds containing one or more 1,2,4-triazole rings have been shown to contain some of the most potent antifungal properties. However, there is rising evidence of intrinsic and developed resistance to azole antifungals among several *Candida* species. (Whaley *et al*., 2017) While *C. albicans* is the most common perpetrator of *Candida* infections, other *Candida* non-albican (NAC) species are becoming increasingly more common among medical case reports.

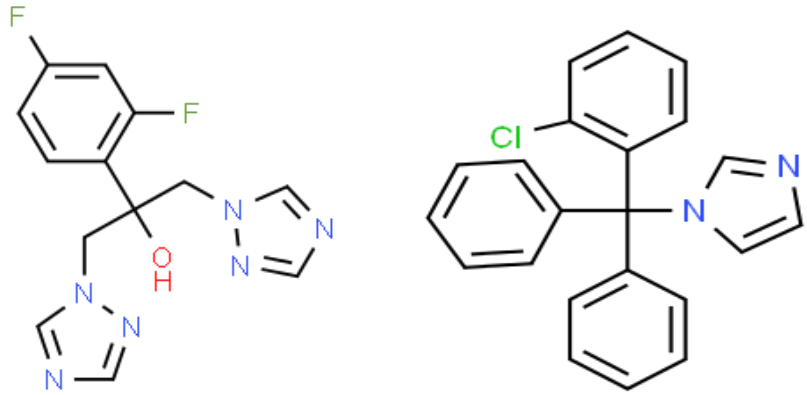

Fig. 1. Structure of Fluconazole and Clotrimazole (Data taken from ChemSpider.com, CSID:2710, Accessed May 25, 2020)

1.1.2 Polyenes

The polyene antifungal drugs consist primarily of amphotericin B and nystatin. These drugs demonstrate a wide spectrum of antifungal activity against common fungal infections, such as candidiasis, aspergillosis, mucormycosis, and cryptococcosis. The primary mode of their antifungal activity results from binding to ergosterol. This binding forms channels in the cell membrane, altering its permeability and causing leakage of Na^+, K^+, and H^+ ions. Resistance to the polyenes is associated with a replacement of ergosterol with other sterols in the fungal plasma membrane.

Amphotericin B is an antifungal obtained from *Streptomyces nodosus*, an actinomycetes found in the soil. It is a member of the polyene family of antibiotics, so called because their structure contains a large lactone (macrolide) ring with numerous conjugated double bonds. The polar hydroxylated portion and the nonpolar hydrocarbon sequence lend an amphophilic character to the molecule. This polyene exerts its activity mainly by binding to ergosterol in fungal cell membranes, developing holes in the membrane and allowing cell components to leak out, causing cell death. It exerts either fungistatic or fungicidal activity depending on the concentration of the drug, the pH, and the fungus involved. Peak activity occurs at a pH between 6.0 and 7.5. Amphotericin B has a broad spectrum of antifungal activity and is effective against several fungal organisms including *Candida* species. (Kuriyama, 2014)

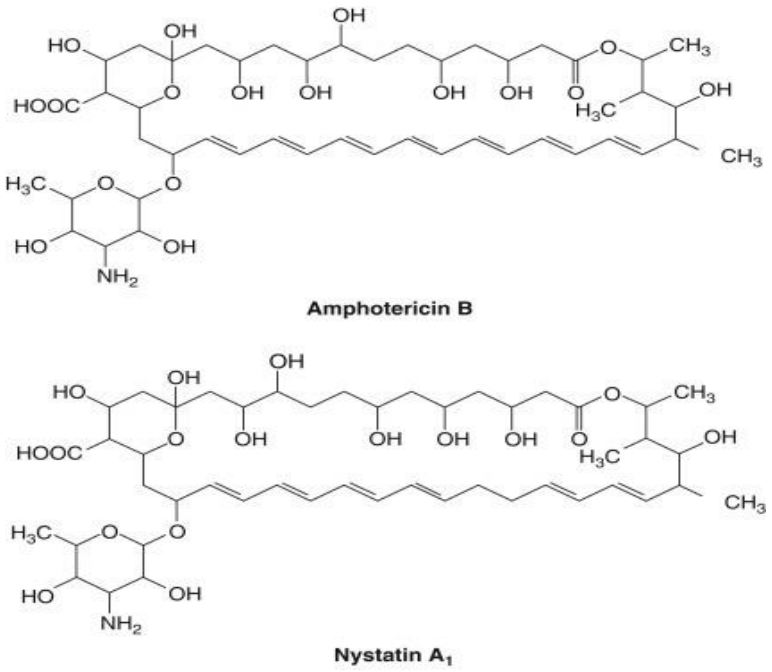

Fig. 2. Structural formulas of Amphotericin and Nystatin (DeRosa, 2006).

Nystatin is synthesized by the bacterium *Streptomyces noursei*, its spectrum of activity is not as wide as that of amphotericin B but it is still active against a number of species of *Candida*, *Histoplasma*, *Cryptococcus*, *Blastomyces*, and other fungi. As with amphotericin B, nystatin is either fungistatic or fungicidal depending on the concentration of the drug present, the pH of the surrounding medium, and the nature of the pathogen. The mechanism of action of nystatin is very similar to that of amphotericin B. It works by damaging the fungal cell membrane and leading to a rapid increase in its permeability to small ions. This results in depletion of cellular K+ ions and inhibition of respiration and glycolysis leading to cell demise. Nystatin works best under acidic pH, with peak activity being registered at pH 4. (Marini, 1961).

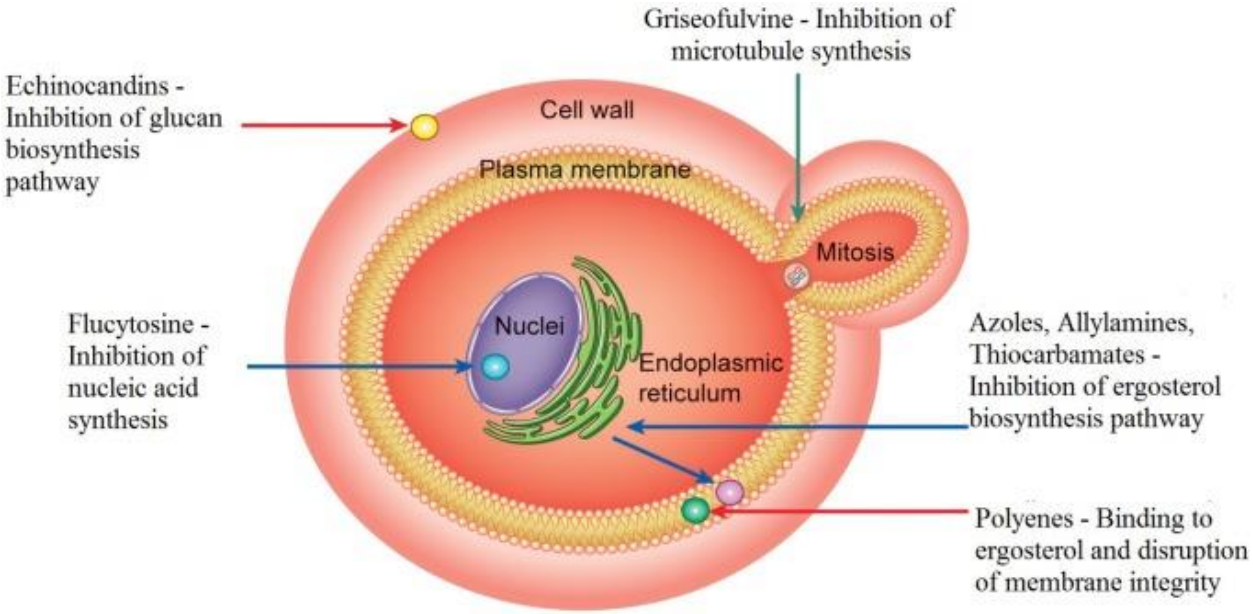

Fig. 3. Mechanisms of action of traditional antifungal agents on cellular targets, including Azoles targeting the biosynthesis ergosterol, and Polyenes disrupting the cell membrane. (Oliveira Santos *et al.*, 2018)

1.1.3 Biochemistry of naturally-occurring compounds

1.1.3.1 Tetrandrine

Tetrandrine (TET) is a bis-benzylisoquinoline alkaloid derived from the radix of *Stephania tetrandra*, which acts as a calcium-channel blocker. It has been used in traditional Southeast-Asian medicine as an anti-inflammatory herb. It is of special interest in this study due its recently discovered anti-oncogenic properties including reversal of MDR in cancer cells. It has also been known to inhibit biofilm formation, one of *Candida*'s main defensive mechanisms against

traditional antifungals, and to prevent hyphal growth in various species of yeast. *C. albicans* cells in biofilms display severe resistance to a wide variety of clinical antifungal agents, including amphotericin B and fluconazole. Using RT-PCR it was found that TET down-regulated the expression of hypha-specific genes *ECE1, ALS3* and *HWP1,* while simultaneously affecting the expression of *EFG1* and *RAS1* genes, regulators of hyphal growth. It has been theorized that the anti-biofilm activity of TET was associated with Ras/cAMP pathway (Liu *et al*., 2013). Apart from demonstrating a significant biofilm-suppressing mechanism of its own, TET has also been known to exhibit synergistic effect with ketoconazole against drug resistant *C. albicans* and synergism with econazole against *Trichophyton mentagrophytes.* An in-vitro study implementing flow cytometry and fluorescent dye spectrometry (Zhang *et al*, 2009) on different *C. albicans* strains aimed at examining the joint effect of fluconazole with TET, revealed a statistically-significant decrease in drug efflux in the presence of TET. The main mechanism responsible for azole resistance in yeast is the efflux pump system, and the administration of TET showed repression of this system even in strains with overexpressed protein pumps. The inhibition of *C. albicans'* drug efflux system in the given study is linked to inhibition of expression of the efflux pump genes *MDR1, FLU1, CDR1,* and *CDR2*. These results make Tetrandrine a very promising new player in the fight against MDR and call for new investigations into its potent antifungal qualities.

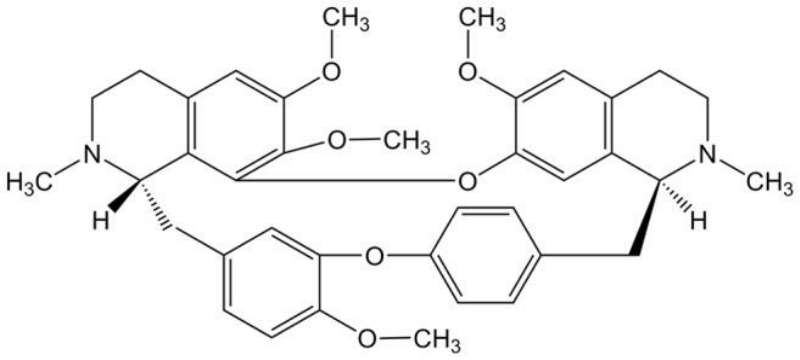

Fig.4. Chemical structure of Tetrandrine (Zhao, 2013).

1.1.3.2 Octanoic acid

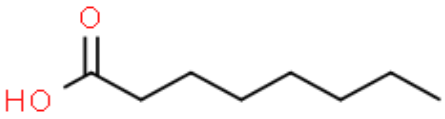

Fig.5. Chemical structure of Octanoic acid (Takahashi et al., 2012)

Octanoic acid, also known as Caprylic acid ($C_8H_{16}O_2$) is a medium-chain saturated fatty acid derived from coconuts with known antibacterial, antifungal, and anti-inflammatory properties. It is also known as a fermentation inhibitor in *S. cerevisiae.* It accumulates mainly in the cells walls of yeast and causes β-oxidative damage to the cells. The anti-fungal effects of certain fatty acids have been demonstrated by several investigators (Neuhauser, 1954, Hoffman 1939). A study conducted by Tsukahara, 1961, revealed that octanoic acid-resin complex exerted an excellent inhibition of growth of *Candida albicans* in vitro as well as in the treatment of severe intestinal candidiasis. Fatty acids exhibit numerous mechanisms for their antifungal action, the main target being the fungus' cell membrane. They cause an increase in membrane fluidity, which results in leakage of the intracellular components and eventual apoptosis. Other targets include interference in protein synthesis, fatty acid metabolism as well as topoisomerase activity. (Pohl et al., 2011). The primary biochemical pathway with which antifungal fatty acids directly interact with the fungal cell is through insertion into the lipid bi-layer of the fungal membranes and physical disturbance to the membrane, resulting in its increased fluidity. These elevations in membrane fluidity cause a generalized disorganization of the cell membrane that leads to conformational changes in membrane proteins, the expulsion of intracellular components, disruption of the cytoplasm and cell disintegration. (Avis and Bélanger, 2001).

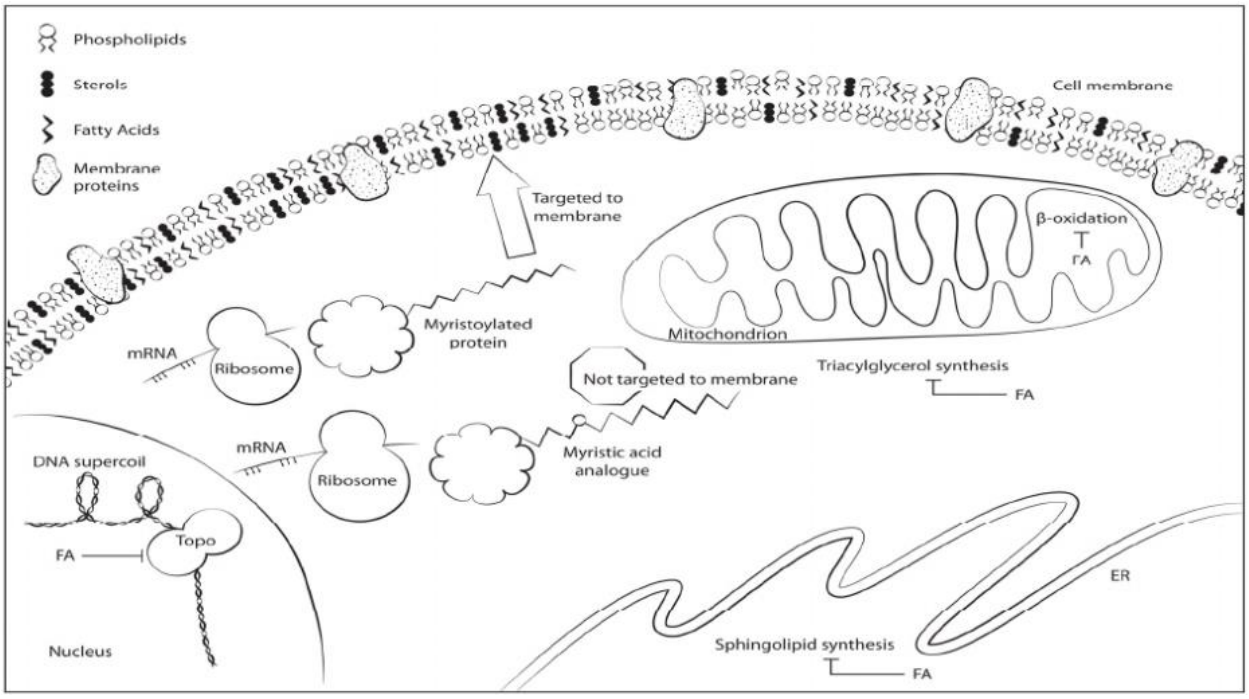

Fig.6. Proposed antifungal mechanisms of free fatty acids: Disruption of cell membrane, inhibition of beta-oxidation, possible inhibition of protein myrisoylation, triacylglycerol synthesis , sphingolipid synthesis and topoisomerase activity. (Pohl, 2011)

1.2 MDR mechanisms in yeast

1.2.1 MDR efflux pumps

Resistance to antifungals in *C. albicans* is a major problem in modern medicine and a barrier to efficient treatment of fungal infections. MDR is usually encountered in inherently resistant yeast strains with a reduced sensitivity to antifungal medications, and in strains that acquire resistance during therapy. The molecular mechanisms behind reduced sensitivity include altered drug affinity and target abundance, low intracellular drug concentrations caused by protein pump efflux and formation of biofilms. (Cowen *et al*., 2015) Multidrug resistance pumps, otherwise known as multidrug efflux pumps are components of the cell membrane present in animals, fungi and bacteria responsible for the expulsion of foreign substances out of the cell membrane. Efflux pumps are involved in a wide range of cellular activities including intercellular communication, biofilm formation, and extrusion of toxic metabolic by-products and antibiotics. The two major classes of MDR pumps responsible for the extrusion of antifungals in yeast are the ATP-binding cassette (ABC) superfamily and Major facilitator superfamily (MFS). Extrusion of compounds by protein pumps is energy-dependant. ABC transporters derive energy from the hydrolysis of ATP, while MFS pumps function using proton-motive force. (Chitsaz, 2017)

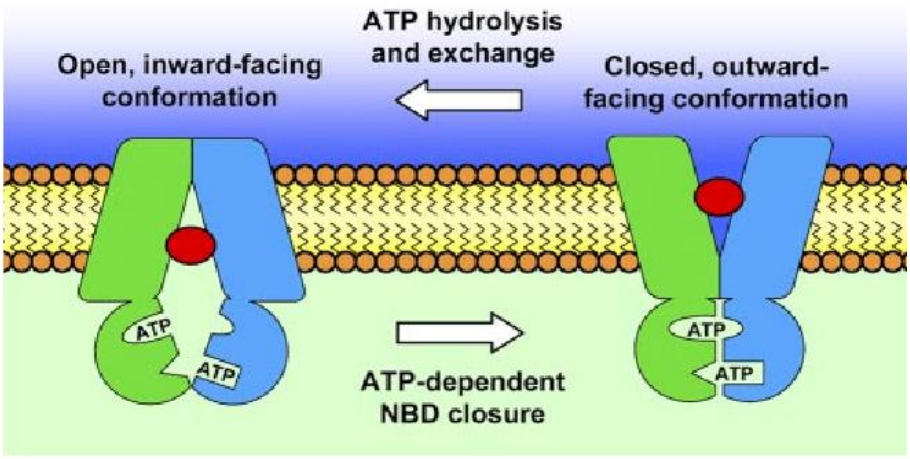

Fig.7. Molecular structure and transport mechanism of an ABC protein pump (Procko, 2009)

ATP-dependent closure/dimerization of NBDs provides energy to change TMD conformation. Substrate molecule enters membrane domain (TMD) cavity. Two ATP molecules bind to the two ABC domains causing the outer opening of the domain to open and releasing the substrate molecule from the membrane. ATP is utilized through hydrolysis of two H_2O molecules releasing 2ADP+Pi.

ABC proteins contain two types of domain, nucleotide-binding domains (NBDs) and transmembrane domains (TMDs). NBDs are involved in the binding and hydrolysis of ATP which provides the energy for substrate translocation. TMDs comprise six transmembrane spans (TMSs) and are believed to be constituents of a substrate channel through the membrane. Active extrusion of foreign molecules is regulated by transmembrane transporter proteins which are composed of four modules: two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs). The MDR mechanism of the cell is thus regulated by the TMDs recognizing and translocating the substrates, and the NBDs providing energy for the required conformational changes. Conformational changes in the TMDs are regulated by association and dissociation of NBDs and powered by nucleotide binding and hydrolysis. Opening of NBDs allows for exchange of nucleotides. Binding of ATP molecules causes association of NBDs and change in TMD conformation from inward-facing to outward-facing. (Ernst et al., 2009) One strategy in overcoming MDR resistance is to inhibit the efflux pumps and chemo-sensitize resistant yeast strains to azoles. Ever since the detection of expression of human ABC protein ABCB1 (also known as MDR1 and P-gp) in a number of different human cancers (Goldstein, 1989), researchers have looked for inhibitors or modulators of ABC transporters. Three generations of ABCB1 inhibitors have since been developed but discontinued in practice due to various reasons such as in vivo toxicity, undesirable side-effects and reduced efficiency in clinical trials. (Kathawala, 2015). However, research into ABCB1 inhibitors for controlling human cancer cells has provided a scaffold for similar research into specific inhibitors of fungal efflux proteins, and further

investigations into manipulating the expression of efflux pumps promises more insights into battling MDR. (Holmes *et al*., 2016)

1.2.1.1 Pdr5 MDR transporter in *S. cerevisiae*

The plasma membrane of yeasts contains a variety of ABC transporters and efflux pump protein systems that act together against structurally unrelated antibiotics. One of these pump systems is the pleiotropic drug resistance (PDR) family. Pdr5 is the most abundant ABC transporter *in S. cerevisiae* which provides resistance to several unrelated drugs. It is a functional homologue of p-glycoproteins in mammals. It is also highly homologous to azole-resistance-mediating multidrug transporters in fungal pathogens. (Ernst *et al*., 2009) Like other members of the PDR family, Pdr5 has an inverted efflux pump structure (NBD‑TMD‑ NBD‑TMD). An important feature of MDR efflux pumps is their very diverse spectrum of substrates. This is explained by the presence of two or more independent substrate binding sites that have been reported to exist in pdr5. Studies on the exact nature of the multifunctionality of efflux pumps have been inconclusive and it is not completely clear how they function. Pdr5p has oligomycin- and vanadate-sensitive ATPase activity. Inactivation of pdr5 is not lethal to yeast cells unexposed to drugs and does not affect their metabolism, but results in a drug-hypersensitive phenotype. Overproduction of Pdr5 results in resistance to antimicrobials like cycloheximide, fluconazole and several unrelated compounds. (Kolaczkowski *et al*. 1996) Since it belongs to the same family of ABC transporters as the mammalian p-glycoproteins, it also shares a number of common substrates and inhibitors with them. P-glycoproteins have been widely studied due to their impact on nullifying the effects of chemotherapy and other anti-cancer treatment in cancer patients. Their similarity to fungal pdr5 makes it easier to study the MDR mechanism in yeast. Due its multi-substrate affinity and activity against a wide variety of compounds, further research into the inactivation of the expression of pdr5 and testing of non-interactive compounds could pave the way for development of a new generation of highly effective antifungals.

1.2.2 Azole resistance

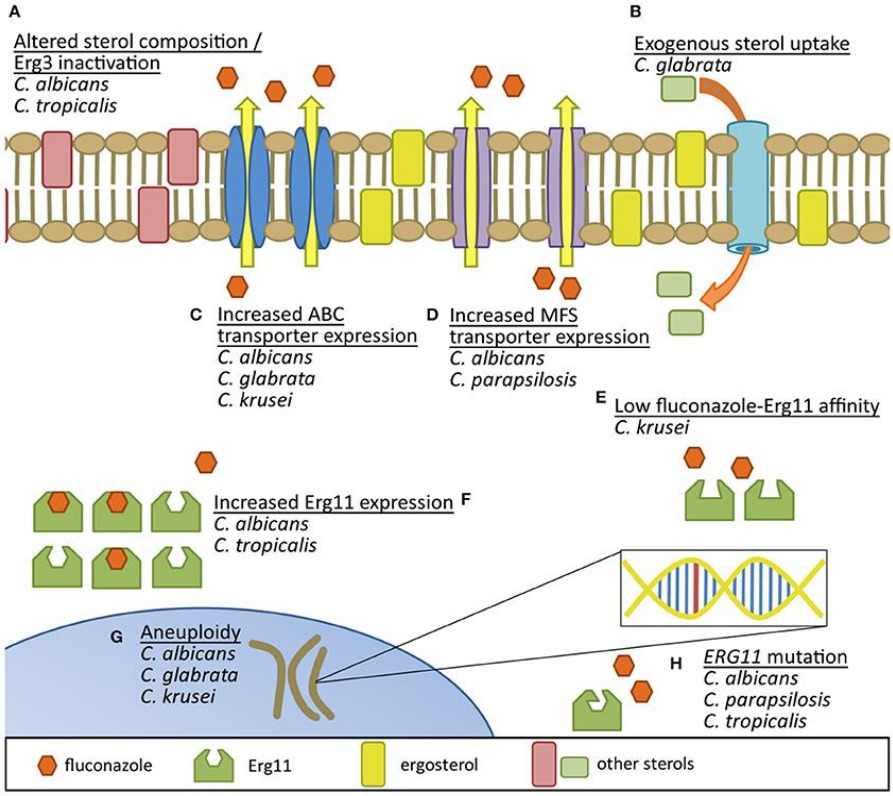

Fig.8. Mechanisms responsible for azole resistance in various *Candida* species. **(A)** *ERG3* inactivation **(B)** Uptake of exogenous sterols interfering with endogenous sterol production inhibition. **(C)** Overexpression of ABC efflux pumps and **(D)** MFS pumps **(F)** Increased expression of Erg11 protein **(H)** Mutations in *ERG11* (Whaley et al., 2017)

There are three types of antifungal resistance- primary or intrinsic resistance that exists prior to antifungal exposure, acquired resistance that occurs after antifungal exposure and may be reversible and clinical resistance developed as a result of low-dose administration of azoles as a prophylactic measure against possible infection. (Cowen et al., 2015; Siikala et al., 2010). Resistance against azoles can develop through several mechanisms- (i) changes in the biosynthesis of sterols, resulting in their substitution for ergosterol; (ii) overexpression of the target enzyme, leading to inefficient action of the antifungal compound; (iii) overexpression of drug efflux pumps that reduce the intracellular concentration of the drug; and (iv) changes in the target gene sequence, leading to reduced binding capacity between the target protein and compound molecule (Ksiezopolska, 2018). Ergosterol mutations in the *ERG3* gene prevent the conversion of 14-α-methylfecosterol to 14-α-methyl-3,6-diol thus blocking the adhesion of azoles to the fungal cell wall.(Sanguinetti et al., 2015).The second mechanism of resistance involves modification of the

target enzyme encoded by the *ERG11* gene, also known as cytochrome P450 lanosterol 14 α-demethylase (Cyp51) supressing azole binding to the enzyme sites (Marichal et al., 1999; Flowers et al., 2015). The third mechanism is related to induction of multi-drug pumps, which decrease the concentration of drug available for the target enzyme, 14-α-demethylase, in fungal cells (Kanafani and Perfect, 2008) There are two types of active transporters in *C. albicans*- encoded by the *Candida* drug resistance-*CDR* genes (Cdr1 and Cdr2), and those encoded by the multidrug resistance-*MDR1* genes. Cdr1- and Cdr2-type pumps ABC transporters, and Mdr1 is an MFS-type pump that transports solutes from different sides of the cell membrane. Overexpression of transporters encoded by *CDR* genes results in resistance to various azole-derived compounds, while overexpression of MDR1 genes is responsible for fluconazole resistance. Heightened expression of these transporters prevents accumulation of the drug in the cytoplasm. (Oliveira Santos, 2018; Pfaller, 2012) Despite widespread azole-resistance in pathogenic *Candida* species, new, more effective triazoles are being developed. These include ravuconazole, albaconazole, and isavuconazole. Preliminary studies have demonstrated low toxicity, decent pharmacokinetics and anti-*Candida* activity even among fluconazole-resistant strains. (Fera, 2014)

1.2.3 Polyene resistance

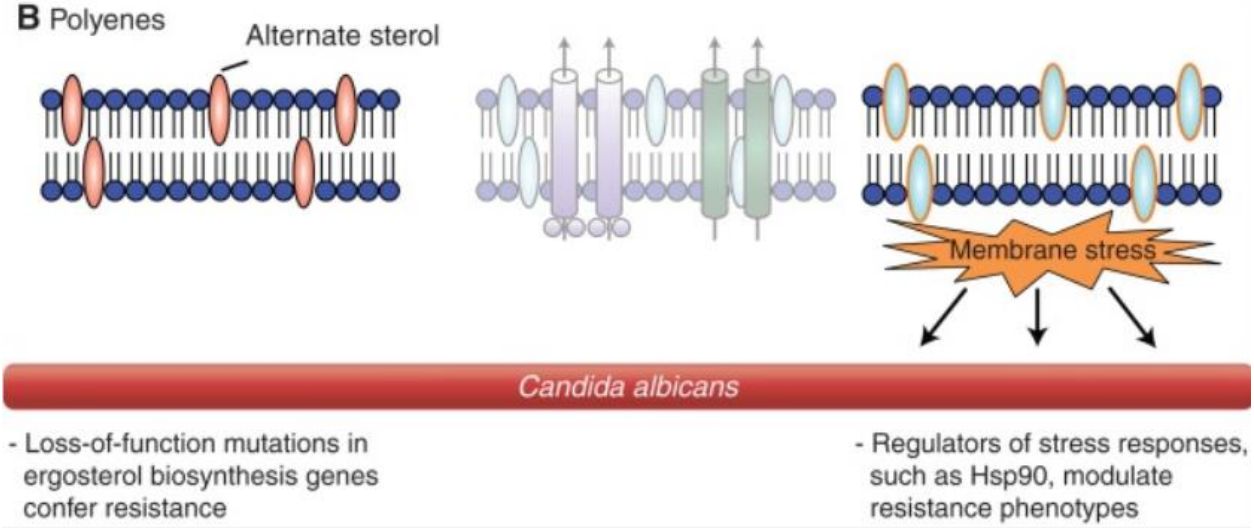

Fig.9. Polyene resistance: Reduction of target ergosterol due to mutations in ergosterol biosynthetic genes. (Oliveira Santos, 2018)

Resistance to polyenes is not nearly as widespread as azole resistance. Some cases of intrinsic resistance to polyenes have been reported in *Candida lusitaniae* and *Trichosporon beigelii* (Pfaller, 1994; Walsh, 1990). Similar to azole resistance in *C. albicans*, polyene tolerance is mainly linked to the substitution of ergosterol with a precursor molecule or an overall reduction of sterols in the cell membrane. (Kanafani & Perfect, 2008) Mutations in the *ERG3* gene involved in

erogosterol biosynthesis lead to accumulation of other sterols in the fungal membrane. As a result, polyene-resistant *Candida* isolates have low ergosterol content, compared with that of polyene-sensitive isolates. (Dick *et al*., 1980) Enzymes such as Δ5,6-desaturase, encoded by the *ERG3* gene, and C-8 sterol isomerase, encoded by *ERG2* gene contribute to ergosterol biosynthesis and are responsible for the main alterations related to polyene resistance. An example of this is the mutation of Δ5,6-desaturase, leading to the conversion of fecosterol to episterol, which has low affinity with Amphotericin B. Polyene-resistant *Candida* species (mainly *C. krusei* and *C. glabrata*) display mutations in *ERG2, ERG3, ERG5, ERG6* , and *ERG11* genes, which encode enzymes involved in ergosterol synthesis. Another possible polyene resistance pathway is the reduction of oxidative damage through increased catalase activity in peroxisomes. (Kanafani & Perfect, 2008).

II. MATERIALS AND METHODS

2.1 Research subjects, materials, reagents and equipment

2.1.1 Research subjects

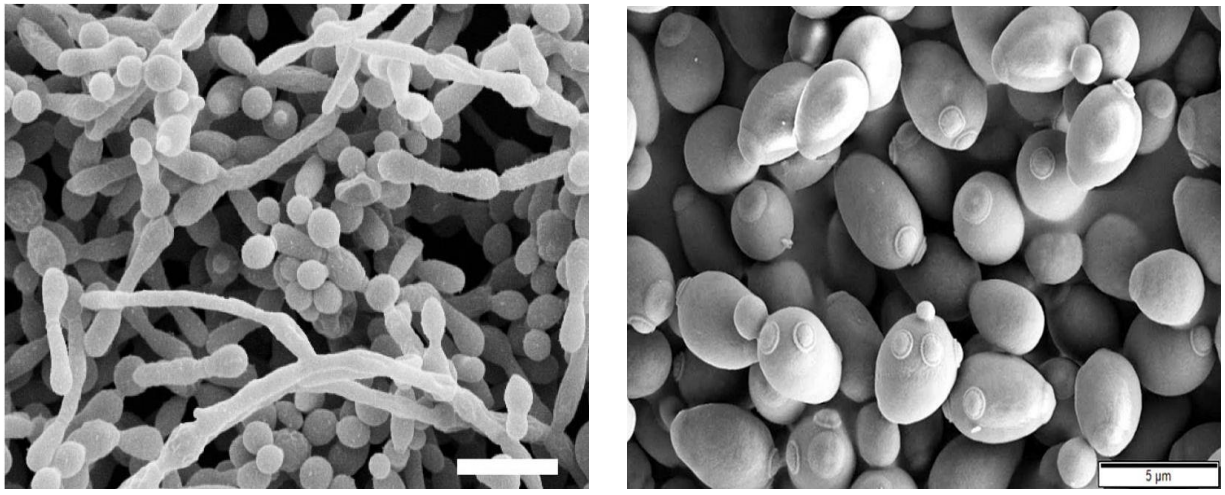

Fig.10. *Candida albicans* SEM (Ramage, 2015); *Saccharomyces cerevisiae* SEM (Murtey, 2016)

The main subject of the research is the pathogenic yeast from the *Saccharomycetaceae* family *Candida albicans*. The two strains used for the research were laboratory strain ATCC10231 and

clinical isolate 110717, isolated from a bladder infection. ATCC10231 is a commonly used reference strain whose purposes include assay of amphotericin B fungizone, nystatin fungicidin, haloprogin, assay of antimicrobial preservatives, media testing, sterility testing, membrane filter testing, preparatory test control, etc. Brewer‘s yeast *Saccharomyces cerevisiae* from the *Saccharomycetaceae* family was investigated as a model organism that has been extensively researched in MDR studies. The strains investigated were Wild Type and pdr5, with an overexpressed pdr5 protein pump.

2.1.2 Growth Media

For preliminary research and overnight culture preparation, YEPD media was used, which is a growth medium consisting of 1% yeast extract, 2% peptone, 2% D-glucose and deionised water. After preparation, YEPD is sterilised in an autoclave. For the MIC experiments with *C. albicans* the medium used was RPMI 1640 with the addition of 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer and glucose, brought to pH 7 by addition of NaOH using a pH meter. RPMI 1640 is a pre-prepared powder containing the following, per litre: Glucose (2 g), pH indicator (phenol red, 5 mg), salts (6 g sodium chloride, 2 g sodium bicarbonate, 1.512 g disodium phosphate, 400 mg potassium chloride, 100 mg magnesium sulfate, and 100 mg calcium nitrate), Amino acids (300 mg glutamine; 200 mg arginine; 50 mg each asparagine, cystine, leucine, and isoleucine; 40 mg lysine hydrochloride; 30 mg serine; 20 mg each aspartic acid, glutamic acid, hydroxyproline, proline, threonine, tyrosine, and valine; 15 mg each histidine, methionine, and phenylalanine; 10 mg glycine; 5 mg tryptophan; and 1 mg reduced glutathione),Vitamins (35 mg i-inositol; 3 mg choline chloride; 1 mg each para-aminobenzoic acid, folic acid, nicotinamide, pyridoxine hydrochloride, and thiamine hydrochloride; 0.25 mg calcium pantothenate; 0.2 mg each biotin and riboflavin; and 0.005 mg cyanocobalamin). Being so rich in amino acids and vitamins, maintaining strict sterility while working with RPMI to avoid contamination by external microbes is essential to the viability of the experiment. It cannot be sterilised in the autoclave due to its high amino acid content, therefore, the chosen method of sterilisation is filtration. For MIC experiments with *S. cerevisiae,* YEPD media was used.

2.1.3 Antifungal Compounds

Table 1. Antifungals and manufacturers

Compound	**Manufacturer**
Amphotericin B	Alfa Aesar, Germany
Nystatin	ACROS organics, USA
Fluconazole	Sigma-Aldrich, USA
Clotrimazole	Alfa Aesar, Germany

Octanoic acid	Sigma-Aldrich, USA
Tetrandrine	Sigma-Aldrich, USA

For preparation of the stock solution of compounds, the dry compounds are weighed and dissolved in a solvent. The solvent of choice was DMSO as it dissolves both, polar and non-polar compounds. Initial volume of compounds to be added to the microplate is determined by the C1V1=C2V2 formula, where C1 is the concentration of the compound in the stock solution, V1(the unknown) is the volume of compound to be added to the plates, C2 is the required initial concentration of the compound, and V2 is the volume of the well (200 µl).

2.1.4 Tools and materials

Table 2. Equipment and manufacturers

Equipment tool	**Manufacturer**
Autoclave	Steriltechnik AG, Germany
0.22 µm polypropylene syringe filters	Nerbe Plus, Germany
0.5-1000 µl pipettes	Eppendorf Research (Sigma-Aldrich), USA
96-well round-bottom cell culture plate	Nerbe Plus, Germany
Thermostat	Memmert GRIDA, Germany
Shaker/ incubator	ES-20 BioSan, Latvia
Multiplatereader, FluorGENiosPro programme	TECAN Group Ltd., Switzerland
pH meter	inoLab WTW, Xylem Analytics, USA

2.2 Research Methods

2.2.1 Cell culture preparation

Before commencing the experiment, it is necessary to prepare the cell culture. This is done by introducing the cells from a laboratory-prepared stock into sterile room-temperature liquid media using a sterile inoculation loop. The cells are then incubated at 37° C for *C. albicans* and 30° C for *S. cerevisiae* for 18 hours. Alternatively, the cells can first be streaked onto an agar plate from the stock, incubated for 18 hours and then transferred into liquid media and incubated again. Using cells that were streaked onto agar is preferable, as it allows to choose desirable colonies from the several that have grown on the plate. Incubation of cells in liquid media is carried out in a shaker-thermostat to ensure the homogeneity of the cell culture, and to prevent the cells from settling at the bottom of the flask and coagulating.

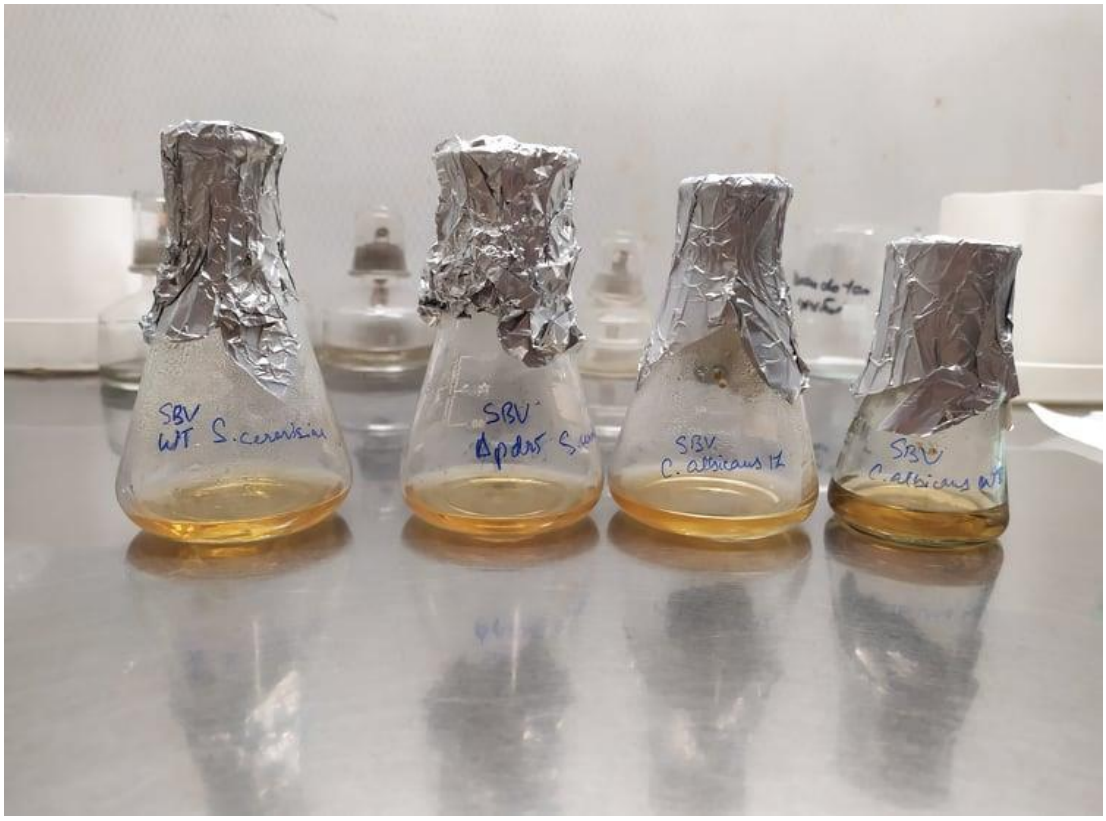

Fig.11. Cell night culture preparation in YEPD media (retrieved from personal archive)

2.2.2 Minimum Inhibitory Concentration (MIC)

In the field of microbiology, the minimum inhibitory concentration (MIC) is the lowest concentration of a drug, in the case of this research, an antimycotic compound, which inhibits the growth of a pathogen. MIC depends on the microorganism, mutations to combat antimicrobials that it may have developed and on the properties of the compound itself. The MIC is determined by preparing solutions of the drug in vitro at increasing concentrations, incubating the solutions with the separate batches of cultured yeast, and measuring the results using broth microdilution or Etest (McKinnon *et al*., 2015). The method used in these experiments was Broth microdilution. By carrying out serial two-fold dilutions in a 96-well cell culture round-bottom plate, the lowest concentration of antifungal drug that is sufficient to inhibit fungal growth is determined, and that is the MIC. The reagents necessary to run this assay are the media, antimicrobial agents, and cells from the night culture being tested. The cells added to the microplate must come from the same colony-forming unit, and must be at the correct concentration, which is determined by calculating the reference optical density (OD). In the case of *C. albicans*, the desired OD was 0.01, and 0.1 OD for *S. cerevisiae*. The yeast inoculates the plate and are incubated for 16‑24 hours. After

incubation, MIC is determined by measuring absorbance using a microplate reader and graphing the obtained results.

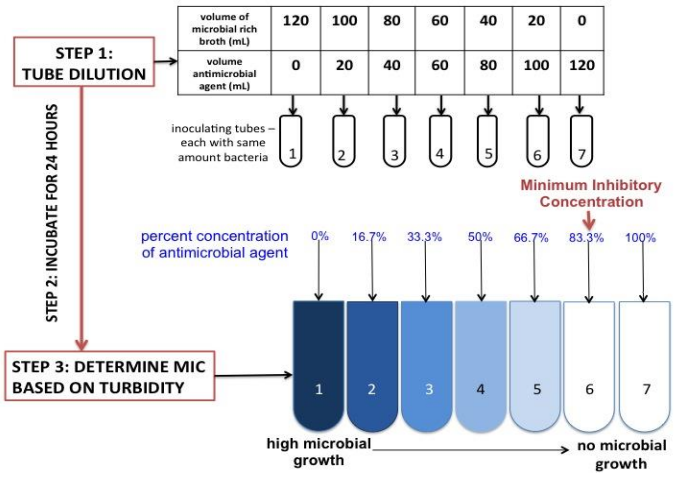

Fig.12. Broth microdilution scheme (Decaussin, 2016).

2.2.3 Obtaining Results

There are two steps to evaluating the results of the experiment. The main conclusions are derived from measuring light absorbancy in the plate using a microplate reader and graphing the results. High absorbancy indicates high cell volume, meaning that the compound at the given concentration is not effective at inhibiting cell growth. Another way to evaluate preliminary results is by visual observation of the turbidity (a measure of cloudiness) of the plates. High turbidity indicates higher cell growth in the plate.

III. RESULTS

Concentration-dependent susceptibility tests for antifungal compounds were performed. The minimal growth inhibitory concentration was established to determine the antifungal activity of the compounds using the BMD (broth microdilution) method. Initial concentrations of compounds used were based on identified MIC values from literature. Experiments were performed in 96-well microplates. There were $3*10^4$ of cells in every well except control with medium.

3.1 Determination of minimal growth inhibitory concentration in *C. albicans*

MIC of antifungal compounds was determined by graphing the absorption values in the microplate using a microplate reader. MIC of pure azoles and polyenes was contrasted with combining them with tetrandrine and octanoic acid. The growth response of *C. albicans* strains Wild Type (ATCC 10231) and clinical isolate (C.i 110717) was compared.

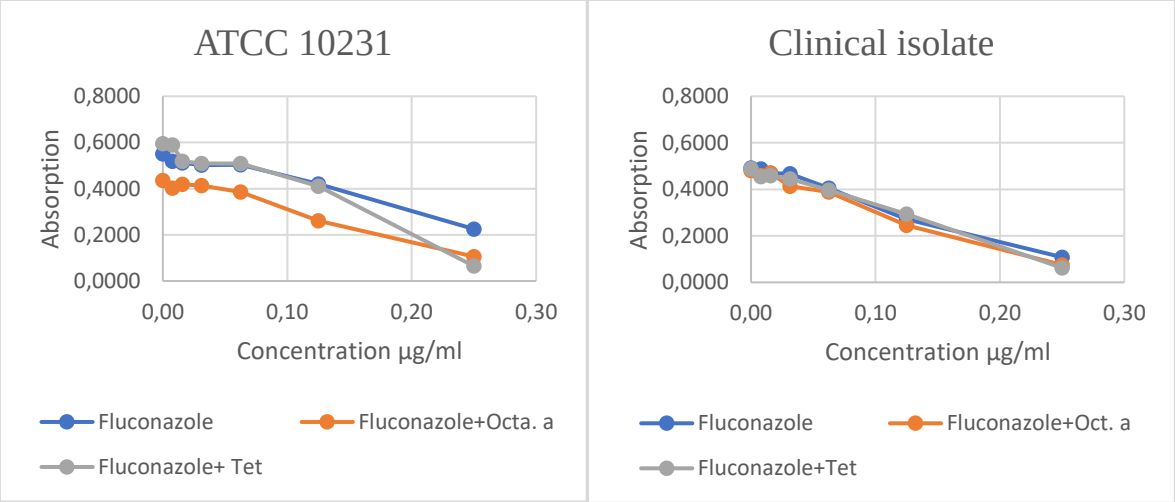

Fig.13. Sensitivity of *C. albicans* cells to fluconazole, fluconazole+ octanoic acid and fluconazole+ tetrandrine. The initial concentrations used were: 0.25 µg/ml fluconazole and 32 µg/ml of TET and octanoic acid.

The sensitivity of ATCC 10231 and clinical isolate to pure fluconazole, fluconazole in combination with TET and fluconazole with octanoic acid was examined. From the presented growth curves, the lowest growth inhibitory concentrations can be determined by the absorption dependence on varying concentrations of the compounds. The MIC values with ATCC 10231 were: (i) Fluconazole MIC_{80} = 0.25 µg /ml, (ii) Fluconazole+ Octanoic acid MIC_{90} = 0.25 µg/ml,

(iii) Fluconazole + Tetrandrine MIC_{90} = 0.25µg/ml. With the clinical isolate: (i) Fluconazole MIC_{90} = 0.25 µg/ml, (ii) Fluconazole+ Octanoic acid MIC_{90} = 0.25 µg/ml, (iii) Fluconazole + Tetrandrine MIC_{90} = 0.25µg/ml. Fluconazole is slightly more active in combination with octanoic acid against ATCC 10231.

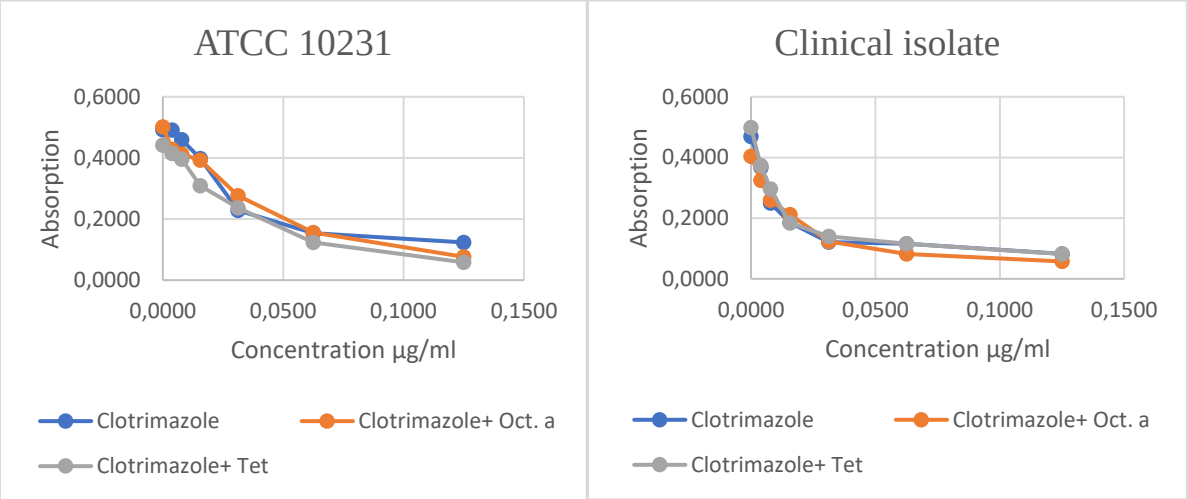

Fig.14. Sensitivity of *C. albicans* to clotrimazole, clotrimazole+ octanoic acid and clotrimazole+ tetrandrine. The initial concentrations used were: 0.125 µg/ml clotrimazole and 32 µg/ml of TET and octanoic acid.

The growth response of ATCC 10231 and clinical isolate to pure clotrimazole, clotrimazole in combination with TET and clotrimazole with octanoic acid was investigated. The MIC values with ATCC 10231 were: (i) Clotrimazole MIC_{90} = 0.125 µg /ml, (ii) Clotrimazole+ Octanoic acid MIC_{90} = 0.125 µg/ml, (iii) Clotrimazole + Tetrandrine MIC_{90} = 0.125µg/ml. With the clinical isolate: (i) Clotrimazole MIC_{90} = 0.125 µg/ml, (ii) Clotrimazole+ Octanoic acid MIC_{90} = 0.0625 µg/ml, (iii) Clotrimazole + Tetrandrine MIC_{90} = 0.125µg/ml. C.i 110717 was more sensitive to the combination of clotrimazole with octanoic acid. From the two azoles investigated, clotrimazole showed much stronger inhibitory action than fluconazole.

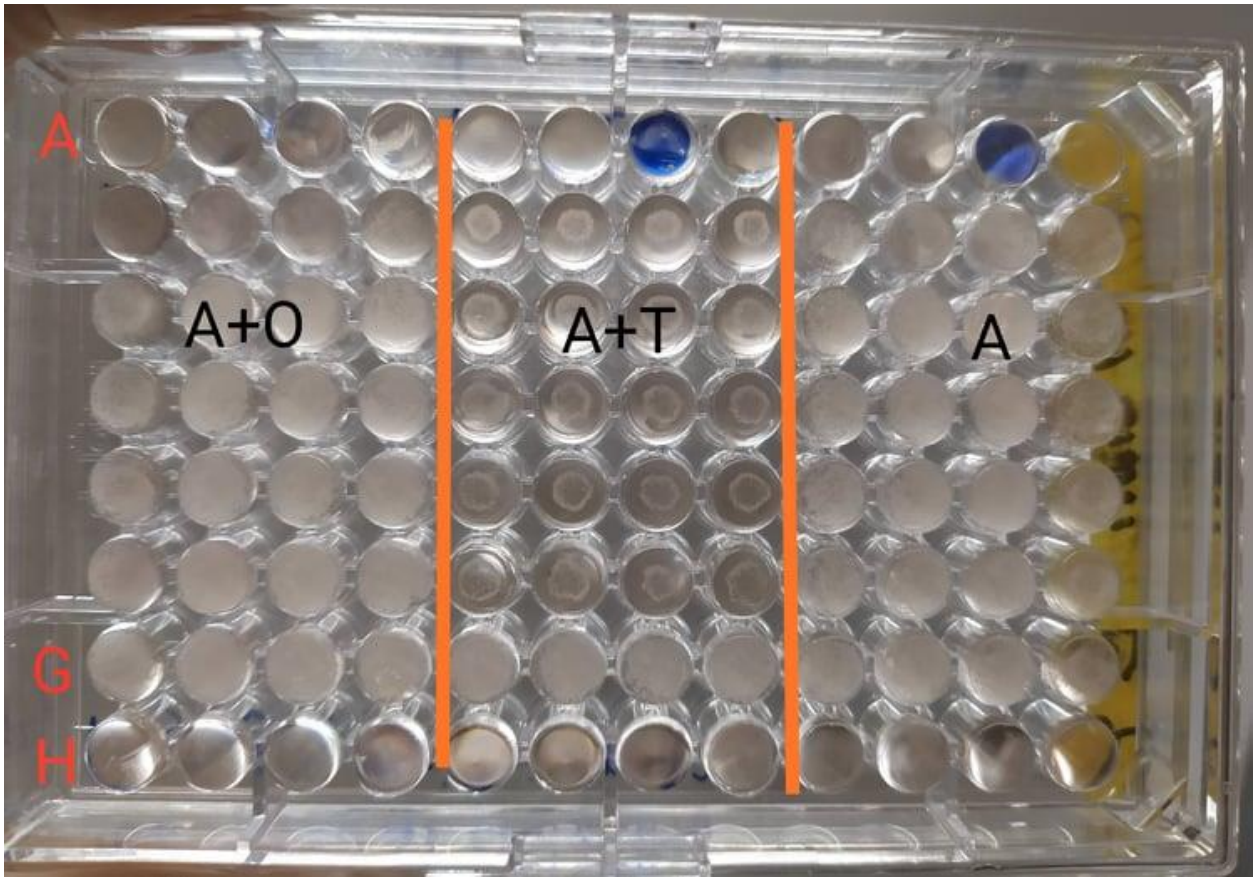

Fig.15. Preliminary visual observation of turbidity of the microplate after incubation- Clinical isolate 110717 with Amphotericin+ Octanoic acid, Amphotericin + Tetrandrine and pure Amphotericin. **(A)** - row with the highest concentration of compounds. **(G)** - Control with only cells. **(H)** - Negative control with only medium. Strong synergistic inhibitory action via combination of amphotericin with TET can be observed.

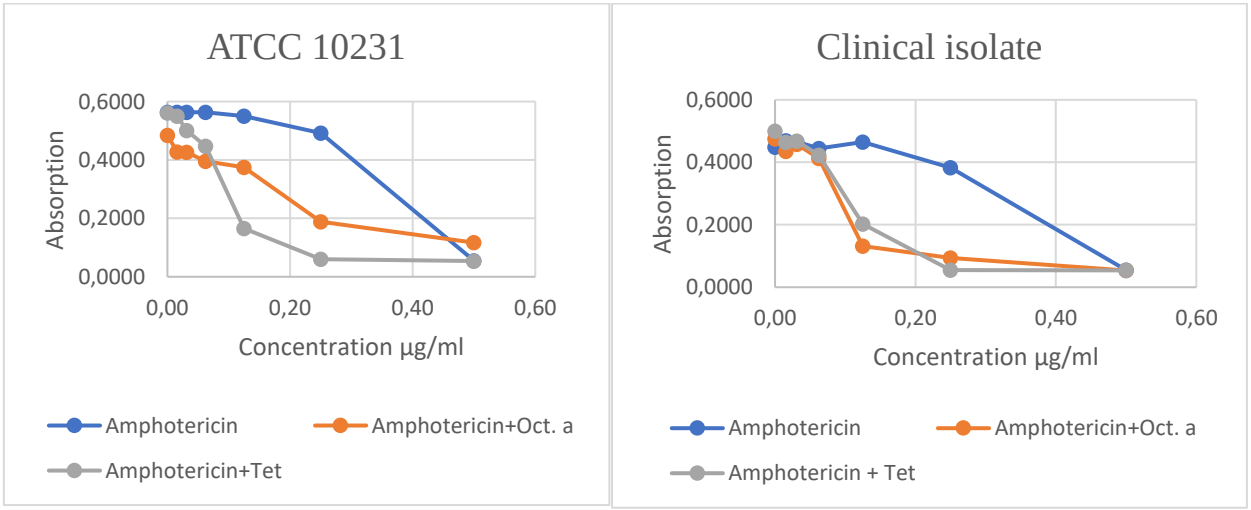

Fig.16. Sensitivity of *C. albicans* to amphotericin, amphotericin+ octanoic acid and amphotericin+ tetrandrine. The initial concentrations of the compounds were: 0.5 µg/ml of amphotericin and 32 µg/ml of TET and octanoic acid.

The MIC values with ATCC 10231 were found to be: (i) Amphotericin MIC_{90} = 0.5 µg /ml, (ii) Amphotericin+ Octanoic acid MIC_{90} = 0.5 µg/ml, (iii) Amphotericin + Tetrandrine MIC_{100} = 0.25 µg/ml. With the clinical isolate: (i) Amphotericin MIC_{100} = 0.5 µg/ml, (ii) Amphotericin+ Octanoic acid MIC_{90} = 0.25 µg/ml, (iii) Amphotericin + Tetrandrine MIC_{100} = 0.25 µg/ml. There was a clearly observable synergistic action between amphotericin and tetrandrine, reducing the MIC by half against both strains. Octanoic acid also demonstrated a strong inhibitory action with amphotericin, but to a lower degree than tetrandrine. Clinical isolate demonstrated a higher sensitivity to the combination of compounds.

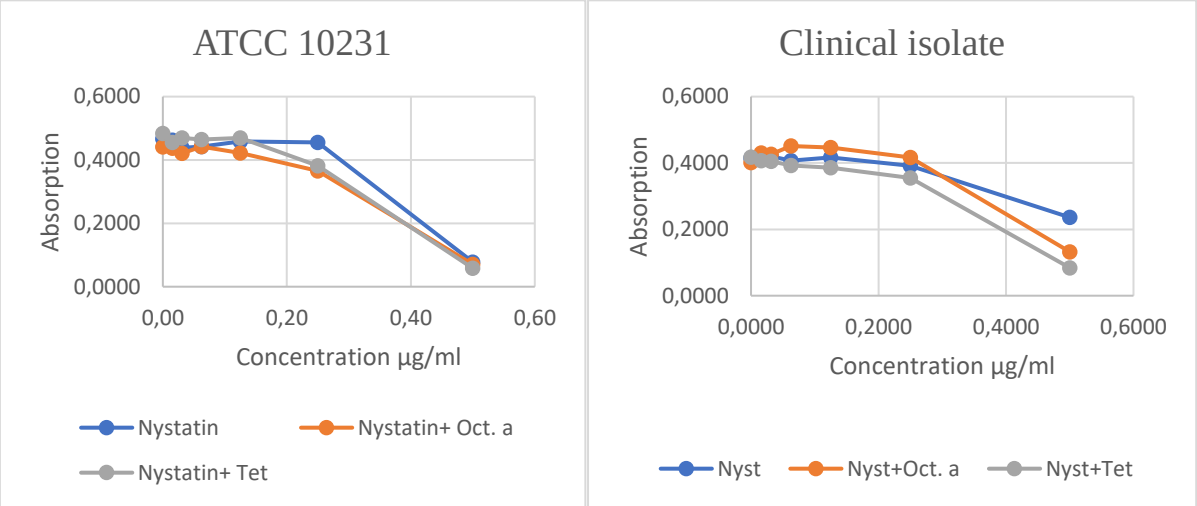

Fig.17. Sensitivity of *C. albicans* cells to nystatin, nystatin+ octanoic acid and nystatin+ tetrandrine. The initial concentrations of the compounds were: 0.5 µg/ml of nystatin and 32 µg/ml of TET and octanoic acid.

The MIC values of Nystatin and compound combinations for ATCC 10231 were as follows: (i) Nystatin MIC_{90} = 0.5 µg /ml, (ii) Nystatin+ Octanoic acid MIC_{90} = 0.5 µg/ml, (iii) Nystatin + Tetrandrine MIC_{90} = 0.5 µg/ml. With the clinical isolate: (i) Nystatin MIC_{80} = 0.5 µg/ml, (ii) Nystatin+ Octanoic acid MIC_{90} = 0.5 µg/ml, (iii) Nystatin + Tetrandrine MIC_{90} = 0.25 µg/ml. The clinical isolate appears to be slightly less sensitive to nystatin than the ATCC 10231 strain. Of the two polyenes, amphotericin exhibits a much stronger inhibitory effect on cell growth than nystatin.

3.2 Determination of minimal growth inhibitory concentration in *S. cerevisiae*

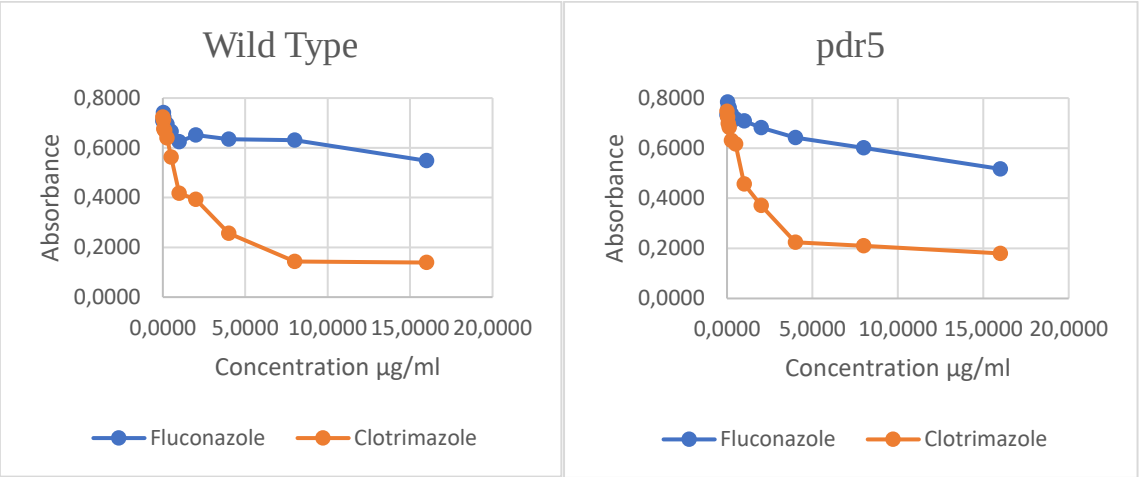

Fig.18. Sensitivity of *S.cerevisiae* cells to fluconazole and clotrimazole.

The sensitivity of Wild Type and pdr5 strains of *S. cerevisiae* was determined to antifungals from the azole family - fluconazole and clotrimazole. From the presented growth curves, the lowest growth inhibitory concentrations can be determined by the absorption dependence on varying concentrations of the compounds. The MIC values with Wild Type were observed to be: Fluconazole MIC_{60} = 16 µg / ml, clotrimazole MIC_{90} = 10 µg / ml; with pdr5: Fluconazole MIC_{60} = 16 µg / ml, clotrimazole MIC_{90} = 10 µg / ml. These results show the development of resistance in *S. cerevisiae* to fluconazole.

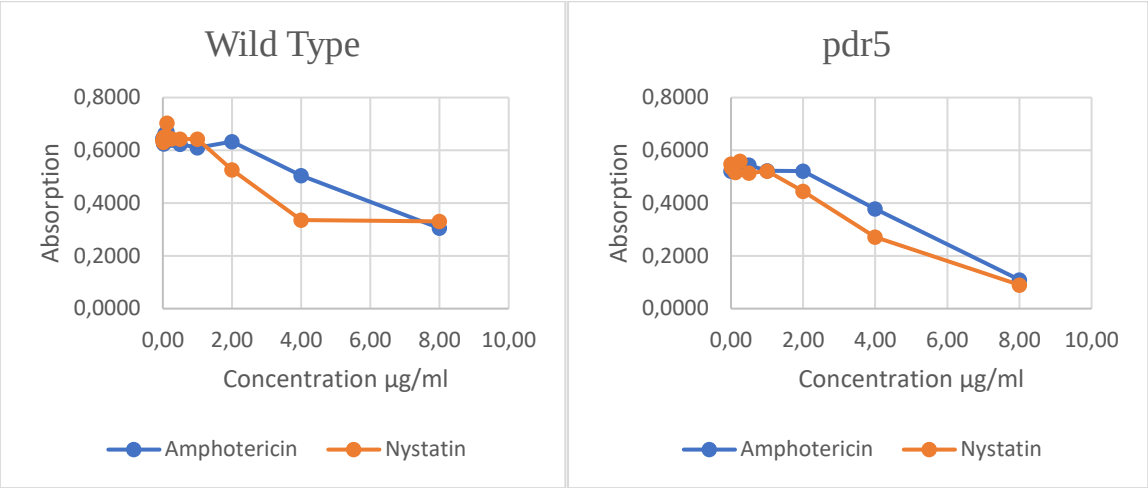

Fig.19. Sensitivity of *S. cerevisiae* cells to amphotericin and nystatin.

Here Wild Type and pdr5 strains were tested against antifungals from the polyene family – amphotericin and nystatin. From the presented growth curves, the lowest growth inhibitory concentrations can be determined by the absorbtion dependence on varying concentrations of the compounds. The MIC values with Wild Type were: Amphotericin MIC_{80} = 4 µg / ml, Nystatin MIC_{80} = 8 µg / ml; with pdr5: Amphotericin MIC_{90} = 8 µg/ml, Nystatin MIC_{90} = 8 µg / ml. Pdr5 strain shows a markedly higher extrusion of the compounds as indicated by the steep curve and increase in absorbance. From this data, it can be concluded that the pdr5 strain with an overexpressed Pdr5p pump is much less sensitive to polyenes than the Wild Type strain.

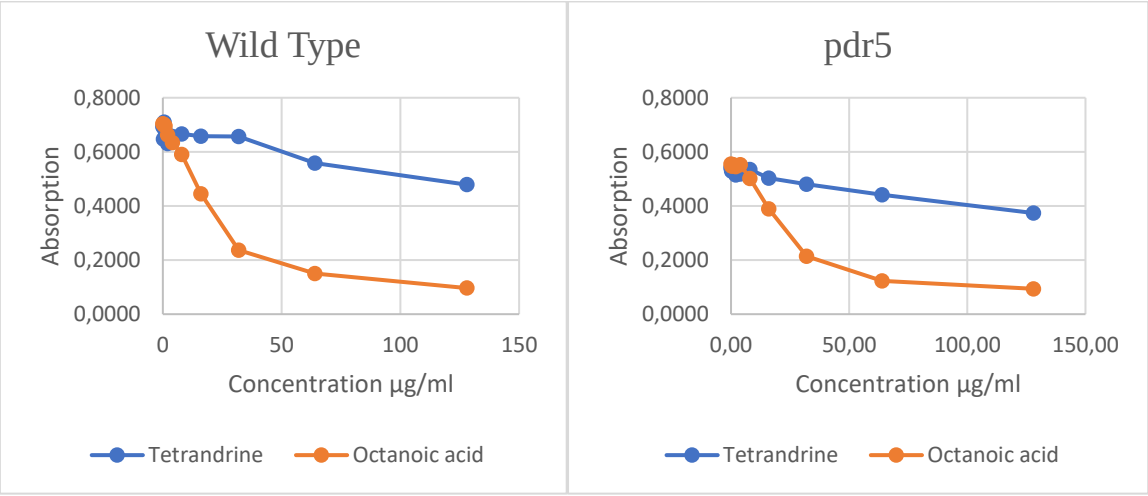

Fig.20. Sensitivity of *S. cerevisiae* cells to tetrandrine and octanoic acid.

The sensitivity of Wild Type and pdr5 to Tetrandrine and Octanoic acid was tested. The MIC values obtained with Wild Type were as follows: Tetrandrine: MIC_{40} = 128 µg / ml, Octanoic acid

MIC_{90} = 64 μg / ml; with pdr5: Tetrandrine MIC_{40}= 128 μg / ml, Octanoic acid MIC_{90} = 64 μg / ml. The given results suggest that *S. cerevisiae* is not very sensitive to Tetrandrine even at high concentrations. Octanoic acid, however, as confirmed by the review of scientific literature, appears to have a noticeable inhibitory effect on both strains.

CONCLUSIONS

1. Fluconazole, clotrimazole and nystatin effectively inhibited growth of both tested *C. albicans* strains. Neither tetrandrine, nor octanoic acid had any effect on sensitivity of *C. albicans* cells to these antifungals.

2. A strong synergistic effect was observed between amphotericin and tetrandrine or octanoic acid. These compounds could useful be for fighting MDR in *C. albicans* cells.

3. *S. cerevisiae* have rather low sensitivity to fluconazole and tetrandrine, but octanoic acid and clotrimazole effectively inhibited growth of these cells. Pdr5 strain with overexpressed efflux pumps was less sensitive to antifungals than wild type strain.

REFERENCES

1. Alekshun MN, Levy SB. Molecular mechanisms of antibacterial multidrug resistance. Cell. 2007;128(6):1037–1050. [PubMed]
2. Antimicrobial Resistance Global Report on Surveillance. Geneva, Switzerland: World Health Organization; 2014.
3. Bansal D, Sehgal R, Chawla Y, Malla N, Mahajan RC. Multidrug resistance in amoebiasis patients. Indian Journal of Medical Research. 2006;124(2):189–194. [PubMed]
4. Bennett JW, Robertson JL, Hospenthal DR, et al. Impact of extended spectrum beta-lactamase producing Klebsiella pneumoniae infections in severely burned patients. Journal of the American College of Surgeons. 2010;211(3):391–399. [PubMed]
5. Bloland PB. Drug Resistance in Malaria. World Health Organization; 2001.
6. Chethana GS, Hari venkatesh KR, Mirzaei F, Gopinath SM. Review on multidrug resistant bacteria and its implication in medical sciences. Journal of Biological Scientific Opinion. 2013;1(1):32–37.
7. Cortez KJ, Maldarelli F. Clinical management of HIV drug resistance. Viruses. 2011;3(4):347–378. [PMC free article] [PubMed]
8. Cowen, Leah E. "Microevolution of Antifungal Drug Resistance." Handbook of Antimicrobial Resistance, 2017, pp. 345–368., doi:10.1007/978-1-4939-0694-9_19.
9. CSID:370, http://www.chemspider.com/Chemical-Structure.370.html
10. Cuenca-Estrella M, Gomez-Lopez A, Mellado E, Buitrago MJ, Monzón A, Rodriguez-Tudela JL. Scopulariopsis brevicaulis, a fungal pathogen resistant to broad-spectrum antifungal agents. Antimicrobial Agents and Chemotherapy. 2003;47(7):2339–2341. [PMC free article] [PubMed]
11. Doliwa C, Escotte-Binet S, Aubert D, et al. Sulfadiazine resistance in Toxoplasma gondii: no involvement of overexpression or polymorphisms in genes of therapeutic targets and ABC transporters. Parasite. 2013;20(19):1–6. [PMC free article] [PubMed]
12. Dzidic S, Suskovic J, Kos B. Antibiotic resistance mechanisms in bacteria: biochemical and genetic aspects. Food Technology and Biotechnology. 2008;46(1):11–21.
13. Fallon PG, Doenhoff MJ. Drug-resistant schistosomiasis: resistance to praziquantel and oxamniquine induced in Schistosoma mansoni in mice is drug specific. The American Journal of Tropical Medicine and Hygiene. 1994;51(1):83–88. [PubMed]
14. Fishbain J, Peleg AY. Treatment of Acinetobacter infections. Clinical Infectious Diseases. 2010;51(1):79–84. [PubMed]

15. Greenberg RM. New approaches for understanding mechanisms of drug resistance in schistosomes. Parasitology. 2013;140(12):1534–1546. [PMC free article] [PubMed]
16. Hamilton KW, Fishman NO. Antimicrobial stewardship interventions: thinking inside and outside the box. Infectious Disease Clinics of North America. 2014;28(2):301–313. [PubMed]
17. He X, Li S, Kaminskyj SG. Using Aspergillus nidulans to identify antifungal drug resistance mutations. Eukaryotic Cell. 2013;13(2):288–294. [PMC free article] [PubMed]
18. Holmes, Ann R, et al. "Targeting Efflux Pumps to Overcome Antifungal Drug Resistance." *Future Medicinal Chemistry*, vol. 8, no. 12, 2016, pp. 1485–1501., doi:10.4155/fmc-2016-0050
19. Howard SJ, Arendrup MC. Acquired antifungal drug resistance in Aspergillus fumigatus: epidemiology and detection. Medical Mycology. 2011;49(1):S90–S95. [PubMed]
20. Hurt AC. The epidemiology and spread of drug resistant human influenza viruses. Current Opinion in Virology. 2014;8:22–29. [PubMed]
21. Khalilzadeh S, Boloorsaz MR, Safavi A, Farnia P, Velayati AA. Primary and acquired drug resistance in childhood tuberculosis. Eastern Mediterranean Health Journal. 2006;12(6):909–914. [PubMed]
22. Ksiezopolska, Ewa, and Toni Gabaldón. "Evolutionary Emergence of Drug Resistance in Candida Opportunistic Pathogens." *Genes*, vol. 9, no. 9, 2018, p. 461., doi:10.3390/genes9090461.
23. Kunjachan S, Rychlik B, Storm G, Kiessling F, Lammers T. Multidrug resistance: physiological principles and nanomedical solutions. Advanced Drug Delivery Reviews. 2013;65(13-14):1852–1865. [PMC free article] [PubMed]
24. Lee CR, Cho IH, Jeong BC, Lee SH. Strategies to minimize antibiotic resistance. International Journal of Environmental Research and Public Health. 2013;10(9):4274–4304. [PMC free article] [PubMed]
25. Li XZ, Nikaido H. Efflux-mediated drug resistance in bacteria: an update. Drugs. 2009;69(12):1555–1623. [PMC free article] [PubMed]
26. Liu, Y., Zhao, Y.-Q., Wang, L.-P., Ma, C., Zhao, K., & Feng, N.-P. (2013). Preparation and characterization of tetrandrine-phospholipid complex loaded lipid nanocapsules as potential oral carriers. *International Journal of Nanomedicine*, 4169. doi: 10.2147/ijn.s50557
27. Loeffler J, Stevens DA. Antifungal drug resistance. Clinical Infectious Diseases. 2003;36(1):S31–S41. [PubMed]
28. Lurain NS, Chou S. Antiviral drug resistance of human cytomegalovirus. Clinical Microbiology Reviews. 2010;23(4):689–712. [PMC free article] [PubMed]

29. Marks SM, Flood J, Seaworth B, et al. Treatment practices, outcomes, and costs of multidrug-resistant and extensively drug-resistant tuberculosis, United States, 2005–2007. Emerging Infectious Diseases. 2014;20(5):812–821. [PMC free article] [PubMed]
30. Margeridon-Thermet S, Shafer RW. Comparison of the mechanisms of drug resistance among HIV, hepatitis B, and hepatitis C. Viruses. 2010;2(12):2696–2739. [PMC free article] [PubMed]
31. McFadden DC, Tomavo S, Berry EA, Boothroyd JC. Characterization of cytochrome b from Toxoplasma gondii and Q(o) domain mutations as a mechanism of atovaquone-resistance. Molecular and Biochemical Parasitology. 2000;108(1):1–12. [PubMed]
32. McKinnon, PS and Davis, SL. Pharmokinetic and pharmacodynamic issues in the treatment of bacterial infectious diseases. in: VL Yu, G Edwards, PS McKinnon, C Peloquin, G Morse (Eds.) Antimicrobial therapy and vaccines, volume II: antimicrobial agents. ESun Technologies, Pittsburgh, PA; 2005: 5–19)
33. Moehring RW, Anderson DJ. Antimicrobial stewardship as part of the infection prevention effort. Current Infectious Disease Reports. 2012;14(6):592–600. [PubMed] 1. Kuchler, K., Sterne, R., and Thorner, J. (1989) EMBO J. 8, 3973–3984 2. Doige, C. A., and Ames, G. (1993) Annu. Rev. Microbiol 47, 291–319 3. Higgins, C. F. (1994) Cell 79, 393–395 4. Gottesman, M. M., Currier, S., Bruggemann, E., Lelong, I., Stein, W., and Pastan, I. (1994) Curr. Top. Membr. Transp. 41, 3–17
34. Mohapatra S. Drug resistance in leishmaniasis: newer developments. Tropical Parasitology. 2014;4(1):4–9. [PMC free article] [PubMed]
35. Muzny CA, Schwebke JR. The clinical spectrum of Trichomonas vaginalis infection and challenges to management. Sexually Transmitted Infections. 2013;89(6):423–425. [PubMed]
36. Nagamune K, Moreno SNJ, Sibley LD. Artemisinin-resistant mutants of Toxoplasma gondii have altered calcium homeostasis. Antimicrobial Agents and Chemotherapy. 2007;51(11):3816–3823. [PMC free article] [PubMed]
37. Nikaido H. Multidrug resistance in bacteria. Annual Review of Biochemistry. 2009;78:119–146. [PMC free article] [PubMed]
38. Olasehinde GI, Ojurongbe O, Adeyeba AO, et al. In vitro studies on the sensitivity pattern of Plasmodium falciparum to anti-malarial drugs and local herbal extracts. Malaria Journal. 2014;13(article 63) [PMC free article] [PubMed]
39. Orozco E, López C, Gómez C, et al. Multidrug resistance in the protozoan parasite Entamoeba histolytica . Parasitology International. 2002;51(4):353–359. [PubMed]

40. Ouellette M, Légaré D, Papadopoulou B. Multidrug resistance and ABC transporters in parasitic protozoa. Journal of Molecular Microbiology and Biotechnology. 2001;3(2):201–206. [PubMed]
41. Owens RC., Jr. Antimicrobial stewardship: concepts and strategies in the 21st century. Diagnostic Microbiology and Infectious Disease. 2008;61(1):110–128. [PubMed]
42. Pohl, Carolina & Kock, Johan & Thibane, Vuyisile. (2011). Antifungal free fatty acids: A Review. Science Against Microbial Pathogens: Communicating Current Research and Technological Advances.
43. Popęda M, Płuciennik E, Bednarek AK. Proteins in cancer resistance. PostępyHigienyiMedycynyDoświadczalnej. 2014;68:616–632. [PubMed]
44. Qi L, Cui J. A schistosomiasis model with praziquantel resistance. Discrete Dynamics in Nature and Society. 2013;2013:13 pages.945767
45. Rodero L, Mellado E, Rodriguez AC, et al. G484S amino acid substitution in lanosterol 14-α demethylase (ERG11) is related to fluconazole resistance in a recurrent Cryptococcus neoformans clinical isolate. Antimicrobial Agents and Chemotherapy. 2003;47(11):3653–3656. [PMC free article] [PubMed]
46. Santos, Giselle C. De Oliveira, et al. "Candida Infections and Therapeutic Strategies: Mechanisms of Action for Traditional and Alternative Agents." *Frontiers in Microbiology*, vol. 9, Mar. 2018, doi:10.3389/fmicb.2018.01351.
47. Singh V. Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education. Vol. 1. Formatex Research Center; 2013. Antimicrobial resistance; pp. 291–296.
48. Strasfeld L, Chou S. Antiviral drug resistance: mechanisms and clinical implications. Infectious Disease Clinics of North America. 2010;24(2):413–437. [PMC free article] [PubMed]
49. Suppiah J, Zain RM, Nawi SH, Bahari N, Saat Z. Drug-resistance associated mutations in polymerase (p) gene of hepatitis B virus isolated from malaysian HBV carriers. Hepatitis Monthly. 2014;14(1):7 pages.e13173 [PMC free article] [PubMed]
50. Tenover FC. Mechanisms of antimicrobial resistance in bacteria. The American Journal of Medicine. 2006;119(6):S3–S10. [PubMed]
51. Tsukahara, Tohru. "Fungicidal Action Of Caprylic Acid Forcandida Albicans." *Japanese Journal of Microbiology*, vol. 5, no. 4, 1961, pp. 383–394., doi:10.1111/j.1348-0421.1961.tb00217.x.
52. Ullman B. Multidrug resistance and P-glycoproteins in parasitic protozoa. Journal of Bioenergetics and Biomembranes. 1995;27(1):77–84. [PubMed]

53. Vanaerschot M, Dumetz F, Roy S, Ponte-Sucre A, Arevalo J, Dujardin JC. Treatment failure in leishmaniasis: drug-resistance or another (epi-) phenotype? Expert Review of Anti-Infective Therapy. 2014;12(8):937–946. [PubMed]
54. Whaley, S. G., Berkow, E. L., Rybak, J. M., Nishimoto, A. T., Barker, K. S., & Rogers, P. D. (2017). Azole Antifungal Resistance in Candida albicans and Emerging Non-albicans Candida Species. Frontiers in Microbiology, 7. doi: 10.3389/fmicb.2016.02173
55. World Health Organization. Antimicrobial resistance. 2014.
56. Wu CP, Ohnuma S, Ambudkar SV. Discovering natural product modulators to overcome multidrug resistance in cancer chemotherapy. Current Pharmaceutical Biotechnology. 2011;12(4):609–620. [PMC free article] [PubMed]
57. Wutzler P. Antiviral therapy of herpes simplex and varicella-zoster virus infections. Intervirology. 1997;40(5-6):343–356. [PubMed]
58. Yang Z, Li C, Miao M, et al. Multidrug- resistant genotypes of plasmodium falciparum, Myanmar. Emerging Infectious Diseases. 2011;17(3):498–501. [PMC free article] [PubMed]
59. Zhang, L., Geng, Y., Duan, W., Wang, D., Fu, M., & Wang, X. (2009). Ionic liquid-based ultrasound-assisted extraction of fangchinoline and tetrandrine fromStephaniae tetrandrae. Journal of Separation Science, 32(20), 3550–3554. doi: 10.1002/jssc.200900413

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to the supervisor of my thesis, Prof. Habil. Dr. Rimantas Daugelavičius for affording me the opportunity to work at the department of Biochemistry and for correcting and reviewing my thesis.

I am very grateful to Neringa Kuliešienė for her careful guidance, exceptional patience and immense professionalism in familiarizing me with the scientific method and teaching me to deliver my work with quality, precision and purpose. It was a great privilege to collaborate with her.

I would like to thank the reviewer of my thesis, Erika Juškaitytė for screening and assessing my thesis.

A big, hearty thank you to Tomas, Deimantė and Simona from the Department of Biochemistry for being very kind and attentive and imparting me with practical knowledge that greatly accelerated my lab experience.