COMBAT RESISTANCE IN PROKARYOTIC AND EUKARYOTIC CELLS

Ph.D. thesis

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Szeged

2009

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ABBREVIATIONS

5-FU 5-fluorouracile

AACs aminoglycoside acetyltransferases

ABC transporters ATP-binding cassette transporters

AMP Ampicillin

ANTs aminoglycoside nucleotidyltransferases

AO acridine orange

APH aminoglycoside phosphotransferases

ATP adenosine triphosphate

B. fragilis *Bacteroides fragilis*

BPTs biphenyl tetrazoles

C. albicans *Candida albicans*

ccc DNA covalently closed circular DNA

CE collision energy

CFU colony forming unite

CPZ Chlorpromazine

D. pulchra *Delisea pulchra*

DMSO dimethyl sulfoxide

DNA deoxyribonucleic acid

EB ethidium bromide

E. coli *Escherichia coli*

E. faecium *Enterococcus faecium*

e.g. exempli gratia

EMB eosin methylene blue

ERY Erythromycin

ESBLs extended spectrum beta-lactamases

FAR Fluorescence Activity Ratio

FDA Food and Drug Administration

FQ Fluoroquinolone

FIC fractional inhibitory concentration

GENT Gentamycin

HPLC High Perormance Liquid Chroamtography

IDA Information Dependent Acquisition

LB Luria-Bertani

M. tuberculosis *Mycobacterium tuberculosis*

MATE multidrug and toxic compound extrusion

MDR multidrug resistance

MFP periplasmic membrane fusion protein

MFS major facilitator superfamily

MIC minimum inhibitory concentration

MSSA methicillin-sensitive *Staphylococcus aureus*

MRM Multiple Reaction Monitoring

MRSA methicillin resistant *Staphylococcus aureus*

MTT 3-(4,5-dimethylthiazyl)-2,5-diphenyltetrazolium bromide

MTY Minimal Trypton-Yeast

OD optical density

OMP outer membrane protein

OXY Oxytetracycline

P. aeruginosa *Pseudomonas aeruginosa*

PBP penicillin binding protein

PBS phosphate-buffered saline

P-gp P-glycoprotein

QS quorum sensing

R 123 rhodamine 123

RNA ribonucleic acid

RND resistance-nodulation cell division

S. cerevisiae *Saccharomyces cerevisiae*

SDS sodium dodecylsulfate

SMR small multidrug resistance family

S. pneumoniae *Streptcoccus pneumoniae*

TSA Tryptic Soy Agar

TSB Tryptic Soy Broth

TZ Thioridazine

YTB Yest Extract-Tryptone Broth

1. INTRODUCTION

1.1 Bacterial resistance

The optimism of the early period of antimicrobial discovery has been tempered by the emergence of bacterial strains with resistance to these therapeutics. Antimicrobial resistance threatens the effectiveness of successful treatment of infections and is a public health issue with local, national and global dimensions. Antimicrobial resistance thus can result in increased morbidity, disease burden and mortality. Resistance of bacteria to antimicrobials provide not only a physiological threat but is also costly from economic viewpoint [1,2].

Resistant bacterial strains appeared soon after the introduction of antibiotics in the therapy. These strains initially showed up in hospitals, where most antibiotics were used, causing clinical difficulties for nosocomial treatment on a global scale [3,4,5]. More serious therapeutical obstacle is caused by bacteria resistant to more than one antibiotic. This phenomenon is called multidrug resistance (MDR). This multiple drug resistance of bacteria was first experienced among enteric bacteria i.e. *Escherichia coli*, *Shigella* and *Salmonella* species in the 1950s and 1960s [6]. This attitude changed in the 1970s when *Haemophilus influenzae* and *Neisseria gonorrhoeae*, that cause respiratory and genitourinary disease respectively, appeared with single and multiple resistance [1].

Emergence and spread of MDR strains of human pathogenic bacteria render the therapy more precarious and sometimes unsuccessful. Notable global examples include hospital and community MDR strains of *Mycobacterium tuberculosis*, *Enterococcus faecium*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Beside the growing rate of methicillin resistant *S. aureus* (MRSA), a steadily increasing proportion of MRSA also shows low-level resistance to vancomycin, of which resistance trait is mainly transferred from vancomycin-reistant *enterococci* [1,7,8]. The Centers for Disease Control and Prevention announced the emergence of extensive resistance strains of *M. tuberculosis* raising concerns of a future epidemic of a virtually untreatable tuberculosis [8,9,10].

Antibiotic resistance has been unavoidable from an evolutionary perspective, since the antibiotic pressure provides the potential for resistant bacteria to acquire an important advantage. Discretionary use of antibiotics lead to the spread of resistant strains, which causes the most expressed problem at nosocomial circumstances, mainly in immunocompromized patients. Important contributing factors to community acquired infection with resistant bacteria may be the poor hand-hygiene among healthcare workers and device-associated

infections [11]. Antibiotics can be disseminated into the environment from agricultural sources as well, of which importance has recently been recognized and studied extensively. Antibiotics are widely used as growth promoters in veterinary practice in sub-clinical concentrations and this poses a potential risk for the selection of resistant bacterial strains, which may be transmitted to humans [12].

Since the growing rate of bacterial resistance is of great medical and public concern, there is an urgent need to develop appropriate methods and protocols to overcome or at least reduce the incidence and spread of resistant strains.

1.2 Mechanisms of resistance in bacteria

The indiscriminate use of antibiotics facilitates the appearance and further development of resistance in bacteria, of which mechanisms show a versatile picture. The timeline for the development of clinically significant resistance is multifactorial and depends on several parameters, such as the quantity of antibiotic used, how widely it is prescribed, the frequency with which sub-therapeutic levels of antibiotic are used to select for resistant organism, the reservoirs of existing resistance mechanisms, the number of mutations required for resistance to emerge in a killing target and the fitness of the resistant organisms.

However certain bacterial strains have intrinsic resistance against antimicrobial agents (e.g. intrinsic sulfonamide resistance of *Enterococci*, efflux mediated intrinsic resistance of *Pseudomonas*, macrolide resistance of Gram-negatives, aminoglycosides in strict anaerobes), acquired resistance against antimicrobial chemotherapy possess a larger risk of therapeutical failure. The genetic determinants encoding antimicrobial resistance can be located on the bacterial chromosome or on plasmids, which may replicate independently from the chromosome. Normally susceptible populations of bacteria may become resistant to antimicrobial agents through mutation and selection, or by acquiring from other bacteria the genetic information that encodes resistance. The former is called vertical evolution and the latter horizontal evolution. Horizontal evolution may occur between strains of the same species or between different bacterial species or genera. The acquisition of such genetic elements may occur through genetic mechanisms *i.e.* conjugation, transduction and transformation. For each of these processes, transposons may facilitate the transfer and incorporation of the acquired resistance genes into the host's genome or into plasmids. During conjugation, a Gram-negative bacterium transfers plasmid-containing resistance genes to an adjacent bacterium, via an elongated proteinaceous structure termed pilus, which joins the two organisms. During transduction, resistance genes are transferred from one bacterium to

another via bacteriophage. Finally, transformation (the process whereby bacteria acquire and incorporate DNA segments from other bacteria that have released their DNA into the environment after cell lysis) can move resistance genes into previously susceptible strains. Mutation and selection, together with the mechanisms of genetic exchange, enable many bacterial species to adapt quickly to the introduction of antibacterial agents in their environment [13].

Soil–dwelling bacteria produce and encounter a myriad of antibiotics, evolving corresponding sensing and evading strategies. The presence of antibiotics in the environment has promoted the acquisition or independent evolution of highly specific resistance elements in the absence of innate antibiotic production (such as vancomycin resistance in *Streptomyces coelicolor*, *Paenibacillus* and *Rhododcoccus*). The soil thus could serve as an underrecognized reservoir for resistance that has already emerged or has the potential to emerge in clinically important bacteria [14].

Antibiotic resistance in bacteria can be divided into the following major groups based on the mechanism involved: Resistance may develop due to the presence of an enzyme that inactivates (beta-lactamases) or modifies the antibiotic (aminoglycoside, due to the presence of an alternative enzyme for that inhibited by the antibiotic), modification of the antibiotic-target site, reduced permeability and active efflux may also lead to resistance [15].

Enzymatic degradation and modification: A great deal of antibiotics has hydrolytically susceptible chemical bonds, whose integrity central to biological activity. There are several enzymes that have evolved to target and cleave these vulnerable bonds and as a result, provide a means of destroying antibiotic activity. The first reported antibiotic resistance was the production of penicillinase by pathogenic *E. coli*. Beta-lactamases are widely prevailing enzymes among Gram-negative human pathogenic bacteria, which are able to hydrolyze the beta-lactam ring and render the antibiotic inactive before it reaches the penicillin binding protein (PBP) target. The underlying structural kinship that beta-lactamases share with PBPs allows these enzymes to bind acylate and use a strategically located water molecule to hydrolyze and thereby inactivate the beta-lactam. To date, over 530 beta-lactamase enzymes have been reported. These enzymes may be encoded by the bacterial chromosome, plasmids or transposons. Two major classification systems exist in order to group the great number of these globular proteins: the Ambler classification system and the Bush-Jacoby-Medeiros system, according to the substrate specificity or chemical structure (amino acid sequence) of the enzymes. It is important to emphasize that a single microorganism usually harbours more than one beta-lactamase. As soon as new beta-lactame antibiotics have been introduced into

clinical practice, ″novel″ beta-lactamases i.e. plasmid encoded AmpC beta-lactamases, ESBLs (extended spectrum beta-lactamases) and carbapenemases emerged [16,17,18].

Macrolid esterases also belong to the group of enzymes that are able to inactivate the antibiotic via hydrolizing the macrocyclic structure. While not a common drug resistance mechanism, the presence of erythromycin esterases results in very high levels of resistance in *E. coli* [18].

The most diverse, and consequently the largest, family of resistance enzymes is the group-transferases. These enzymes covalently modify antibiotics resulting in structural alteration that impair target binding. Aminoglycoside resistance is often mediated by modifying enzymes, which transfer a functional group to the aminoglycoside structure thereby inactivating the antibiotic. The structural modifications may be related to the following enzymes: aminoglycoside nucleotidyltransferases (ANTs), aminoglycoside acetyltransferases (AACs), aminoglycoside phosphotransferases (APH). Structural modification of chloramphenicol and streptogramin is carried out also by acetyltransferases [19].

Glycosyltransfer is so far not a widespread mechanism of antibiotic resistance, although it does play a role in self-protection in antibiotic-producing organisms. Macrolide glycosylation in *Streptomyces lividans* is the prototype of this class [20].

Active efflux and reduced uptake: Antibiotic efflux as a resistance mechanism was first recognized for tetracycline in the late 1970s [21]. Since that time, efflux-mediated resistance to a wide range of antibacterial agents, including antibiotics, biocides and solvents, has been reported in many bacteria. Although some are drug-specific, many efflux systems accommodate multiple drugs and thus contribute significantly to bacterial intrinsic and acquired MDR. Although drug efflux pumps are found in Gram-negative and Gram-positive bacteria, efflux-mediated resistance in Gram-negative bacteria is a more complex problem due to the molecular architecture of the cell envelope. Drug efflux systems pump out a broad range of and structurally unrelated compounds from bacteria in an energy-dependent manner, without drug alteration or degradation. Bacterial drug efflux transporters are currently classified into five families: the major facilitator superfamily (MFS), adenosine triphosphate (ATP)-binding cassette superfamily (ABC transporters), the small multidrug resistance family (SMR), the resistance-nodulation cell division (RND) superfamily and the multidrug and toxic compound extrusion (MATE) family. Efflux transporters can be further classified into single- or multi-component pumps. Multi-component pumps, found in Gram-negative organisms, function in association with a periplasmic membrane fusion protein (MFP) component and an outer membrane protein (OMP) component. In most cases multidrug efflux

transporters are chromosomally encoded and therefore not readily transferable between bacteria. There are, however, examples in both Gram-positive and Gram-negative bacteria where these genes are found on mobile elements [22,23].

In the presence of some antibiotics, bacterial pathogens switch off or decrease the expression of the porins that provide entrance for certain hydrophilic antimicrobials resulting in a porin-deficient phenotype. Causality between loss of porins and antibiotic resistance has been suggested in several reports. The outer membrane barriers and multidrug efflux mechanisms are important co-determinants of intrinsic resistance in Gram-negative bacteria, and mutation-driven changes in the outer membrane permeability barrier and efflux activity are responsible for episodes of acquired resistance in these bacteria [24,25].

Target site modification: This group of resistance mechanisms may be based on mutation or extrachromosomal and acquired genetic elements (e.g. plasmids). Streptomycin resistance in about one half of *M. tuberculosis* isolates is associated with the missense mutations in the *rpsL* gene coding for ribosomal protein S12 or nucleotide substitutions in the 16S rRNA gene. Amino acid replacements in the S12 protein affect the higher-order structure of 16S rRNA and confer to streptomycin resistance [26]. Production of altered PBPs with reduced affinity for β-lactams is a common mutational resistance mechanism in several bacterial species (*Streptococci*, *Staphylococci*, *Pseudomonas aeruginosa*, *Helicobacter pylori*) [27,28,29]. One important mechanism of resistance in Gram-positive bacteria is the *erm* genes encoded methylase, which is responsible for the N^6-dimethylation of an adenine residue of the 23S rRNA and that prevents macrolide binding and allows protein synthesis [30,31].

1.3 Attempts to overcome bacterial resistance

The past two decades have witnessed the rapid gowth of bacterial resistance. One approach to meet this challenge is the continued, incremental improvement of existing classes of antibiotics (e.g. beta-lactams that retain their activity against low affinity PBPs) and the development of antibiotics with new structure and target. Traditionally, antibacterial drug discovery relied on the identification of compounds that killed bacteria, followed by the elucidation of the mechanism. However, the completion of the sequences of the genomes of a large number of bacteria has made possible a new approach to drug discovery – target-based screening.

Knowledge of molecular mechanism and structure of antibiotic degrading enzymes is essential to develop approaches to ultimately prevail over resistance. A great number of antibiotics were developed in order to avoid the effects of degrading or modifying enzymes.

Inhibitors of these enzymes would serve an aid in the fight against antibiotic resistance. This approach has been highly successful as a strategy to overcome resistance to the penicillinases where the mechanism-based inactivators of beta-lactamases clavulanic acid, sulbactam and tazobactam have been introduced to the clinical practice [18].

The combination of trimethoprim and sulphamethoxazole involves a mutual interference of two sequential steps in the bacterial folate biosynthetic pathway. Sulphamethoxazole competitively inhibits the dihydropteroate synthetase, while trimethoprim inhibits dihydrofolate reductase, involved in the next step in the folic acid pathway [32,33].

Antibiotics that have recently been taken into clinical practice. Within the last ten years, a number of new antibiotics have been introduced with more or less success. Linezolid (Zyvox, Pharmacia) was the first oxazolidinone type antibiotic, which was introduced into clinical practice for vancomycin resistant *Enterococcus faecalis* infections, nosocomial pneumonia and complicated skin and skin-structure infections. Oxazolidinones were the first class of antimicrobial agents that have been introduced since 1980. Linezolid is a totally synthetic agent, thus, there is a low probability of pre-existing, naturally occurring resistance mechanisms, such as those that might exist in antibiotic producing microorganisms or in organisms that share the same environment with an antibiotic producer. Oxazolidinones target an early step of protein synthesis involving the binding of N-formylmethionil-tRNA to the ribosome. Oxazolidinones also inhibit the formation of the initiation complex in bacterial translation systems [34,35,36]. Quinupristine/dalfopristin is a mixture of two streptogramin antibiotics. Individually, these antibiotics are bacteriostatic against Gram-positive bacteria, but in combination, bactericidal activity can be observed. This combination was the first drug approved by the Food and Drug Administration (FDA) in the USA for the treatment of serious infections caused by vancomycin resistant *E. faecium*. The appearance of side effects, the limitation of administration and emerging resistance has led to abandonment of quinupristin/dalfopristin [37,38]. Glycylcyclines (new generation tetracyclines) have been developed to overcome bacterial resistance mechanisms that emerged to earlier members of the tetracycline class introduced between 1950s and 1970s. Tigecycline (Tigacyl, INN) of glycylcyclines was found to be effective against MDR Gram-negative pathogenes, however it was poorly active against *P. aeruginosa* because it is the substrate for the MexAB/OprM efflux pump [39,40]. Daptomycin, the fermentation product of *Streptomyces roseosporum*, is a novel lipopeptide antibiotic with activity against Gram-positive microorganisms. It was discovered in the 1980s but clinical trials were stopped due to toxicity and clinical development was re-initiated only in 1999 with new dosing schedule. Its mechanism of action

involves insertion into and disruption of the Gram-positive plasma membrane without entering the cytoplasm in a Ca^{2+}-dependent manner. Daptomycin oligomerizes in the membrane and causes efflux of potassium from the bacterial cell [41,42,43]. Vancomycin and teicoplanin resistance stimulated the search for new glycopeptide antimicrobials for the treatment of staphylococcal and streptococcal infections. Dalbavancin, oritavancin and telavancin are hemisynthetic products of natural origin. Dalbavancin is derivative of teicoplanin; oritavancin and telavancin are derivatives of vancomycin. The presence of lipophilic tail on these molecules results in them haveing a prolonged half-life. They share the same mechanism of action with their ancestors [44,45]. Telithromycin is the first member of the ketolide class, a new family of antimicrobials structurally related to macrolides. Telithromycin is a semisynthetic derivative of the 14-membered ring macrolide, erythromycin. Ketolides act via the inhibition of protein synthesis; they interact with the 50S ribosomal subunit near the peptidyl transferase site to inhibit the translation of rRNA and to prevent the elongation step of protein synthesis. In addition, they also interact with the partially assembled 50S subunit precursors to block the formation of a functional subunit. On the basis of clinical trial data, telithromycin possesses a spectrum of activity against the common and atypical respiratory pathogenes, including penicillin- and macrolide-resistant strains of *S. pneumoniae* [46]. The challenge in overcoming target site mutations is to develop agents that are unaffected or at least less impacted by alterations in the genes that code for resistance mechanisms. In fluoroquinolone (FQ) resistance, the alteration of DNA gyrase or topoisomerase IV causes the cessation of FQ activity. Newer FQs, such as sitafloxacin and clinafloxacin are highly active against bacteria carrying individual mutations in gyrA or parC that adversely impact on the activity of other FQs. This appears to be due to the fact that these agents target both gyrase and topoisomerase IV equally well and mutations in both genes are required for resistance [47,48]. As new antimicrobials are discovered, there is a need to assess their potentials in combination therapies with old antibiotics that have been rendered ineffective by the development of resistant strains.

New targets for antibiotic development. Sortases promote the covalent anchoring of surface proteins to the cell wall envelope. These enzymes catalyze a transpeptidation reaction by first cleaving a surface protein substrate at the cell wall sorting signal. Since, the elements of transpeptidation and cell-wall sorting are conserved, sortases that catalyze that reaction might also be found conserved in different bacterial species. Compounds that inhibit sortase may be used as anti-bacterial therapies for the treatment of human infections caused by Gram-positive microbes [49,50]. The non-mevalonate pathway (DOXP/MEP pathway, 1-deoxy-D-xilulose-

5-phosphate/2-C-methyl-D-erythritol-4-phosphate pathway) of isoprenoid biosynthesis is absent in mammals, but occurs in serious human pathogenes. Enzymes of the DOXP/MEP pathway present attractive targets for development of broad spectrum antimicrobial drugs, since isoprenoid precursors are important for the biosynthesis of cell wall elements [51,52,53].

Genomics made a new potential target-rich therapeutic area, since genetic investigations revealed the subset of essential bacterial genes that would be a priori candidates for killing sites by inhibitors. Comparative bacterial genomics allow further prioritization of open reading frames that are conserved in pathogens but absent in higher eukaryotes, and for which a function can often be predicted by bioinformatics [54, 55].

Resistance modifiers. Pharmacons, which reduce the resistance of bacteria or enhance the activity of an antibiotic, are called resistance modifiers or MDR inhibitors. These compounds do not evidently exert antimicrobial properties, however they might be able to modify bacteria so that they will gain susceptible phenotype applied in combination with antibiotics. These agents may be existing medicines applied in a therapeutical area other than antiinfectives, newly synthesised molecules that target resistance mechanisms in order to reverse MDR and compounds of natural origin that have the potential to modify antibiotic resistance. Resistance modifiers may target different mechanisms e.g. efflux systems, quorum sensing systems, cell membrane permeability, antibiotic degrading enzymes; these agents also may act on genetic level e.g. elimination of plasmids that carry antibiotic resistance genes. In Gram-negative bacteria, outer membrane forms a permeability barrier, which explains the enhanced resistance of Gram-negatives versus Gram-positives. Recent data indicate that the outer membrane barrier as a determinant of resistance is only significant in the context of additional resistance mechanisms that work synergistically with it to promote resistance [56]. It is expected therefore, that compromising this barrier by its permeabilization would be an effective approach to combating antimicrobial resistance [57,58]. A variety of compounds which are employed in the management of pathological conditions of a non-infectious aethiology have also been shown to modify cell permeability and to exhibit broad-spectrum antimicrobial activity *in vitro* against bacteria. These compounds are called non-antibiotics. Synergy between conventional antibiotics and non-antibiotics has been supported by a number of investigations. The compounds that have produced such synergy against a wide range of bacterial species are phenothiazines and tricyclic antidepressants and other membrane stabilizing agents employed in the management of psychoses, pain etc.

Unfortunately, *in vivo* studies have only rarely been initiated upon the *in vitro* potential advantages [59].

Chlorpromazine (CPZ), a phenothiazine employed for the management of psychosis, has long been known to have *in vitro* activity against a wide spectrum of bacteria. However, *in vitro* activities require concentrations of the drug that are well beyond those clinically achievable. Nevertheless, sporadic reports have appeared over the years suggesting that clinical doses of CPZ are effective for the management of bacteriaemia [60,61]. The *in vivo* activity is apparently the result of the drug being concentrated by macrophages that contain the phagocytosed organism [62,63]. It was demonstrated that physiological concentrations of CPZ present in the medium could enhance the killing of *M. tuberculosis* that had been phagocytosed by macrophages and this is the case for thioridazine (TZ) as well [64,65]. Thioridazine below the concentrations that are achieved in the plasma of TZ-treated patients have been shown to kill tuberculosis (TB), methicillin-sensitive *S. aureus* (MSSA) and MRSA intracellularly in macrophages that have low killing activity against the bacterium [66,67]. Since, TZ has been safely used for decades, it is anticipated to be a complementary agent in the treatment of persistent and recurrent intracellular infections.

Efflux pump inhibitors: Active efflux systems are present in all living cells. They participate in the detoxifying process expelling various harmful compounds and xenobiotics. The emergence of active efflux as a major causative factor in antibiotic resistance has been one of the most significant trends in anti-infective chemotherapy over the past decades. The phenomenon affects virtually all classes of antibiotics and frequently results in multi-drug resistant phenotypes [68]. Efflux activity and pump components are putative targets for the development of new molecules. To specifically block the activity of drug efflux pumps, several possibilities are available: create an obstruction in the outer membrane channel, generate competition in the inner membrane pump, alter the pump assembly or collapse the energy component of the mechanism. The recent demonstration of drug capture in the periplasm and outer-membrane pumping out, termed 'periport', offers the possibility to use periplasmic transit blockers to obstruct the AcrB-pump central cavity [69]. Several compounds have been assessed on diverse drug resistant Gram-negative bacteria. These compounds include PAβN (potentiates levofloxacin activity in resistant *P. aeruginosa*), carbonyl cyanide *m*-chlorophenylhydrazone (an energy uncoupler that collapses the membrane energy), quinolines and arylpiperazine derivatives that reverse MDR in bacteria [70]. For specific efflux pumps, many putative inhibitors that share tetracycline-analogue structural properties have been screened to combat the tetracycline efflux mechanism. Some

of them demonstrate an interesting reversal capacity and these studies indicate that tetracycline derivatives, identified by their ability to block the Tet(B) efflux protein, can restore tetracycline activity against resistant bacteria bearing either of the two known resistance mechanisms (efflux and ribosomal-protecting resistance mechanism) [71].

Plasmid curing agents: The genetical basis of resistance is often the R-plasmid, which can be transferred to other bacteria in the environment of the recipient, and these extrachromosomal DNA sequences can be responsible for the emergence of multiple resistance to antibiotics. Plasmid may be lost spontaneously in a very low frequency (10^{-5} to 10^{-7}), but certain effects can increase the probability of plasmid loss, which is the basis of artificial plasmid elimination. In early studies, acridine orange (AO), ethidium bromide (EB) and later sodium dodecylsulfate (SDS) were found to be powerful plasmid eliminators [72,73]. Their toxicity did not allow their *in vivo* testing for their antiplasmid effect. Molnar *et al.* investigated the effects of many tricyclic drugs on plasmid replication and found that two drugs applied in everyday practice exerted antiplasmid activity [74]. Chlorpromazine and promethazine eliminated the tetracycline, chloramphenicol, streptomycin and sulfonamide resistance of an *E. coli* strain. That finding resulted in systematically synthesized phenothiazine and acridine derivatives being studied for their biological activity and a relevant proportion of the tested molecules proved to have an antiplasmid effect [75,76,77,78,79]. The possible mechanism of action is complex formation with the guanine-cytosine-rich regions of the plasmid DNA in the covalently closed circular (ccc) form of DNA, which are necessary for normal plasmid replication in an uncomplexed form [80]. The use of phenothiazine drugs as resistance modifiers is restricted due to their toxicity, because the concentrations required for antiplasmid effects are beyond that may be clinically achievable, therefore only a limited number of data are available about their resistance-modifier activity *in vivo*. However, promethazine was studied *in vivo* in children with frequently recurring pyelonephritis, in combination with gentamycin, and the result was positive, the combination reduced the number of recurrences in urinary tract infections as compared with the control group [81]. In another *in vivo* study, some of the patients recovered from the urogenital infections in spite of the fact that plasmid elimination in the urine did not occur and the causative agents were resistant to gentamycin. These findings suggest that promethazine may affect the specific pili mediated and plasmid encoded adhesion or multiplication of bacteria on epithelial cells [82]. Promethazine and imipramine were investigated in the inhibition of adhesion of nephropathogenic *E. coli* strain in tissue culture by the evaluation of scanning electronmicroscopy. It was found that besides the direct antibacterial effect it can be

presumed that low concentration of promethazine and imipramine can inhibit the reversible and irreversible attachment of bacteria to epithelial cells, since both drugs interfere with the function of the microfilaments of cells and bacteria via membrane effects [83,84].

Blocking degrading/altering enzymes: There have been considerable efforts to discover inhibitors of β-lactamases and, in particular, molecules that target the emerging metallo-β-lactamases. Metallo-β-lactamases confer resistance to the majority of commercially used antibiotics and are present in problematic pathogens. Mercaptoacetic acid thiol ester and mercaptophenylacetic acid analogues were reported to have *in vitro* effect against metallo–β–lactamases with greatest potency demonstrated against the *S. maltophylia* L1 enzyme [85,86]. Biphenyl tetrazoles (BPTs) are a structural class of potent competitive inhibitors of metallo-β-lactamase identified through screening and predicted using molecular modelling of the enzyme structure. Inhibition of metallo-β-lactamase by BPTs *in vitro* correlates well with the antibiotic sensitization of resistant *B. fragilis* [87]. Class C β-lactamases are related to cephalosporin resistance when hyperproduced. Several penems inhibit AmpC enzymes, but none has been developed commercially. 1,5 dihydroxy-4-pyridon monobactam, which inhibits AmpC β-lactamases but not other types, lacks antibacterial activity on its own. However, in the presence of conalbumin, as an iron chelator, it potentiated the antimicrobial effects of different antibiotics applied in combination against AmpC producers *in vitro* [88]. Some of the more recently described β-lactamase inhibitors are broad-spectrum, being active against Class A and C enzymes [89].

Observations that aminoglycoside derivatives that are poor substrates for modifying enzymes are more active against resistant bacteria provided a new approach in overcoming resistance mediated by modifying enzymes. In this vein, a number of novel aminoglycosides have been described whose activity is unaltered in resistant versus sensitive strains. A recently described Kanamycin A variant exhibits spontaneous loss of the phosphate group donated by *O*-phosphotransferases (APH), rendering it effectively resistant to APH-mediated inactivation. Although the compound is inherently much less potent than kanamycin A, these studies suggest a potentially useful approach to develop enzyme-resistant aminoglycosides [27].

Quorum sensing inhibitors: Quorum sensing (QS) is a generic regulatory mechanism used by many bacterial species to perceive and respond to different factors in a population density-dependent manner. This communication system is based on the production, release, detection and response to small hormone-like signal molecules, the so called autoinducers. Quorum sensing mechanisms play crutial role in the production of virulence factors, formulation of biofilms, entry into stationary phase, conjugal transfer of plasmid DNA, spore formation and

transformation competence of human pathogenic bacteria, therefore it seems an appropriate target in the development of new antimicrobials or adjuvant agents. Several strategies aiming at the interruption of the bacterial quorum sensing circuits are possible, including inhibition of signal generation, signal dissemination and signal reception [90]. *S*-adenosil methionine analogues have been demonstrated to be potent inhibitors of QS signal synthesis and some macrolide antibiotics may also compromise the synthesis of QS signals in *P. aeruginosa* at sub minimum inhibitory concentration (MIC) concentration [91,92]. Certain bacteria produce enzymes that degrade QS signal molecules, which phenomenon has a great significance from clinical and ecological viewpoint. *Bacillus* species were found to produce enzymes that catalyze the hydrolysis of acetylated homoserine lactones, which are the main type of autoinducers among Gram-negative bacteria [93]. These enzymes would serve as models for the development of chemical agents that would act via the inhibition of QS-signal dissemination. Blocking of quorum-sensing signal transduction can be achieved by an antagonist molecule capable of competing or interfering with the native signal for binding to the receptor.

QS mechanisms were reported to regulate the expression of efflux pumps that mediate resistance to structurally unrelated compounds, therefore, the parallel intervention in the QS and efflux mechanisms could potentiate the antimicrobial and resistance modulation [94,95].

Biofilm formation is an example of microbial community behaviour and is based on QS mechanisms. Bacteria present in biofilms have characteristics distinct from those of free-swimming (planktonic) bacteria of the same species, including a significantly increased tolerance to antimicrobial therapies and the host immune response. In modern clinical microbiology, the establishment of bacterial biofilm is often considered a pathogenicity trait during chronic infections [96]. Since, QS is linked to virulence factor production and biofilm formation, virulent microorganisms could potentially be rendered non-pathogenic by inhibition of their QS systems.

Plant derived modulators: Medicinal plants have always provided a stable source for medicines. Not only the herbs themselves but certain plant derived compounds have served as lead molecules for further chemical modulation and natural products still continue to play a highly significant role in drug discovery and development process [97]. Herbal drugs have the advantage that they were used on a regular basis in the past and those products are still available in the same drug formulation (teas, lotions, powders, ointments, emollients, oils, dressings, cleansers) and plants, especially those with ethnopharmacological uses, have been the primary sources of medicines for early drug discovery and there should be an abundance

of drugs, remaining to be discovered in these plants, which are hidden in undiscovered areas [98,99,100]. Since, plants synthesise a great deal of compounds to fight against bacterial infections, this property might be of potency against human infections as well. Antimicrobial compounds may be secondary metabolites of versatile chemical structure. Flavonoids are able to form complex with extracellular soluble proteins and bacterial cell walls, furthermore flavonoids i.e. galangin and 3,7-dihydroxyflavone were shown to restore the vancomycin sensitivity of *E. faecalis* and *E. faecium*, by lowering the MICs to the level of vancomycin sensitive strains. It is suggested that the alternatively synthesized disaccharide peptide (ala-lac) production might be initially inhibited by the presence of flavonoid [101,102,103]. Catechins act via DNA gyrase inhibition by binding to the N-terminal fragment of the enzyme [104]. Furthermore, catechins are able to restore the susceptibility of resistant bacteria to different antibiotics e.g. tetracyclines and β-lactames [105,106]. The crude extract of *Salvia officinalis* was found to reduce the MIC of aminoglycosides in vancomycin resistant *enterococci*, then the effective compound was isolated. Carnosol, the active compound showed weak antimicrobial activity and greatly reduced the MICs of various aminoglycosides [107]. The sesquiterpenoid farnesol, which is naturally found in the essential oil of citrus fruits is able to compromise biofilm formation of bacteria and fungi. Some studies indicated a possible interaction of farnesol with cell membranes of certain bacterial species including *Streptococcus mutans*. This natural compound has the potential to inhibit the development of dental caries via the inhibition of biofilm formation [108]. It was also successful at enhancing the antibacterial efficacy of antibiotics to which *S. aureus* strains were somewhat susceptible, which raises the possibility for farnesol to be an adjuvant agent in the therapy of skin infections [109,110,111].The Australian red macroalga *Delisea pulchra* produces a range of halogenated furanone compounds that display antimicrobial properties. It is hypothesized that furanones of *D. pulchra* constitute a specific means of eukaryotic interference with bacterial signalling process via competing with the quorum-sensing signals. Some derivatives of these compounds were shown to repress quorum sensing in *P. aeruginosa* and reduce virulence factor expression [90,112].

Novel drug delivery systems. Another strategy to overcome resistance is to improve the delivery or otherwise enhance the accessibility of antibiotics to their sites of action. Liposomal preparations of hydrophobic antibiotics such as ethambutol for treatment of mycobacterial infections have been reported [113]. Another approach is the linking of two different classes of antibiotics in order to have a more specific effect.

There are clear trends to show that the mainstream in pharmaceutical research is moving away from single molecule or single target approach to combination and multiple target approaches [114].

1.4 Efflux mediated resistance in tumor cells

Cancer is a major health issue all over the world and one of the main difficulties in the treatment of that complex disease is the increasing rate of resistance. Resistant tumors are usually found to be cross-resistant to a broad but well defined spectrum of structurally unrelated cytotoxic drugs. Numerous mechanisms of MDR exist in cancer cells, however, energy dependent drug transporters are the major factors in the background of chemoresistance. Membrane transporters have been classified into two major families: ATP-binding cassette (ABC) transporter family and solute carrier transporter family [115]. There are 49 genes that have been identified as ABC transporters in human genome and these members of the superfamily were further divided into seven subfamilies, i.e., ABCA (12 members), ABCB (11), ABCC (13), ABCD (4), ABCE (1), ABCF (3) and ABCG (5). Although, these ABC transporters share a common structure of nucleotide-binding domain and transmembrane domain, their different primary sequences determine their specific structures and functions [116]. One major type of MDR is linked to the overexpression of a 170 kDa plasma membrane glycoprotein, known as the P-glycoprotein (P-gp). P-gp mediates resistance to a wide array of chemotherapeutical agents and also appears to play a role in the protection of tissues against toxic compounds (P-gp is present in the apical surface of many epithelial cells and the endothelial cells of the blood-brain barrier) [117]. Compounds called chemosensitizers reverse MDR *in vitro*, resulting in decreased drug efflux and increased cellular drug accumulation. The design of P-gp inhibitors received great attention and progressed through three distinct generations. These compounds are currently used drugs with other indication (verapamil, cyclosporin A, quinidine) or analogues of the first generation modulators (dexverapamil, valspodar, cinchonine). These drugs could be administered in combination with the conventional chemotherapeutics in order to enhance the affectivity of the given treatment. This approach, however, holds the potential of increasing the appearance of unwanted and toxic effects due to reduced drug-efflux from normal cells. This problem could be circumvented by the development of tumor-specific drug formulations [118].

2. AIMS OF THE THESIS

Since the discovery of antibotics and their uses as chemotherapeutic agents, there was a belief in the medical fraternity that this would lead to the eradication of infectious diseases. However, diseases and infective agents that were once thought to have been controlled by antibiotics are returning in resistant form, which is an inevitable process from evolutionary viewpoint. Alexander Flemming warned in his 1945 Nobel prize acceptance lecture: ″Penicillin is to all intents and purposes non-poisonous so there is no need to worry about giving an overdose and poisoning the patient. There may be a danger, though, in under-dosage. It is not difficult to make microbes resistant to penicillin in the laboratory exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body″ [119]. Confronted with a possible shortage of new antimicrobials, there is a need to ensure a careful use of our available drugs or seeking drugs that block resistance mechanisms. This is the case for malignant neoplastic diseases as well, where multidrug resistance of tumor cells causes the insufficiency of chemotherapy. Inhibition of MDR, therefore enhancing the antitumor potency of a given therapy could be an attractive approach. Our aim was to investigate possible candidates among plant derived compounds and chemical agent for the reversal of resistance in bacteria and in tumor cells in the following procedures.

- Antiplasmid activities of organic silicon (SILA) compounds were tested on *E. coli* K12 LE 140, E. coli P673 (hemolysine plasmid containing) and pBR322 transformed *E. coli* AG100 and AG100 A bacterial strains.
- Essential oils were studied in order to determine their antibacterial and antifungal activities. Gram-positive and Gram-negative bacterial strains and yeast strains were selected to define the MIC values in the microdilution method.
- Since, resistant bacteria often carry the genes responsible for resistant phenotype on plasmids, there is a possibility that plasmid curing agents would be potential candidates for the complementation of antibiotic therapy. Therefore, the previously studied essential oils and their components were tested for the plasmid curing activity on the methabolic plasmid of *E. coli* F'lac K12 LE140 laboratory strain.
- One of our aims was to study the effects of selected essential oils on the quorum sensing signal mechanisms of bacteria in order to model new target mechanisms for the development of antibiotics.

- Thioridazine derivatives were tested on *M. tuberculosis* H37Rv laboratory strains to evaluate their antibacterial activity with the BACTEC 450 respirometrical method.
- Essential oil components were investigated in the antiproliferativity assay on human MDR1 gene transfected mouse lymphoma cell line and its parental, sensitive cell line in order to determine the activity of the cells on cell proliferation.
- Essential oil components were tested for their MDR reversal activity on human MDR1 gene transfected mouse lymphoma cell line in the rhodamine exclusion test; the MDR reversal activity was based on the inhibition of P-gp in the cell membrane.
- Interactions between essential oil components and cytostatic doxorubicin were studied in the checkerboard method in 96-well plates in order to classify the quality of interaction between the two compounds.
- Anastasia Black (Russian black sweet pepper, *Capsicum annuum* L, Solanaceae) pepper was extracted with solvents of different polarity and the extracts were further fractionated by column chromathography. The extracts and fractions were tested for their MDR reversal activity on human MDR1 gene transfected mouse lymphoma cells.
- Anastasia Black extracts and fractions, which were found to be active in the biological assay, were analysed with High Perormance Liquid Chroamtography (HPLC) and Liquid Chromatography-Mass Spectometry (LC-MS-MS) methods in order to determine the possible active agents.
- Four essential oil components were selected based on the flowcytometry assay for studying their properties in the apoptosis induction. Dual staining with acridine orange-ethidium bromide and the annexin V-FITC methods were applied.

3. MATERIALS AND METHODS

3.1 Chemicals

Natural compounds

- **Essential oils**: Orange oil (*Citrus sinensis* L., Myrtaceae), eucalyptus oil (*Eucalyptus globulus* L., Myrtaceae), fennel oil (*Foeniculum vulgare* Mill., Lamiaceae) geranium oil (*Geranium robertianum* L., Geraniaceae), juniper oil (*Juniperus communis* L., Cupressaceae), peppermint oil (*Mentha piperita* L., Lamiaceae), rosemary oil (*Rosmarinus officinalis* L., Lamiaceae), purified turpentine oil (distilled from Pinus species), thyme oil (*Thymus vulgaris* L., Lamiaceae), rose oil (*Rosa damascena* L., *Rosaceae*), lavender (*Lavandula angustifolia* L., *Labiatae*), chamomile (*Matricaria recutica* L., *Asteraceae*). These essential oils suit the requirements of the Hungarian Pharmacopoeia (Ph.Hg. VIII.), they are distributed by the Phoenix Pharma Plc., Hungary. Australian tea tree oil (*Melaleucae alternifoliae* Cheel, Myrtaceae)was purchased from Main Camp Marketing Pty Ltd, Ballina, NSW, Australia. In the experiments the the essential oils were dissolved in dimethyl sulfoxide (DMSO).
- **Origanum essential oils:** Essential oils of *Origanum vulgare* ssp. *hirtum* (Link) Ietswaart, cultivated in the Experimental Station of the Budapest University of Economic Sciences and Public Administration, Budapest (**sample A, B, C**) and in the experimental field of the Institute of Ecology and Botany of the Hungarian Academy of Sciences, Vácrátót (**sample D**), Hungary. The extraction procedure was carried out at the Department of Pharmacognosy, Faculty of Pharmacy, University of Szeged.
- **Essential oil components:** The following essential oil gas-chromatography standard components were donated by the Department of Pharmacognosy, Faculty of Pharmacy, University of Szeged (Sigma-Aldrich Ltd., Budapest, Hungary): *p*-cymene, eucalyptol, beta-cariophyllene, carvacrol, limonene, linalool, alpha-pinene, beta-pinene, sabinene, alpha-terpinene, gamma-terpinene, borneol, cariophyllene-oxide, thymol, menthol.
- **Russian black sweet pepper extracts and fractions**: Dry powdered Anastasia Black (*Capsicum annuum* L. var. *angulosum* Mill., Solanaceae) (400 g) was successively extracted with hexane, acetone, methanol (MeOH) and 70% MeOH at room temperature. After evaporation of the solvent *in vacuo*, the hexane extract [**H0**] yielded 5.75 g, the acetone extract [**A0**] 7.27 g, the MeOH extract [**M0**] 69.3 g and the 70% MeOH extract [**70M0**] 49.1 g. Initially, an aliquot of the hexane extract [**H0**] (4.5 g) was subjected to silica gel column chromatography, with step-wise elution with hexane and hexane-acetone (9:1) [**H1**] (3.03 g), hexane-acetone (4:1) [**H2**] (0.66 g), hexane-acetone (3:2) [**H3**] (0.19 g) and hexane-acetone (2:3) [**H4**] (0.06 g).

  The acetone extract [**A0**] (4.8 g) was applied to a silica gel chromatographic column, and was then eluted step-wise with benzene [**A1**] (0.30 g), benzene-EtOAc (9:1) [**A2**] (0.11 g), benzene-EtOAc (4:1) [**A3**] (0.13 g), benzene-EtOAc (3:2) [**A4**] (1.37 g), benzene-EtOAc (2:3) [**A5**] (1.36 g) and EtOAc [**A6**] (1.23 g).

  The MeOH extract [**M0**] (10 g) was applied to a silica gel chromatographic column and was eluted step-wise with trichloromethane ($CHCl_3$) and $CHCl_3$-MeOH (49:1) [**M1**] (0.05 g), $CHCl_3$-MeOH (24:1) [**M2**] (0.09 g), $CHCl_3$-MeOH (9:1) [**M3**] (0.43 g), $CHCl_3$-MeOH (4:1) [**M4**] (1.58 g), $CHCl_3$-MeOH (3:2) [**M5**] (0.23 g) and MeOH [**M6**] (6.48 g).

  Finally, 70% MeOH extract [**70M0**] (10 g) was applied to an octadecylsilane (ODS; C_{18}) column and was eluted step-wise with MeOH-H_2O (1:4) [**70M1**] (6.22 g), MeOH-H_2O (2:7) [**70M2**] (1.36 g), MeOH-H_2O (1:3) [**70M3**] (0.21 g), MeOH-H_2O

(1:2) [**70M4**] (0.24 g), MeOH-H_2O (1:1) [**70M5**] (0.49 g), MeOH-H_2O (2:1) [**70M6**] (0.41 g) and MeOH [**70M7**] (0.93 g).

Synthetic compounds

- **Organosilicon compounds**: Patented compounds 1,3-Dimethyl-1,3-bis(4-fluorophenyl)-1,3-bis(3-morpholino-propyl)-disiloxan-dihydrochlorid (**Sila 409**) and, 1,3-Dimethyl-1,3-bis(4-fluorophenyl)-1,3-bis{3-[1(4-buthyl-piperazinyl)]-propyl}-disiloxan-tetrahydrochlorid (**Sila 421**) were synthesised by Hegyes *et al.* [120]. Compounds 409 and 421 have received patents, Brevet Europeen n^0 0099150.6, PCT/DE00/04110.
- **Thioridazine derivatives:** Fourteen substituted thioridazine derivatives were synthesised by György Hajós at the Chemical research Center of the Hungarian Academy of Sciences (See Annex 1.).
- **Other chemicals useds:** promethazine (Pipolphen, EGIS Pharmaceuticals, Budapest), penicillin (Biogal-Teva Pharma Rt., Budapest, Hungary), ampicillin (AMP), tetracycline hydrochloride (Sigma Aldrich, St. Louis, MO, USA), oxytetracycline (OXY), gentamycin (GENT) (Chinoin/Sanofi-Synthelabo, Budapest, Hungary), erythromycin (ERY) (Gedeon Richter Ltd., Budapest, Hungary), fluconazole (Diflucan, Pfizer, Amboise, France), dimethylsulfoxid (DMSO) (SERVA, Feinbiochemica, Heidelberg, Germany), and 3-(4,5-dimethylthiazyl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich, St. Louis, MO, USA) (dissolved in phosphate buffered saline to 5 mg/mL concentration).

3.2 Prokaryotic and eukaryotic cells

- **Bacterial strains:** *E.* F'lac K12 LE140 (tsx, str, Δlac, su^-, λ^r, mal^-), *E. coli* AG100 (proton pump carrier) and *E. coli* AG 100A (acrAB deleted mutant), originally prepared by Professor Dr. H. Nikaido, *E. coli* $AG100_{TET}$ and *E. coli* AG $100A_{TET}$ were induced to high level tetracycline resistance (8 μg/mL) (gradually exposed to increasing concentrations of tetracycline until their resistance to tetracycline increased, Amaral L. *et al*.) [121], *E. coli* $AG100_{TET}$ pBR322 and *E. coli* AG $100A_{TET}$ pBR322 (the tetracycline-induced strains were transformed with pBR322 plasmid), *E. coli* P673 nephropathogenic strain isolated from swine (harbors two plasmids: pCW1-tetracycline resistance, pCW2 – hemolysin production, *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus epidermidis* (clinical isolate). *Chromobacterium violaceum* CV026 (obtained from Prof. Thomas J. Burr, Cornell University, Geneva, NY), *E. coli* ATTC 31298 and the partially characterized Ezf 10/17 isolated from a grapevine crown gall tumor, both control strains for producing QS signals.
- **Yeast strains:** *Candida albicans* ATCC 10231, *C. albicans* ATCC 14053, *Saccharomyces cerevisiae* 0425 52C (Grand, mitochondria containing strain) and *S. cerevisiae* 0425 δ/1 (Petit, mitochondria deficient strain).
- **Tumor cell lines:** Human MDR1 gene transfected mouse lymphoma cell line (MDR, L5178) and its parent cell line (PAR, L5178Y). Human cervical adenocarcinoma cells (HeLa).

3.3 Culture media

- **Culture media for bacterial and fungal strains**
  LB (Luria-Bertani) broth and agar: 0.5% Yeast extract, 1.0% Trypton, 1.0% NaCl, 2.0% agar; **MTY (Minimal Trypton-Yeast) broth and agar:** 0.1% NH_4Cl, 0.7% K_2HPO_4, 0.3% NaH_2PO_4 x $2H_2O$, 0.2% NaCl, 1.0% Trypton, 0.1% Yeast extract, 1.5% Agar; **Eosin methylene blue** (EMB) (BioMérieux): 3.6% EMB; **TSB and TSA (Tryptic Soy Broth and Agar):** 3.0% TSB, 2% Agar (Scharlau Chemie S.A. Barcelona, Spain); **YTB (Yest extract-Tryptone Broth):** 0.5% Yeast extract, 1.0% Tryptone, 0.5% NaCl; **Blood-agar:** 4.42% Columbia medium for blood agar (Biolab PLC., Hungary), 5.0% sterile defibrinated sheep blood (Phylmaster Ltd., Hungary); **2xYTB (Yeast Peptone, Dextrose):** 1.0% Yeast extract, 2.0% Peptone, 2.0% Glucose, 1.3% Citric acid, 1.4% Na_2HPO_4
- **Culture media for tumor cells**
  Media for L5178 and L5178Y mouse lymphoma cell lines: Modified McCoy's 5A medium (Gibco BRL, Grand Island, NY, USA) (supplemented with streptomycine, nystatine, 200 mM L-glutamine, 10% heat-inactivated horse serum)
  The human cervical adenocarcinoma cells (HeLa) were cultivated in Eagle's MEM (Gibco BRL, Paisley, UK) supplemented with 10% heat-inactivated FBS, 1% non-essential amino acids and an antibiotic-antimycotic mixture.

3.4 Assays for determination of antimicrobial effects

Determination of bacteriostatic activity by agar diffusion method: Precultures of bacteria and yeasts (bacteria: overnight at 37 °C, yeasts: for 24 h at room temperature) were diluted 10-100-fold in physiological saline solution (0.9% w/v), and 100 µL of the dilution was then plated on nutrient agar plates. Wells 5 mm in diameter were punched into the agar and filled with 20-30 µL of the tested solution and control solution. After incubation, the antimicrobial activity was evaluated by measuring the diameters of the inhibition zones observed (given in mm).

Determination of MIC values: The MIC values were measured in sterile 96- well microtitre plates. 100 µL aliquots of solutions of tested compounds and solvent blank control samples were pipetted into the wells in the first column of the plate, and 50 µL of physiological saline solution into the wells in columns 2-12. The samples of the compounds in the first column were diluted with a multibarrel pipette with 50 µL amounts from the 1^{st} column to the 12^{th}. The precultures of the microbes were diluted and from these suspensions, 50 µLs were added to each well. After incubation (bacteria: overnight at 37 °C, yeasts: for 24 h at room temperature), 10 µL of MTT solution was pipetted into each well to stain the living cells. The MIC was defined as the lowest concentration of the test sample that resulted in a complete inhibition of growth.

3.5 Assays for plasmid elimination activity

F'lac plasmid elimination: An overnight preculture of *E. coli* F'lac K12 LE140 was diluted 10^4-fold in physiological saline solution and inoculated in 0.05 µL aliquots (approximately $5x10^3$ cells) into 5.0 mL of MTY nutrient broth. Various concentrations of the samples and promethazine control were added. The tubes were incubated at 37 °C for 24 h without shaking. Two dilutions, with 10^4 and 10^5-fold concentrations, were prepared from tubes showing growth and plated in 100 µL amounts on EMB agar. The plates were incubated at 37 °C for 24 h, and were then counted for lac^- plasmidless (pink) and lac^+ plasmid-containing (deep-violet) colonies. The percentage (%) of plasmid elimination was counted. Promethazine is used as a positive control.

Elimination of the pCW2 plasmid of *E. coli* P673: An overnight preculture of *E. coli* P673 was diluted 10^4-fold in physiological saline solution and inoculated in 0.05 µL aliquots (approximately $5x10^3$ cells) into 5.0 mL of MTY nutrient broth. Various concentrations of the samples and promethazine control were added. The tubes were incubated at 37 °C for 24 h without shaking. Two dilutions, with 10^4 and 10^5-fold concentrations, were prepared from tubes showing growth and plated in 100 µL amounts on blood agar. The plates were incubated at 37 °C for 24 h and the colonies with and without haemolytic zones were counted and the percentage of plasmid elimination was determined.
Replica plating: *E. coli* $AG100_{TET}$ pBR322 and *E. coli* AG $100\underline{A}_{TET}$ pBR322 strains were cultured overnight and were diluted to 10^{-4} and 1 mL aliquots inoculated into 100 mL of MTY broth. Various concentrations of SILA 409 and SILA 421 were added to 5 mL of the diluted cultures, and were incubated at 37 °C for 24 h. Dilutions of 10^{-4} and 10^{-5} were prepared from tubes showing growth and plated on MTY agar. The plates were incubated at 37 °C for 24 h. The velvet replica from master plates were prepared onto plates containing antibiotics (8 µg/mL of tetracycline and 100 µg/mL of ampicillin) and after 24 h incubation the master and replica plates were compared and the ratio of plasmid elimination was determined [122].

3.6 Transformation of pBR 322 plasmid into E. coli AG 100_{TET} and AG $100A_{TET}$

Primary culture of *E. coli* AG 100_{TET} and AG $100\underline{A}_{TET}$ was cultured at 37 ºC overnight in YTB medium supplemented with tetracycline (8 µg/mL) for both strains and kanamycin (100 µg/mL) for *E. coli* AG $100\underline{A}_{TET}$. 1 mL of the primary culture was added to 50 mL YTB medium and was cultured for further 4 h at 37 ºC in order to reach an optical density (OD) of 0.25-0.30 (at 540 nm). The cultures were kept on ice for 10 min and centrifuged with 3500 rpm for 10 min. Supernatant was removed and bacteria were resuspended in cold 0.1 M $MgCl_2$ solution. After centrifuging the suspension, cold 0.1 M $CaCl_2$ solution was added and the cells were kept at 0 ºC for 1 h. 5 µL of the purified pBR 322 plasmid was pipetted to 200 uL competent cell suspension. The mixture was kept on ice for further 45 min and 2 min long heat shock (42 ºC) was applied. Transformed bacteria were cultured at 37 ºC for 1h, centrifuged, resuspended in YTB broth and plated on antibiotic (8 µg/mL tetracycline, 100 µg/mL ampicillin for both transformed strains and further 100 µg/mL kanamycine was applied in case of the *E. coli* AG $100\underline{A}_{TET}$) containing YTB plates.

3.7 Checkerboard microplate method as a model for combination therapy

The microdilution checkerboard method is the technique used most frequently to assess antimicrobial/antitumor/antiproliferative combinations *in vitro*. Dilutions of studied samples and the antibiotics/cytostatics were made for evaluation of the interactions. Dilutions from the logarithmic-growth phase of the bacterial culture/tumor cell culture were prepared and distributed into microtitre trays containing varying concentrations of the different drugs and the examined compound. The inoculated trays were incubated at 37 °C for 24 h for bacteria and at 37 °C (5.0 % CO_2 atmosphere) for 72 h for tumour cells. At the end of the incubation period, 20 μ L of MTT (thiazolyl blue tetrazolium bromide) solution (5 mg/mL stock) was added to each well. After incubation at 37 °C for 4h, 100 µL of sodium dodecyl sulfate (SDS) solution (10 %) was measured into each well and plates were further incubated at 37 °C overnight. The bacterial/cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Dynatech MRX vertical beam ELISA reader. The results of the combined effects of the antibiotics/cytostatics and studied samples were categorized as synergism, addition, indifference or antagonism. In order to assess the activities of combinations, fractional inhibitory concentration (FIC) indices were calculated as FIC_A + FIC_B, where FIC_A and FIC_B are the minimum concentrations that inhibited the bacterial/tumor growth for samples A and B, respectively.

$$FIC_A = \frac{MIC_A \text{ combination}}{MIC_A \text{ alone}} \qquad FIC_B = \frac{MIC_B \text{ combination}}{MIC_B \text{ alone}}$$

The mean FIC index was calculated as: $FIC_{index} = FIC_A + FIC_B$, and the results were interpereted as follows: synergistic (<0.5), additive (0.5-1.0), indifferent (>1), or antagonistic (>4.0).

3.8 Inhibition of QS mechanisms by essential oils

To detect autoinducer production of tested bacteria, the biosensor strain *Chromobacterium violaceum* CV026 was used. This *Tn5* mutant strain produces the purple pigment violacein upon induction with externally added short-chain autoinducers [123] and it has been widely used for the detection of *N*-acyl homoserine lactones or quorum sensing inhibitors [124,125,126]. *E. coli* ATTC 31298 and the partially characterized Ezf 10/17 isolated from a grapevine crown gall tumor (Szegedi, unpublished) were used as AHL producers. Both strains induce pigmentation of CV026 and therefore they can be used to monitor AHL-induced pigment production by *C. violaceum* CV026.

The QS inhibitory effects were investigated in agar diffusion method. Pair combinations of the sensor strain CV026 and the producer strains ATTC 31298 or Ezf 10/17 each at an MIC equivalent inoculum were swabbed on LB plates as approx. 5 cm line cultures at a 5 mm distance from each other. Whatman 3MM filter paper discs, 7 mm in diameter, impregnated with 10 μl of concentrated or DMSO-diluted oils and placed on the inoculated line cultures. Discs containing acridine orange and 5-fluoro-uracil served as positive controls and that containing DMSO (10 μl) served as the solvent control. Signal transmission was assessed by the effect of the agent present on the disc that did not affect the development of purple colour defined as „No QS inhibitory activity" and by the absence of colour and the size of the colourless zone defined as „QS activity" after 24 and 48 hr incubation period.

3.9 Drug susceptibility assays by the BACTEC 460-TB radiometric proportion method and determination of MICs of thioridazine and its derivatives by the broth (BACTEC) radiometric method

Absolute control vials and drug containing vials were inoculated with approximately 10^5 to 10^6 colony forming unite (CFU) of *M. tuberculosis* H37Rv strain. *M. tuberculosis* strains were cultured in Middlebrook 7H9 broth, Bactec 12B vials (Becton-Dickinson Diagnostic Instrument Systems), until maximum growth was achieved. From these cultures, aliquots were transferred to vials and optical density adjusted to 0.5 McFarland standard. These vials served as the source for the inoculation of triplicate Bactec 12B vials that contained concentrations of each thioridazine derivative that ranged from 0.0 to 20.0 mg/L. 1:2 and 1:100 dilutions of the adjusted inoculum was made and transferred to Bactec 12B vials that contained no drug; this served as the proportional control as defined by the recommended Bactec proportional method for *Mycobacteria* [8,127,128]. The vials were incubated at 37 °C and the contents periodically assessed by the Bactec 460 TB instrument (Becton-Dickinson Diagnostic Instrument Systems) for $^{14}CO_2$ generated from the metabolism of ^{14}C palmitic acid.

3.10 Reversal of multidrug resistance of tumor cell *(Assay for MDR reversal)*

The L5178 mouse T-cell lymphoma cell lines were transfected with human *MDR1/A* (multidrug resistant) gene. *MDR1*-expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain the expression of *MDR1* phenotype. The L5178 *MDR1* T cell and parent cell lines were grown in McCoy's 5A medium with 10 % heat-inactivated horse serum. The cells were adjusted to a density of $2x10^6$/mL and re-suspended in serum free McCoy's 5A medium. 0,5 mL aliquots of the cells were distributed into

Eppendorf centrifuge tubes. Then the tested compounds were added and incubated for 10 minutes at room temperature. 10 µl rhodamine 123 (R123) indicator was then added and incubated for further 20 minutes at 37 °C. After washing twice and re-suspending in 0,5 mL phosphate-buffered saline (PBS), the fluorescence of the cell population was measured by flow cytometry, using Beckton Dickinson FACScan instrument (cell sorter). Verapamil was used as positive control in the R123 accumulation tests. The R123 accumulation was calculated from the fluorescence intensity of the samples. The percentage of control of untreated mean fluorescence activity was calculated for parental and *MDR1* cell lines and compared to the fluorescence of treated cells. *MDR1* reversal activity was calculated by the following equation.

$$\text{Fluorescence Activity Ratio} = \frac{\text{MDR treated / MDR control}}{\text{Parental treated/ parental control}}$$

3.11 LC-MS-MS method for detection of phenolic compounds

The LC-MS-MS analyses of the phenolic compounds present in the sweet pepper extracts were performed on an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Canada) equipped with a TurboIonspray source in negative mode. The operating parameters were as follows: capillary voltage (IS) -4500 V; declustering potential (DP) -60 V; heating to 400 °C. Chromatographic separation was performed on a Prodigy ODS3 100 Å column (250x4.6 mm, particle size 5 µm) (Phenomenex, CA, USA).

The eluents were the following: (A) water-0.1 % formic acid; (B) CH_3CN-MeOH 60:40 (*v/v*). The gradient program was as follows: 30-40 % B (3 min), 40-45 % B (10 min), 45-50 % B (2 min), 50-55 % B (3 min), 55-60 % B (4 min), 60-80 % B (3 min), 80-90 % B (2 min) and 90-30 % B (3 min), at a constant flow of 0.8 mL/min. The LC flow was split and 0.2 mL/min was sent to the mass spectrometer. The injection volume was 20 µL.

The following components were examined: feruloyl glucopyranoside, sinapoyl glucopyranoside, quercetin rhamnopyranoside glucopyranoside, luteolin glucopyranoside arabinopyranoside, apigenin glucopyranoside arabinopyranoside, quercetin rhamnopyranoside, luteolin-(furanosyl-glucopyranosyl-malonyl)-glucopyranoside, hesperidine, feruyl alcohol-(methylhydroxypropionyl)-glucopyranoside, luteolin arabinopyranoside diglucopyranoside, luteolin glucuronide, ferulic acid, sinapinic acid, quercetin, luteolin and apigenin. (See Annex 2. for list of the precursor ion, the MS/MS fragments and the optimum collision energy for each compound). Phenolic compounds were identified by Multiple Reaction Monitoring (MRM) and Information Dependent Acquisition (IDA) analyses based on the literature data [129].

3.12 HPLC method for detection of carotenoid compounds

The carotenoid components of the Anastasia Black extracts and fractions were separated on an HPLC system, involving a Devosil RP Aqueous C30 column (250x4.6 mm, particle size 5 µm). The eluents were the following: (A) 0.004 % ammonium acetate (solvent: MeOH) and (B) *tert*-butylmethyl ether. The gradient program was as follows: 85-70 % B (5 min), 70-60 % B (5 min), 60-45 % B (5 min), 45 % B isocratic (20 min), 45-20 % B (5 min), and 20-85 % B (5 min), at a constant flow of 1 mL/min. The detection wavelength was 453 nm and the injected volume was 20 µL. Lutein and β-carotene was injected as positive controls.

3.13 Apoptosis assays

Flowcytometric assay for apoptosis

The cells were adjusted to a density of $2x10^5$/mL and were distributed in 1.0 mL aliquots into microcentrifuge tubes. The apoptosis inducer 12*H*-benzo[α]phenothiazine (M627) was added to the samples as a positive control at a final concentration of 5 or 25 μg/mL. The M627 was synthetized by Motohashi *et al.* [130]. In the cases of control cultures, 10 μL DMSO was added. The compounds used for treatment was added to the samples at a final concentration of 2 or 10 μg/mL. After incubation for 24 h at 37 °C, the cells were transferred from a 24-well plate into Eppendorf centrifuge tubes, centrifuged and washed with PBS, and resuspended in 195 μL binding buffer. The samples were mixed and centrifuged and supernatant was removed from each tube. 5 μL AV was added to the tubes. Controls without AV were also prepared. The samples and controls were incubated at room temperature for 10 min in the dark, then centrifuged, washed with PBS, and resuspended in 190 μL binding buffer. Before the measurement of fluorescence activity, 10 μL of 20 μg/mL PI was added to the samples and the apoptosis of the cells was next investigated. The fluorescence activity (FL-1 and FL-2) of the cells was measured and analysed on a Beckton Dickinson FACScan instrument . In each analysis, 10 000 events were recorded, and the percentages of the cells in the different states were calculated by using winMDI2.8 [131].

Ethidium bromide and acridine orange (EB/AO) staining for apoptosis

Staining with EB/AO was carried out in a 96-well plate format after 24 h of treatment in order to visualize the basic morphological events [132]. Plates were centrifuged at 1000 rpm, and 8 μL of staining solution (0.1 mg/mL for both AO and EB in PBS) was added to each well. After 10 min, the cells were washed with PBS, and the cells were viewed with a Nikon Eclipse inverted microscope at 200x magnification with a 500/20 nm excitation filter, a cut-on 515 nm LP dichromatic mirror, and a 520 nm LP barrier filter (Chroma Technology, Rockingham, VT, USA). Pictures were taken with a Nikon Coopix 4500 digital camera (Nikon, Tokyo, Japan).

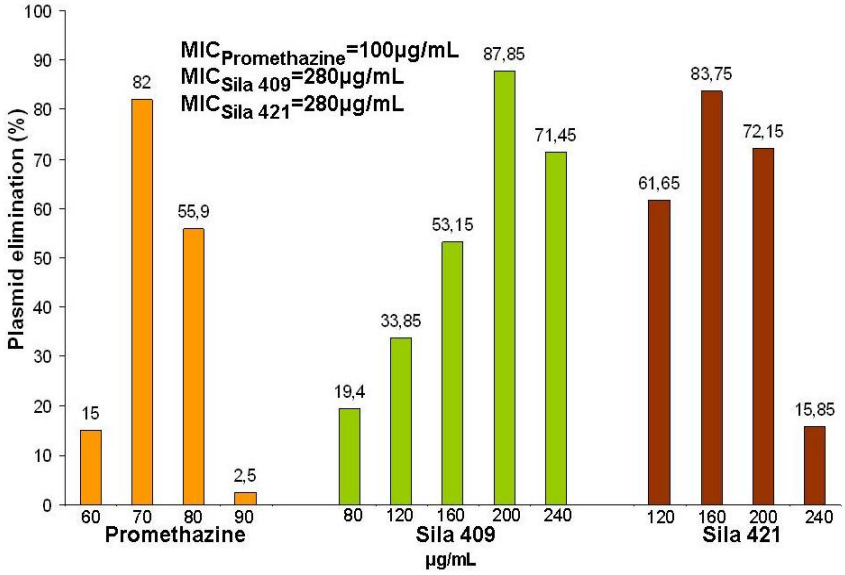
4. RESULTS

4.1 Antibacterial and antiplasmid effects of organic silicon compounds

Sila 409 and Sila 421 patented organosilicon compounds were tested in order to assess their antibacterial and antiplasmid effects on various plasmid carrying strains i.e. *E. coli* F'lac K12 LE 140 (lac operon on plasmid), *E. coli* P673 (Hly plasmid), *E. coli* AG100 and AG100A (previously induced to high concentration tetracycline resistance and transfected with pBR322 plasmid).

The two organosilicon compounds exerted pronounced and stable plasmid-curing activity on *E. coli* F'lac K12 LE 140, the rate of elimination was 87.85% and 83.75% for Sila 409 and 421, respectively. (Figure.1.)

Figure 1. Plasmid elimination of metabolic plasmid of *E. coli* F'lac K12 LE 140 by organic silicon-substituted compounds



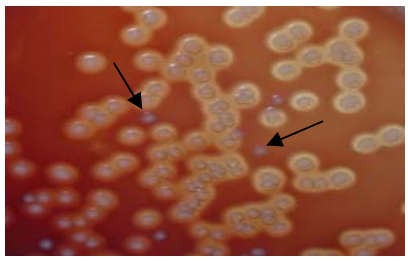
Promethazine was the positive control in this experiment, since this phenothiazine compound exerts reproducible elimination of metabolic and resistance plasmids in a concentration dependent manner.

The two organic silicon compounds were also studied on the nephropathogenic *E. coli* P673. The aim was to interfere with the hemolysin production via curing the pCW2 plasmid.

Sila 421 caused the appearance of low number of non-haemolysing colonies (9.72 %) (Figure 2.).

Tetracycline resistance induced *E. coli* AG 100 (AcrAB wild strain) and *E. coli* AG 100A (AcrAB deleted mutant) strains were transfected with pBR322 plasmid (tet^r, amp^r) and the strains were treated with Sila 409 and Sila 421 and the ratio of plasmid elimination was determined with the replica

Figure 2. Plasmid curing on *E. coli* P673



Lack of haemolytic zone refers to plasmid curing.

plating method. Sila 409 showed activity in this respect on the proton-pump deleted mutant with an elimination rate of 28.9 % at 500 μg/mL concentration, Sila 421 caused the maximum rate of elimination at 6.7 % at 450 μg/mL.

4.2 Antimicrobial and antiplasmid effects of essential oils

The antibacterial and antifungal activities of ten essential oils have been tested that are used in medicine and traditional medicine. The oils were studied on one representative of Gram-positive, Gram-negative bacterial strains and two *S. cerevisiae* 0425 strains. In preliminary experiments the zones of inhibition were assessed, then the MIC values were determined by microdilution method. Results are summarized in Table 1 and 2.

Table 1. Bacteriostatic and fungistatic activities of essential oils on bacteria and yeast strains

	Zones of inhibition (mm)			
Samples	*E. coli* F'lac	*S. epidermidis*	*S. cerevisiae* 0425 52C	*S. cerevisiae* 0425 φ/1
Orange oil	0	0	7	8
Eucalyptus oil	22	15	16	21
Fennel oil	14	15	11	11
Geranium oil	0	14	14	12
Juniper oil	0	0	17	15
Peppermint oil	23	15	12	15
Rosemary oil	15	0	10	10
Turpentine oil	17	15	15	22
Thyme oil	**26**	**24**	**23**	**25**
Tea tree oil	17	14	19	21
Penicillin	24	23	-	-
Gentamycin	32	33	-	-
Fluconazole	-	-	27	19
DMSO	0	0	0	0

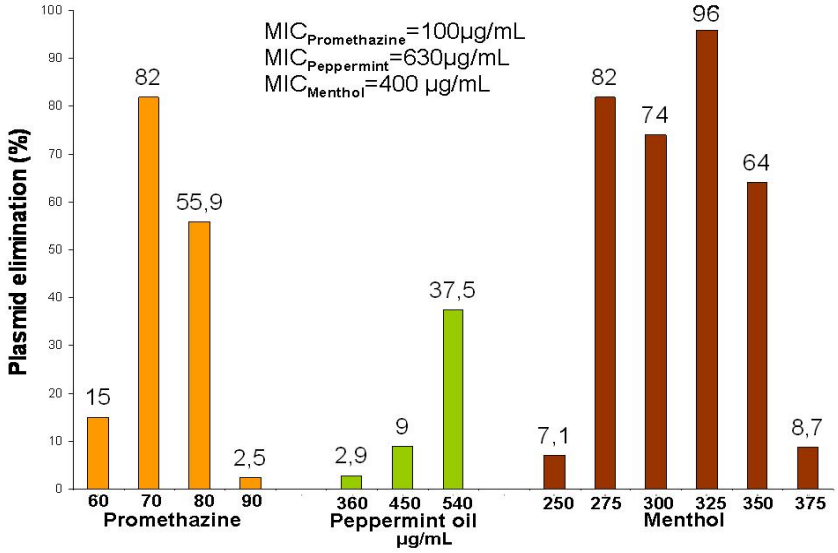
Table 2. MIC values of essential oils on bacteria and yeast strains

	MIC values (mg/mL)			
Samples	*E. coli* F'lac	*S. epidermidis*	*S. cerevisiae* 0425 52C	*S. cerevisiae* 0425 φ/1
Orange oil	-	-	2.8	2.8
Eucalyptus oil	2.8	2.8	0.7	0.7
Fennel oil	3.0	3.0	0.8	0.8
Geranium oil	0.0	5.6	1.4	1.4
Juniper oil	5.4	-	2.7	2.7
Peppermint oil	5.7	5.7	0.4	0.4
Rosemary oil	11.3	11.3	2.8	5.7
Turpentine oil	2.7	2.7	**0.2**	**0.3**
Thyme oil	**1.5**	**1.5**	0.4	0.7
Tea tree oil	5.6	5.6	2.8	2.8
Penicillin	0.008	0.004	-	-
Gentamycin	0.001	0.001	-	-
Fluconazole	-	-	0.064	0.056
DMSO	0.0	0.0	0.0	0.0

Most of the essential oils had antimicrobial effects, however these effects were less pronounced compared to antibiotic controls. Thyme oil showed the most expressed antimicrobial effects in both assays, while orange oil had only a slight antifungal activity without any antibacterial effect.

Those essential oils, which exerted antibacterial effects, were studied in order to investigate their plasmid-curing activity on the metabolic plasmid of *E. coli* F'lac K12 LE 140 (Figure 3.).

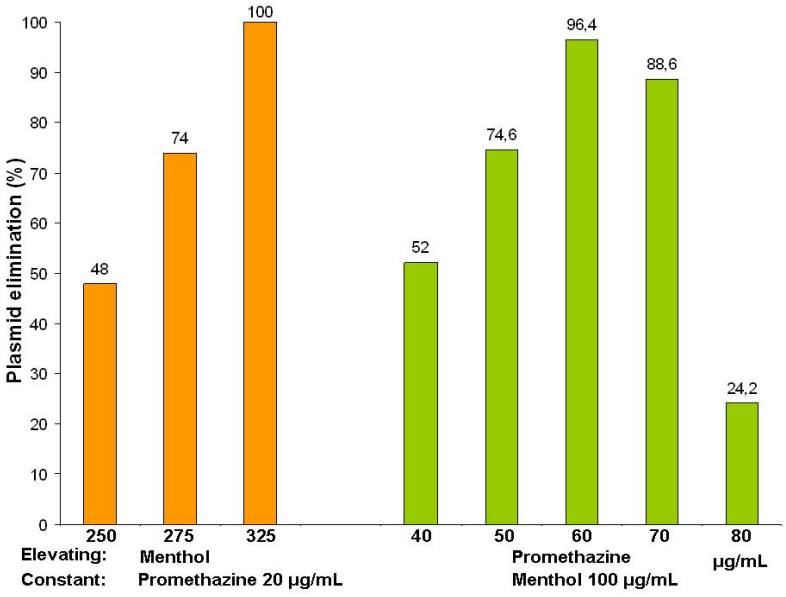
Figure 3. Elimination of F'lac plasmid in *Escherichia coli* K12 LE140



This method serves as a model of the elimination of resistance plasmid from R-plasmid carrying multiresistant pathogenic strains. Promethazine, the H1 receptor antagonist was the positive control. This phenothiazine was found to exert stable plasmid-curing activity in various bacterial strains [133].

Menthol, the major component of peppermint oil was studied in combination with promethazine in order to assess the plasmid curing activities together. One of the components was applied in constant concentration, while the other was added in growing concentrations. The rates of plasmid curing were as high as for

Figure 4. Elimination of F'lac plasmid in *Escherichia coli* K12 LE140 by the combination of menthol and promethazine



menthol alone but the concentrations required to reach this rate was lower (Figure 4.).

Peppermint oil and its major component, menthol was investigated in checkerboard method in combination with various antibiotics on *E. coli* F'lac K12 LE140. The FIX indices were calculated based on the MIC values of the compounds alone and in combination. The FIX values are characteristic to the phenomenon of the interaction. Peppermint oil and menthol showed additive interaction with oxytetracycline and other combinations resulted in indifferent action. Data are shown in Table 3.

Table 3. MIC values, FIC indices and type of interaction of peppermint oil and menthol combined with antibiotics on *E. coli* K12 LE140

Samples	MIC (µg/mL)	FIC index	Type of interaction
Peppermint oil (PEP)	400.0	-	
Menthol (MENT)	525.0	-	
Ampicillin (AMP)	2.6	-	
Erythromycin (ERY)	20.8	-	
Gentamycin (GENT)	10.4	-	
Oxytetracycline (OXY)	1.95	-	
Samples in combination			
Peppermint oil			
+ ampicillin	PEP(0.35)+AMP(1.72)	1.0	Indifferent
+ erythromycin	PEP(0.35)+ERY(18.0)	1.0	Indifferent
+ gentamicin	PEP(0.71)+GENT(7.8)	1.25	Indifferent
+ oxytetracycline	PEP(0.35)+OXY(0.49)	**0.5**	**Additive**
Menthol			
+ ampicillin	MENT(390.6)+AMP(1.95)	2	Indifferent
+ erythromycin	MENT(390.6)+ERY(7.8)	2	Indifferent
+ gentamycin	MENT(195.3)+GENT(3.9)	1	Indifferent
+ oxytetracycline	MENT(97.7)+OXY(0.49)	**0.5**	**Additive**

4.3 Antimicrobial effects of *Origanum vulgare* ssp. *hirtum* essential oils

The essential oils of four lines of *Origanum vulgare* L. ssp. *hirtum* (Link) Ietswaart (designated A, B, C and D) cultivated in Hungary were previously analysed by GC and GC-MS methods (See Annex 3.). The antimicrobial activities of the essential oils and their authentic individual components were tested on Gram-positive and Gram-negative bacterial strains and yeast strains in order to evaluate the difference in antimicrobial action of four lines with different composition of essential oil components. Results are summarized in Table 4-7.

All of the *O. vulgare* subsp. *hirtum* oils exerted broad antimicrobial effects, however the effects of the four lines did not correlate in antimicrobial effects with the composition of components. The most sensitive bacterial strain was *E. coli* AG100A, the proton pump-deficient mutant of AG100.

Table 4. Bacteriostatic activity of *Origanum* essential oils, zones of inhibition

Samples	Concentration of stock solution (v/v%)	*E.coli* F'lac	*E.coli* AG100	*E. coli* AG100A	*S. epidermidis*
A	5	33	25	43	26
B	1	18	28	35	15
	5	-	-	-	-
C	5	26	17	26	28
D	5	26	25	45	34
Penicillin	1 mg/mL	24	24	24	23
Gentamicin	1 mg/mL	32	32	32	33
Fluconazol	2 mg/mL	-	-	-	-
DMSO	100	0	0	0	0

Zones of growth ihibition (in millimeter) of microorganisms on nutrient agar plates

Table 5. Fungistatic activity of *Origanum* essential oils, zones of inhibition

Samples	Concentration of stock solution (v/v%)	*S. cerevisiae* δ/1	*S. cerevisiae* 52C	*C. albicans* 10231	*C. albicans* 14053
A					
B	5	32	38	33	33
C	5	35	27	38	38
D	5	32	33	28	28
Penicillin	1 mg/mL	-	-	-	-
Gentamicin	1 mg/mL	-	-	-	-
Fluconazol	2 mg/mL	19	27	31	40
DMSO	100	0	0	0	0

Zones of growth ihibition (in millimeter) of microorganisms on nutrient agar plates

Table 6. MIC determination of *Origanum* oils on various bacterial strains

Samples	*E.coli* F'lac	*E.coli* AG100	*E. coli* AG100A	*S. epidermidis*
D	0,12	0,20	0,08	0,16
A	0,16	0,24	0,12	0,28
B	0,12	0,16	0,08	0,24
C	0,12	0,16	0,08	0,20
Penicillin	8,0	8,0	1,6	4,0
Gentamicin	1,2	4,0	2,0	0,8
Fluconazol	-	-	-	-

Numbers in the table indicate MIC (µl/mL) as the volumes of oils resulting in complete inhibition

Table 7. MIC determination of *Origanum* oils on various fungal strains

Samples	*S. cerevisiae* δ/1	*S. cerevisiae* 52C	*C.albicans* 10231	*C. albicans* 14053
D	0,12	0,12	0,16	0,16
A	0,08	0,12	0,16	0,16
B	0,12	0,12	0,16	0,16
C	0,12	0,12	0,16	0,16
Penicillin	-	-	-	-
Gentamicin	-	-	-	-
Fluconazol	56,0	64,0	16,0	0,8

Numbers in the table indicate MIC (µl/mL) as the volumes of oils resulting in complete inhibition

4.4 Antimicrobial and antiplasmid effects of selected essential oil components.

Essential oils are complexes of several components that produce the biological effects of the oil, therefore investigating the components separately helps to have an insight into the multiple action of the oil. Fourteen different oil components were studied that occur in *Origanum* essential oils of by the broth dilution method. MIC values were tested and compared. Carvacrol markedly exerted antibacterial and antifungal effects, which explains the antimicrobial activity of the four lines of origanum oils, since all of them belong to the carvacrol group according to Pasquier classification. MIC values are shown in Table 8-9.

Table 8. Antibacterial activities of essential oil components

	E. coli F'lac	*S. epidermidis*	*E. coli* AG 100	*E. coli* AG 100 A
1. p-cymene	20	2.5	7.5	5
2. eucalyptol	20	20	20	5
3. β-cariophylenne	> 20	20	20	1.25
4. carvacrol	0.156	2.5	0.156	0.156
5. limonene	0.625	1.25	0.625	0.313
6. linalool	1.25	5	1.25	0.625
7. α-pinene	0.625	1.25	0.625	0.313
8. β-pinene	1.25	1.25	3.75	0.625
9. sabinene	2.5	2.5	> 5	1.25
10. α-terpinene	2.5	1.25	3.75	1.25
11. γ-terpinene	10	5	> 20	7.5
12. borneol	5	25	2.5	1.25
13. cariophyllene-oxide	> 0.5	0.25	0.25	0.16
14. thymol	1.25	0.313	2.5	0.33
DMSO	> 20	> 20	> 20	> 20

MIC values are shown in **µl/mL** in case of compounds 1-11. and DMSO and in **mg/mL** in case of compounds 12-14.

Table 9. Antibacterial activities of esential oil components

	S. cerevisiae 0425 δ/1	*S. cervisiae* 0425 52C	*C. albicans* ATCC 10231	*C. albicans* ATCC 14053
1. p-cymene	0.625	1.25	2.5	2.5
2. eucalyptol	13.75	7.5	> 20	> 20
3. β-cariophylenne	> 20	> 20	> 20	> 20
4. carvacrol	0.313	0.313	1.25	1.25
5. limonene	0.156	0.156	1.25	0.625
6. linalool	7.5	7.5	7.5	12.5
7. α-pinene	0.156	0.156	1.25	0.625
8. β-pinene	0.313	0.078	0.625	0.625
9. sabinene	0.313	0.156	2.5	2.5
10. α-terpinene	0.625	1.25	> 5	5
11. γ-terpinene	1.25	5	15	8.75
12. borneol	2.5	5	2.5	5
13. cariophyllene-oxide	0.25	0.25	0.25	0.5
14. thymol	0.156	0.078	0.078	0.156
DMSO	> 20	> 20	> 20	> 20

MIC vaues are shown in **µl/mL** in case of compounds 1-11. and DMSO and in **mg/mL** in case of compounds 12-14.

The pure essential oil components were also studied in the plasmid elimination assay on the metabolic plasmid of *E. coli* K12 LE 140 bacterial strain. Two of the selected essential oil components exerted antiplasmid effects, i.e. carvacrol and linalool with 45.5 % and 61.2 % respctively. Results are shown in Table 10.

Table 10. Antiplasmid activity of essential oil components on metabolic plasmid of *E. coli* F'lac K12 LE140 strain

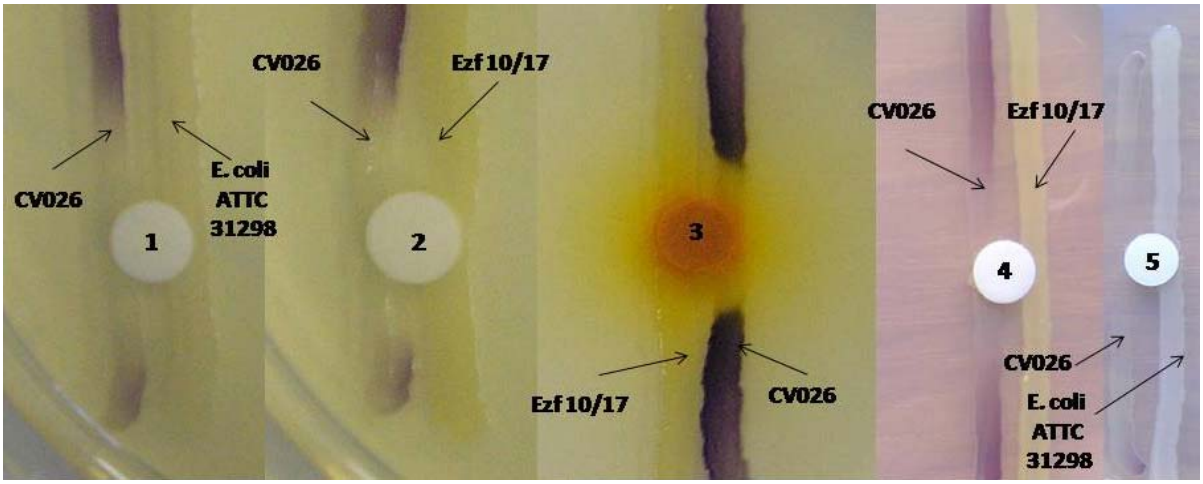
Sample	Concentration	Bacterial growth after 24 h CFU x 10^7	Plasmid elimination (%)
	µg/mL		
Promethazin	60.0	5.6	15.0
	70.0	4.1	82.0
	80.0	1.6	55.9
	90.0	2.3	2.5
	µL/mL		
Carvacrol	0.2	7.4	0
	0.4	1.1	2.9
	0.6	0.8	45.5
	0.8	2.9	0
	1.0	0	-
Linalool	1.0	24.7	0
	2.0	7.5	0.2
	3.0	7.2	39.5
	3.5	9.2	61.2
DMSO	35.0	70,8	0

4.5 Inhibition of QS mechanisms by essential oils

The essential oils tested with the agar diffusion method were compared to positive controls, AO and 5-FU. The use of impregnated disks with various essential oils afforded an understanding of which oils inhibited the sensor response of *C. violaceum* CV026 to AHL released by the bacteria employed. As examples, although the geranium oil was most effective as an inhibitor of the QS response of *C. violaceum* CV026 following induction by the AHL producing strains ATTC 31298 and Ezf 10/17 rose oil also has significant inhibitory activity on QS responses. Rose, lavender and rosemary oils also inhibited colour development to various degrees. Eucalyptus oil inhibited violacein production only after induction with ATTC 31298 while citrus oil reduced colour formation only in the CV026 + Ezf 10/17 combination. As expected they also inhibited bacterial growth.

Figure 5. shows the most effective essential oils that exert QS inhibitory activities.

Figure 5. Inhibition of QS by agar diffusion method. The interference of essential oils with the autoinducer production of *E. coli* ATCC 31298 and *C. violaceum* CV026



Chemicals in the paper discs are as follows: 1. 5-FU (control), 2. 5-FU (control) 3. AO (control), 4. Rose oil, 5. Geranium oil

4.6 Drug susceptibility assays by the BACTEC 460-TB radiometric proportion method and determination of MICs of thioridazine and its derivatives by the broth (BACTEC) radiometric method

Table 11. Antibacterial effects of Thioridazine derivatives on *M. tuberculosis* H37Rv strain

Samples	MIC_{50} Concentration (mg/L)
TZ	2.5
INH	0.08
TZ derivatives	
#1470	10
#1687	20
#1532-2	20
#1868	20
#1869	>20
#1870	10
#1871	>20
#1872	20
#1873	>20
#1874	>20
#1875	5
#1929	>20
#1926	5
#1876	10

The studied fourteen thioridazine derivatives did not reach the MIC in the applied concentrations, nevertheless, they were able to inhibit the growth of *M. tuberculosis* H37 Rv. The most active compound was #1926 with its 61% maximal inhibitory effect at a concentration of 20 mg/L. Compounds numbered 1867, 1869, 1870 and 1875 exerted pronounced inhibitory effects in a concentration range of 1,25-20 mg/L (25-56 %). Other derivatives showed only slight inhibitory effect on $^{14}CO_2$ generated by the culture.

4.7 MDR reversal activities and antiproliferative effects of essential oil components on human MDR1 gene transfected mouse lymphoma cells

Essential oil components that have been investigated on various bacterial and fungal strains were also examined in the rhodamine exclusion assay for evaluating their potential in the modification of chemotherapeutical resistance of tumor cells. The expression of MDR1/A gene in the L5178 mouse lymphoma cell line resulted in MDR, as reflected by the reduced intracellular accumulation of R123. Addition of (±)-verapamil as control reversed the MDR, as reflected by the increase in R123 accumulation. The L5178 mouse lymphoma cells were treated with the aliquots of essential oil components and stained with R123 fluorescent dye and the R123 accumulation was measured by flow cytometry. The fluorescence activity ratios were determined and compared with the verapamil control. Eucalyptol, α-terpinene, borneol and thymol increased the rhodamine accumulation in the cells, however the structural changes in the cells according to FSC and SSC values might be attributed to the toxic activity of the oil components. Results are summarized in Table 12.

The antiproliferative activities of the selected oil components were studied on the L5178 cell line alone and in combination with doxorubicin. The results of the antiproliferativity assay for the compounds alone are in Table 13. The ID_{50} values hardly differ for the cell line that expresses MDR phenotype from the results of parent cell line.

The checkerboard microplate method was applied to study the interaction between multidrug resistance modifiers and doxorubicin. The serial dilutions of doxorubicin and the oil components were adjusted in a 96 well microplate, so that the different concentrations of the cytostatic agent and the oil components could exert antiproliferative effects in various combinations. (Table 14.) The types of interactions varied from indifferent and additive to synergistic. The synergy was observed between α-terpinene and doxorubicine.

Table 12. MDR reversal activity of essential oil components on human MDR1 gene transfected mouse lymphoma cells

Samples	µl/mL	FSC	SSC	Fluorescence Activity Ratio
PAR +R123	-	670,73	427,75	-
PAR +R123	-	662,96	440,25	-
MDR	-	605,22	429,57	-
MDR +R123 mean	-	607,315	433,26	
Verapamil	10 µg/mL	602,29	429,82	2,39
1. p-cymene	0,03	616,99	436,93	1,19
	0,30	644,57	456,45	0,93
2. eucalyptol	0,10	645,47	443,81	3,71
	1,00	623,86	454,09	0,82
3. β-cariophyllene	0,10	641,44	443,40	0,95
	1,00	722,00	449,14	1,03
4. carvacrol	0,06	610,21	446,33	0,74
	0,60	593,33	397,32	1,36
5. limonene	0,008	610,06	433,61	0,76
	0,08	673,12	456,83	0,73
6. linalool	0,02	609,83	441,40	0,88
	0,20	604,19	448,24	0,77
7. α-pinene	0,02	614,07	443,93	0,68
	0,20	769,61	487,75	0,83
8. β-pinene	0,03	601,32	436,51	1,15
	0,30	609,22	438,15	0,87
9. sabinene	0,20	667,7	446,08	0,96
	2,00	665,62	454,14	0.97
10. α-terpinene	0,10	416,58	375,27	2,57
	1,00	692,61	469,98	0,95
11. γ-terpinen	0,10	615,79	438,42	0,60
	1,00	605,23	442,83	1,21
12. borneol	0,20	359,33	533,94	2,76
	2,00	615,00	456,80	0,86
13. cariophyllene-oxide	0,20	329,70	296,28	0,78
	2,00	371,61	336,71	0,65
14. thymol	0,20	617,63	435,89	0,81
	2,00	378,15	322,69	3,65
DMSO control	20,00	614,64	445,66	1,43
MDR	-	609,41	436,94	-

Table 13. Antiproliferative effects of essential oil components on human MDR1 gene transfected mouse lymphoma cells

	ID_{50} values	
Samples	**PAR (L5178 Y)**	**MDR (L5178)**
1. p-cymene	0.23	0.28
2. eucalyptol	1.08	1.02
4. carvacrol	0.63	0.64
5. limonene	0.04	0.03
6. linalool	0.08	0.13
7. α-pinene	0.04	0.04
8. β-pinene	0.11	0.20
9. Sabinene	0.09	0.15
10. α-terpinene	0.02	0.04
11. γ-terpinene	0.59	0.69
12. Borneol	0,435 mg/mL	0,539 mg/mL
13. β-cariophyllene	0,138 mg/mL	0,225 mg/mL
14. thymol	0.69	0.70

ID50 values are in μL/mL for compounds 1-11. and in mg/mL for compounds 12-14.

Table 14. Antiproliferative effects of selected essential oil components in combination with doxorubicin

Samples	**FIX**	**Type of interaction**
1. p-cymene	1.190	Indifferent
2. eucalyptol	1.086	Indifferent
3. β-cariophyllene	0.750	Additive
4. carvacrol	1.150	Indifferent
5. limonene	1.510	Indifferent
6. linalool	2.070	Indifferent
7. α-pinene	0.950	Additive
8. β-pinene	1.150	Indifferent
9. sabinene	0.889	Additive
10. α-terpinene	0.127	**Synergy**
11. γ-terpinen	0.610	Additive
12. borneol	0.930	Additive
13. cariophyllene-oxide	0.829	Additive
14. thymol	0.730	Additive

4.8 Bioactivities of Anastasia Black (Russian Black Sweet Pepper)

Anastasia Black (Russian sweet pepper) of *Capsicum annuum L. var. angulosum* Mill. (Solanaceae) was successively extracted with hexane, acetone, methanol and 70% methanol, and the extracts were further separated into a total of twenty-three fractions by silica gel or octadecylsilane column chromatography. These extracts have been investigated for their MDR reversal activities (Table 15.). One extract and four column fractions displayed higher MDR1-reversal activities as compared with the MDR1 control (fluorescence activity ratio=1) or verapamil control (fluorescence activity ratio=6.13). The 4 μg/mL hexane fraction [**H4**] (fluorescence activity ratio=14.72) had the highest MDR1 reversal activity, followed by [**H2**] (fluorescence activity ratio=12.45) and [**H3**] (fluorescence activity ratio=7.24). The 40 μg/mL hexane fraction corresponded to the highest increase in R123 accumulation [**H4**] (fluorescence activity ratio=81.74), followed by [**H2**] (fluorescence activity ratio=57.95),

[**H0**] (fluorescence activity ratio=26.1), [**A6**] (fluorescence activity ratio=21.68) and [**H3**] (fluorescence activity ratio=20.94).

Table 15. MDR-reversal activity of Anastasia Black extracts and fractions.

Sample	**FSC**	**SSC**	**Fluorescence activity ratio**
PAR control	534.83	127.47	-
MDR control	584.65	144.76	1
MDR +R123(*mean*)	588.09	147.21	-
25% DMSO control	535.24	130.56	0,92
Verapamil 10 μg/mL (positive control)	620.20	156.09	6.13

Anastasia Black	**Concentration (μg/mL)**		**Concentration (μg/mL)**		**Concentration (μg/mL)**	
	4	**40**	**4**	**40**	**4**	**40**
H0	497.21	613.16	149.04	177.33	5.52	**26.1**
H1	571.64	577.30	148.15	140.53	3.73	4.03
H2	579.37	572.08	144.99	158.05	**12.45**	**57.95**
H3	573.74	577.81	139.05	146.25	7.24	**20.94**
H4	578.34	581.55	139.27	150.82	**14.72**	**81.74**
A0	578.84	585.76	137.58	143.40	2.84	7.05
A1	588.46	582.66	140.29	143.85	1.26	1.44
A2	577.97	587.32	154.78	155.81	**22.94**	15.83
A3	577.11	590.23	137.43	144.05	3.72	8.08
A4	608.62	626.99	193.35	182.67	2.22	12.65
A5	625.77	636.51	169.66	172.49	6.72	13.89
A6	619.82	609.02	164.29	157.74	2.46	**21.68**
M0	603.84	608.37	154.32	154.90	0.66	0.64
M1	584.70	597.54	145.94	156.06	0.70	1.65
M2	580.01	549.51	144.16	138.18	2.08	2.49
M3	543.14	540.68	136.66	136.40	4.25	7.82
M4	534.99	530.06	129.78	128.82	0.47	0.46
M5	525.13	514.85	128.32	127.99	0.46	0.45
M6	518.02	517.27	127.26	127.40	0.44	0.42
70M0	595.88	566.05	139.98	139.77	0.79	0.54
70M1	563.79	555.99	142.45	137.96	0.72	0.46
70M2	549.99	547.96	135.34	135.62	0.46	0.41
70M3	543.17	541.60	133.04	135.41	0.42	0.33
70M4	541.98	534.05	129.18	130.62	0.36	0.33
70M5	535.15	528.76	127.93	123.61	0.32	0.31
70M6	526.09	524.07	123.66	124.49	0.28	0.33
70M7	518.30	520.50	121.18	120.88	0.32	0.42

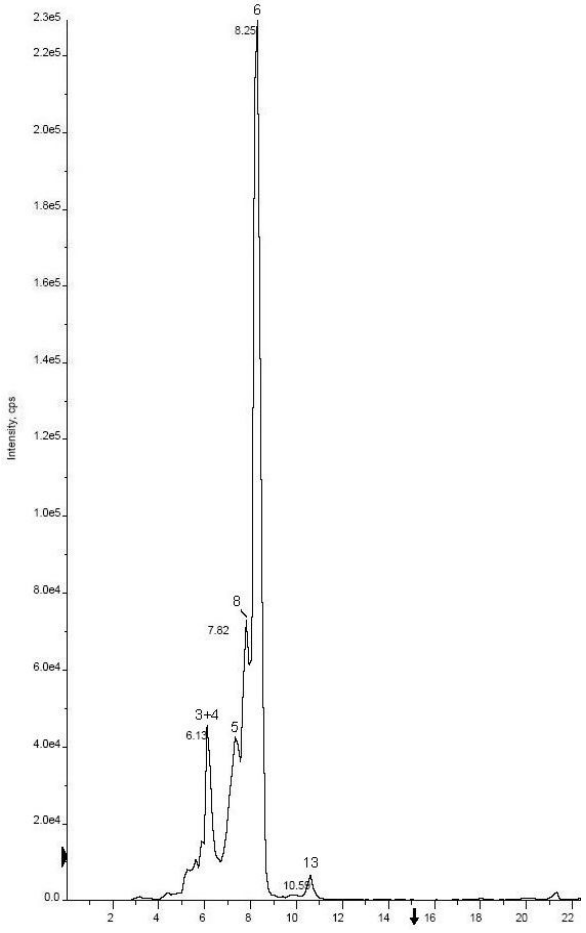
FSC: forward light scatter
SSC: side light scatter

Some acetone fractions exhibited relatively high fluorescence activity ratios: [**A2**] ($FR_{4\ \mu g/mL}$ =22.94), [**A4**] ($FR_{40\ \mu g/mL}$ =12.65), [**A5**] ($FR_{40\ \mu g/mL}$ =13.89). The above results demonstrate that the effect of MDR1 reversal correlates with the polarity and the concentration of the Anastasia Black extracts.

The measured FSCs (forward light scatter) and SSCs (side light scatter) of the Anastasia Black-treated cells did not differ significantly from those of the parent or MDR1 control cells. Thus, it can be stated that the samples had only slight or no cytotoxic activity.

Samples [**A0**], [**M0-M6**] and [**70M0-70M7**] were analysed by LC-MS-MS for phenolic compounds. Sixteen different phenols were investigated, including glycosylated molecules and their aglycones [129]. The results of the qualitative analysis showed that 8 out of the total of 16 phenols generated defined peaks. Six of these compounds had been reported in the literature, while luteolin glucuronide and luteolin arabinopyranoside diglucopyranoside were identified for the first time by IDA (information dependent acquisition) experiments and were then examined in MRM (multiple reaction monitoring) analyses. The extract [**M0**] contained the most of the compounds: seven peaks were noted for the following compounds (with their retention times): quercetin rhamnopyranoside glucopyranoside (**3**) (6.30 min), luteolin glucopyranoside arabinopyranoside (**4**) (6.13 min), apigenin glucopyranoside arabinopyranoside (**5**) (7.33 min), quercetin rhamnopyranoside (**6**) (8.25 min), hesperidine (**8**) (7.50 min), luteolin arabinopyranoside diglucopyranoside (**10**), and luteolin glucoronide **(11)** (10.59 min). The data are illustrated in Figure 6. Fractions [**M2**], [**M5**] and [**M6**] of the [**M0**] extract furnished the peaks of compounds **3**, **4**, **6** and **8**. The more polar the fractions were, the more polyphenols were detected. Five peaks were noted in extract [**A0**]: compounds **3**, **4**, **6**, **8** and **11**. In this case, the retention time of hesperidine (**8**) was 7.36 min. In extract [**70M0**], four different peaks appeared, those of compounds **1**, **3**, **4** and **6**. The LC-MS-MS analysis revealed that the components were eluted in the order of increasing polarity in the fractions of this extract. The peak of feruloyl glucopyranoside (**1**) was noted in fraction [**70M4**], quercetin rhamnopyranoside glucopyranoside (**3**) and luteolin glucopyranoside arabinopyranoside (**4**) were detected in fraction [**70M5**]. In addition to compounds **3** and **4**, quercetin rhamnopyranoside (**6**) was present in fraction [**70M6**]. The most marked peaks were due to quercetin rhamnopyranoside (**6**), the most characteristic flavonoid component of the sweet pepper samples studied.

Figure 6. LC-MS-MS analysis of Anastasia Black methanol extract [**M0**]: peaks and retention times of phenolic compounds[1].



HPLC of samples [**H0**] and [**A0-A6**] allowed for the identification of the carotenoid components through comparison with the peaks of lutein and β-carotene as standards. In extract [**H0**], carotenoids could not be detected and therefore the fractions [**H1-H4**] were not analysed. A peak in extract [**A0**] was assigned to lutein, but this peak was not detectable in the fractions. Most components of [**A0**] appeared in the fraction [**A4**] during benzene-EtOAc elution, but no peak in that fraction corresponded to lutein and other peaks were not definite.

[1]Detected phenolic compounds and retention times: quercetin rhamnopyranoside glucopyranoside (**3**) (6.30 min), luteolin glucopyranoside arabinopyranoside (**4**) (6.13 min), apigenin glucopyranoside arabinopyranoside (**5**) (7.33 min), quercetin rhamnopyranoside (**6**) (8.25 min), hesperidine (**8**) (7.50 min), luteolin arabinopyranoside diglucopyranoside (**10**), luteolin glucuronide **(11)** (10.59 min).

4.9 Apoptosis induction by essential oil components

An evaluation of the capacity of selected essential oils as apoptosis inducers, was demonstrated by Annexin V in flow cytometric analysis and by staining with ethidium bromide and acridine orange, using human *mdr1* gene-transfected mouse lymphoma cells and a human cervical adenocarcinoma cell line (HeLa). The pretreated (24h with the oil components) L5178 mouse lymphoma cells were washed and resuspended in binding buffer and Annexin V-FITC was added. After ten minutes incubation, cells were washed and propidium iodide was given. The fluorescence activity of the cells was measured and analysed by flowcytometry. The percentage of apoptosis and cell death was determined (Table 16.). The tested compounds are moderate apoptosis inducers compared to the control 12H-benzo(α)-phenothiazine. The total apoptosis rate varied between 5.32-8.93 % for the essential oil components.

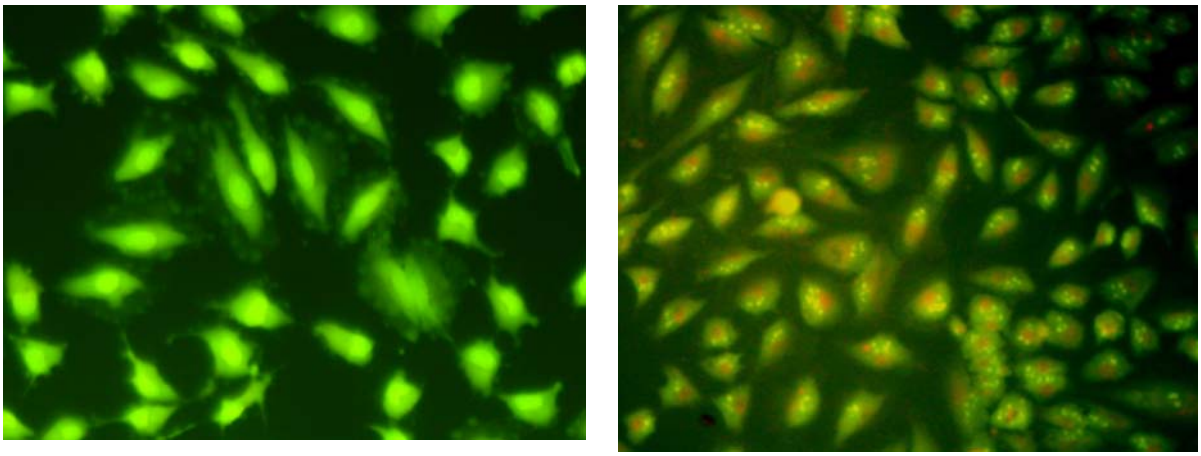
Table 16. Apoptosis induction by essential oil components

Sample	Concentration (µg/mL)	Early apoptosis %	Late apoptosis %	Total apoptosis %	Cell death %
Cell control	-	0.78	0.06	0.84	0.12
Cell control + **PI**[a]	-	0.05	0.07	0.12	3.09
Cell control + **annexin-V** + **PI**	-	2.49	0.96	3.45	4.77
Cell control + **annexin-V** + **PI** + **DMSO** 1.0 v/v%	-	0.68	1.41	2.09	3.42
M-627[b]	5	40.90	58.75	99.65	0.05
	25	5.42	94.16	99.58	0.09
	50	1.86	97.67	99.53	0.34
Eucalyptol	0.92	3.63	1.69	5.32	2.08
	9.20	4.17	2.34	6.51	2.70
Beta-pinene	0.87	3.27	2.38	5.65	2.85
	4.35	**5.04**	3.83	**8.87**	3.73
	8.72	4.67	2.60	**7.27**	2.76
Alpha-terpinene	0.84	4.14	2.77	6.91	3.45
	4.18	3.95	2.51	6.46	2.90
	8.37	3.82	2.95	6.77	3.89
Borneol	0.05	**6.19**	2.74	**8.93**	2.55
	0.50	4.74	3.34	**8.08**	2.76

[a]Propidium iodide
[b]M-627: 12H-benzo(α)-phenothiazine

Apoptosis induction by the selected components was also evaluated by the dual staining method on HeLa cells. Pretreated HeLa cells were stained acridine orange (AO) and ethidium bromide (EB) in order to visualise the basic morphological events. AO permeates all cells and makes nuclei appear green. EB is taken up by cells only when the cytoplasmic membrane integrity has been lost and it stains the nuclei red (Figure 7.)

Figure 7. Dual stained HeLa cells without treatment and treated with 5 µg/mL borneol.



Control Borneol pretreated HeLa cells

5. DISCUSSION

Antibiotic resistance of bacteria and chemotherapeutic resistance of cancer cells are major problems in the treatment of infections and malignant neoplastic diseases. One attempt to overcome resistance would be the use of resistance modifiers that aided the effectivity of the conventional treatment protocols. Our aim was to elucidate the MDR modifying potential of chemically synthesised compounds and natural derivatives in *in vitro* studies on prokaryotic and eukaryotic cells.

Patented organosilicon derivatives were shown to exert antiplasmid activity on various bacterial cultures. Certain chemically synthesised compounds affect the physical and functional characterisistics of the cell envelope of bacteria. At concentrations near their MIC they alter the electrophoretic pattern of proteins extracted from the cell envelope, cause its destruction and inhibit influx- and efflux transport mechanisms [134,135,136]. The elimination of plasmids from *E. coli* by phenothiazines has been attributed to their differential effects on the synthesis of plasmid and bacterium DNA, with greater effects noted on plasmid DNA synthesis. Because these compounds were shown to reverse the resistance to tetracycline due to overexpressed efflux pumps, and therefore are putative inhibitors of the efflux pumps of *E. coli*, the inhibition of efflux pumps by these agents may result in larger amounts of compound reaching plasmid DNA targets during the division of the bacterium. There is the possibility that elimination of plasmids may also invole their physical removal via openings created in the cell envelope. However, SILA compounds do not inhibit the replication at the concentration employed, there is little chance that the exit of the plasmid from the bacterium is due to the creation of openings in the cell envelope by the agents.

The antiseptic properties of aromatic plants and their extracts have been recognised since antiquity and are still used in the medicine, food and cosmetic industry. There appears to be a revival in the use of traditional approach to protecting livestock and food from disease and spoilage in industrial countries. Essential oils are generally isolated from non-woody plants by distillation methods, mainly by steam or hydrodistillation. Essential oils are usually made up of terpenoids, specifically monoterpenes and sesquiterpenes, but diterpenes may also be present and a variety of low molecular weight aliphatic hydrocarbons, acids, alcohols, aldehydes, acyclic esters or lactones, coumarines and homologues of phenylpropanoids. Most terpenes are derived from the condensation of branched five-carbon isoprene units and are categorized according to the number of these units present in the carbon skeleton [137]. The

mechanism of action of terpenes is not fully understood, it is assumed that membrane disruption by the lipophilic components is involved in the antibacterial action. *In vitro* studies proved that increasing the hydrophilicity of kaurene diterpenoids by the addition of a methyl group drastically reduced their antibacterial activities [138]. Terpenoids may serve as an example of lipid soluble agents which affect the activities of membrane-catalysed enzymes, for example their action on respiratory pathways. Certain components of essential oils can act as uncouplers, which interfere with proton translocation over a membrane vesicle and subsequently interrupt ADP phosphorylation. Specific terpenoids with functional groups, e.g. phenolic alcohols or aldehydes, also interfere with membrane-integrated or associated enzyme-proteins, stopping their production or activity [139]. The mode of action also depends on the microorganism and is mainly related to its cell wall structure. Gram-negative bacteria have intrinsic resistance against toxic components, since they have a permeability barrier against toxic agents. Hydrophobic macromolecules, such as essential oil constituents, are unable to penetrate the barrier. On the other hand, essential oils usually express low aqueous solubility, which prevents them from reaching a toxic level in cellular membranes [140]. In case of tea tree oil, the antimicrobial effect is based on the denaturation of membrane proteins, resulting in the outer membrane disruption, subsequent K^+ leakage, respiration inhibition and cell lysis [141,142]. Essential oils are also able to inhibit the synthesis of DNA, RNA, proteins and polysaccharides in fungal and bacterial cells [143].

The oil contents of the *O. vulgare* subsp. *hirtum* lines were high (3.8-7.0% v/w). The compositions of the essential oils were determined by GC and GC-MS techniques. The nineteen identified constituents accounted for 97.1-99.1 % of the oils. These oils were found to contain carvacrol (61.7-84.2 %), γ-terpinene (1.3-16.0 %) and *p*-cymene (4.2-9.5 %) as main constituent. The essential oil of sample C also contained a significant quantity of thymol (3.1 %). The compositional characteristics indicated that all these lines belong to the "carvacrol group" described by Pasqier [144]. The concentrations of the minor components in the four samples varied, but the differences were not significant. All of the *O. vulgare* subsp. *hirtum* oils exerted antimicrobial effects on the tested bacterial and fungal strains. The most sensitive strain among the tested bacteria was the proton-pump deficient *E. coli* AG100A. The MIC values demonstrated a difference in sensitivity between the two *E. coli* AG strains. The proton-pump carrier *E. coli* AG100 displayed MIC values that were 2-3 times as high as those for the proton pump-deficient mutant *E. coli* AG100A. These results suggest that the antimicrobial effects of the tested oils are probably based on proton pump-related

mechanisms. The different compositions of the four samples of *O. vulgare* subsp. *hirtum* did not appear to have any appreciable influence on the antimicrobial effects tested. The antimicrobial activity of the oil is clearly due to the high activity of carvacrol, as was described by Dorman *et al* [137]. These alterations correlate with the ability of hydrocarbons to interact with hydrophobic structures, like bacterial membranes. These findings are based on investigations of *E. coli* and *S. aureus* by transmission electron microscopy, which allows to study the possible bacterial ultrastructural alterations [145].

The role of QS is well known in plant pathology, however it also plays an important role in several human diseases. To cure bacterial diseases through the manipulation of QS regulated processes several natural and synthetic compounds have already been tested. The results obtained in our study show that some essential oils can also inhibit QS. Although at this time we are not suggesting that these compounds be used for the modification of QS in bacterial populations of a host, we do suggest that because these compounds do modify QS at least in the laboratory environment, that the approaches employed in our study may eventually lead to a new form of therapy for some mixed infections of the human which, at this time, are problematic for therapy including cancer [146,147,148].

Phenothiazines have been shown to have *in vitro* activity against antibiotic susceptible and antibiotic resistant strains of *M. tuberculosis* [149,150,151,152]. Although chlorpromazine has received the largest attention experimentally, it is not seriously considered as anti-tubercular drug due to its frequent and severe side effects [153]. Thioridazine, an equally effective neuroleptic drug is essentially devoid of the side effects noted for its parent chlorpromazine [154]. It is also the equal to chlorpromazine with respect to its anti-tubercular properties [152,153,154]. And as is the case for chlorpromazine it is concentrated by macrophages and kills intracellular *Mycobacteria* that have been phagocytosed by macrophages that have little killing action of their own [155]. These latter observations are extremely important for unlike the *in vitro* activity which requires concentrations of the thioridazine well beyond those clinically achievable, the concentration in the medium that affords the killing of intracellular *Mycobacteria* is below that found in the plasma of a patient treated with the same drug. The advent of multi-drug resistance of *M. tuberculosis* (MDRTB) now common throughout the world, coupled to the fact that there are no other effective anti-TB agents other than isoniazid, rifampin, ethambutol, streptomcyin and pryanimazide. And these strains can be killed readily by thioridazine after they have been phagocytosed. Animal

and human studies are now being conducted to determine the effectiveness of thioridazine and derivatives as anti-TB drugs.

MDR in tumour cells is a significant problem in the chemotherapy of many cancers. MDR often originates from elevated expressions of particular proteins, such as cell-membrane transporters, which can result in an increased efflux of the chemotherapeutic agents from the cells, thereby lowering their intracellular concentration [156].

The results of the present study demonstrate that Anastasia Black extracts and their fractions reversed the MDR of mouse T-lymphoma cells. P-gp, which belongs in the ABC transporter family, reduces the cytotoxic activity of anticancer agents in tumor cells, such as etoposide, vinblastin, mitomycin C, doxorubicin and actinomycin D, by pumping them out of the cytoplasm of the cells. In our study, R123 was a model substance of these antitumor agents. The results indicate that samples extracted or eluted with an apolar solvent were more efficient than the polar ones and thus the apolar carotenoids, vitamins and polyphenols of Anastasia Black are probably responsible for the MDR1-reversal activity.

Sixteen different phenolic compounds were studied by LC-MS-MS in Anastasia Black extracts and fractions. The investigated compounds were identified previously in different plant species. Compounds **1-7** and **9** previously isolated from hot pepper (*Capsicum annuum* L. var. Bronowiczka Ostra) and the structures were determined. Quercetin rhamnopyranoside was found for the first time in pepper species in a study by Materska *et al* [129]. This glycosylated flavonoid occurs in many plants and its antioxidant ability has been demonstrated [157,158,159]. The aglycone quercetin is a potent biomolecule due to its antioxidant activity. *In vitro* studies on cells and pure DNA have indicated the possible mechanisms of action. Quercetin can protect against mitomycin C-induced DNA strand breakage and exhibits antiproliferative activity [160,161]. There is an emerging view that flavonoids not only act as conventional hydrogen-donating antioxidants, but may exert modulatory action in cells via the protein kinase and lipid kinase signalling pathways [162].

The MDR-reversal effects were detected in those Anastasia Black extracts and fractions which were eluted by less polar solvents. Lipophilic components, e.g. carotenoids, may therefore be responsible for the P-gp inhibitory effect. Lutein- and β-carotene-based HPLC analisys of samples [**H0**] and [**A0-A6**] was performed in order to investigate the carotenoid content of Anastasia Black. The isolated lutein and β-carotene had previously been investigated for their MDR-reversal activities on human MDR1/A gene-transfected mouse lymphoma cells, MDA MB 231 (HTB-26) human breast cancer cells and Colo 320 human

colon cancer cells *in vitro*. Lutein increased the R123 accumulation of MDR mouse lymphoma cells and colon cancer cells, but β-carotene displayed no modulating activity on the drug sensitivity of mouse lymphoma or breast cancer cells [163,164]. The mechanism of action in mouse lymphoma cells is presumed to be charge-transfer complex formation with P-gp [163].

The sweet pepper samples eluted with less polar eluents exerted higher bioactivity due to their lipophilic components. The MDR-reversing carotenoids were detected in very small amounts in these samples and it may therefore be assumed that other lipophilic compounds are responsible for the MDR-reversal activity. Some of the isolated carotenoids from Anastasia Black pepper are able to act as resistance modifiers.

Several cytostatic drugs, including naturally occurring and pharmaceutical compounds, induce apoptosis in cancer cell [165,166]. Apoptosis is typically induced by the activation of membrane receptors, cell cycle arrest, p53 activation by DNA damaging agents and mitochondria pore transition permeability [167]. The apoptotic process is characterized by particular morphological and ultrastructural features, which can be evidenced by several assays, including FITC-conjugated annexin V method or dual satining assay [167].

The essential oil components that were applied caused moderate increase of apoptotic cells. The annexin V positivity of treated-cells by various compounds could be a consequence of the structural alteration in cell membrane, which results in the translocation of phosphatidylserine molecules from the inside to the outer surface of the membrane. The typical morphological features of apoptosis could be observed in the dual staining method. The apoptosis inducing effect of MDR reversal compounds can have special importance in experimental chemotherapy.

6. SUMMARY

The main aim of the thesis was to elucidate different possibilities in overcoming resistance in prokaryotic and eukaryotic cells by natural and chemically synthesised compounds. Potential resistance modifiers were investigated for their antimicrobial and anti-plasmid activities on various bacterial and fungal strains and the MDR reversal effects on tumour cell cultures.

Organosilicon compounds SILA 409 and SILA 421 were previously proved to exert MDR reversal activities on tumour cells and our studies gave an insight into the effects of the two chemicals on prokaryotic cells. They had a pronounced antibacterial and anti-plasmid activity on different bacterial plasmids.

Various essential oils and essential oil components were proved to have antimicrobial activity. Our aim was to test essential oils on bacterial and fungal strains in order to assess antimicrobial and anti-plasmid activities. Peppermint oil and its main constituent menthol showed a remarkable anti-plasmid action. This fact might suggest that essential oils have a more complex effects on prokaryotic cells than interaction with the cell membrane causing membrane disruption and ion leakage. The antimicrobial effects of four lines of *Origanum vulgare* subsp. *hirtum* were similar, which can be explained by the fact that most of its constituents exert antimicrobial effects and the essential oil content was similar in all lines according to the analysis made by GC methods. The main constituent of all four lines, carvacrol also exerted antiplasmid activity.

Cell-cell interactions are essential between prokaryotic and eukaryotic cells and organisms as well. Important processes are controlled by QS mechanisms in bacteria. The inhibition of these processes might be advantageous in overcoming infections and antibiotic resistance. In our model experiments essential oils had inhibitory effects on QS mechanisms by the inhibition of QS signal-molecule production.

Anastasia Black (Russian Black Sweet Pepper) extracts and fractions had MDR reversal activities. The most active fractions were further analysed by LC-MS-MS and HPLC methods. It may be concluded that phenolic compounds might be responsible for the effects.

As an alternative way of anti-tumour effect, apoptosis induction of selected essential oil components were studied. The tested compounds had moderate apoptosis inducing effect of the L 5178 Y cells during the 1 hour incubation. Typical apoptotic markers were observed after a 24 h treatment with borneol by staining with EB and AO on HeLa cells.

7. ÖSSZEFOGLALÓ

A disszertáció fő célja a rezisztencia visszafordításának tanulmányozása prokarióta és eukarióta modelleken természetes eredetű anyagokkal és kémiailag szintetizált vegyületekkel. Potenciális rezisztencia módosító vegyületeket vizsgáltunk baktériumokon és sarjadzó gombákon, hogy meghatározzuk az anyagok antimikróbás és antiplazmid hatásait. Továbbá tumorsejteken tanulmányoztuk multidrog rezisztenciára gyakorolt hatásukat.

SILA 409 és SILA 421-es szerves szilícium vegyületekről korábbi kísérletek bebizonyították, hogy tumorsejteken képesek a rezisztencia visszafordítására. Vizsgálatainkban a baktériumok növekedésére és különböző plazmidokra kifejtett hatásukat tanulmányoztuk. Mindkét vegyület kifejezett antibakteriális és antiplazmid hatást mutatott.

Számos illóolajról és komponenseiről bizonyították, hogy antimikróbás hatással rendelkeznek. Az antimikróbás hatás mellett célunk volt tanulmányozni különböző illóolajok antiplazmid hatását is. A borsosmenta olaj és legfontosabb összetevője, a mentol mutatta a legkifejezettebb antiplazmid hatást. A plazmidokra gyakorolt hatás alapján arra következtethetünk, hogy az illóolajok prokaryota sejtekre gyakorolt hatása komplexebb annál, hogy csak a sejtmembránnal való kölcsönhatásukkal magyarázzuk antibakteriális hatásukat. Az *Origanum vulgare* subsp. *hirtum* illóolajainak antimikróbás hatása is kifejezett volt. A négy típus hatásában megfigyelhető hasonlóság valószínűleg az összetételbeli hasonlóságban rejlik, amelyet a gázkromatográfiás vizsgálatok támasztanak alá. A négy vonal illóolajainak legfőbb komponense a karvakrol, amely szintén rendelkezik antiplazmid hatással.

A sejtek közötti kommunikációnak óriási jelentősége van mind a prokaryota mind az eukaryota világban. A baktériumok közötti kommunikáció egyik legfőbb eszköze az ún. quorum sensing. Ezen mechanizmusokat támadva specifikus célmechanizmusokat találhatunk, amelyek elősegíthetik új antimikróbás szerek fejlesztését. Kísérleteinkben illóolajok hatását vizsgáltuk a QS szignálmolekulák szintézisére.

Anastasia Black (Orosz fekete paprika) kivonatainak rezisztenciára gyakorolt hatását vizsgáltuk áló Pgp-t expresszegér limfóma sejteken rodamin exklúziós teszttel, majd a hatásos frakciókat analizáltuk LC-MS-MS és HPLC módszerekkel. Megfigyeléseink szerint az aktív hatóanyagok valószínűleg fenolos vegyületek lehetnek.

Az apoptózist indukáló és MDR gátló hatás a gyógyszerkutatás szempontjából két értékes tulajdonság. Az illóolajok komponensei közül kiválasztott anyagok kis mértékben indukáltak apoptózist, amely a borneol esetében 24 órás inkubációt követően volt a legmarkánsabban megfigyelhető.

8. ACKNOWLEDGEMENTS

I would like to express my deep and sincere gratitude to my supervisor, **Professor József Molnár** for his continuous support and encouragement throughout my studies. I also would like to express my thanks for his skilful and understanding guidance.

I express my sincere gratitude to **Professor Yvette Mándi**, the head of the Department of Medical Microbiology and Immunobiology, for providing the opportunity to work at the department.

I would like to give special thanks to **Annamária Molnár, Gabriella Spengler, Mrs Vigyikán Anikó Váradi, Helga Engi, Míra Ágnes Szabó, Zoltán Gábor Varga** and colleagues at the Department of Medical Microbiology and Immunobiology, who supported me in my work and created a pleasant working environment.

I greatly acknowledge to **Professor Judit Hohmann, Erzsébet Varga, Katalin Veres** (Department of Pharmacognosy, Faculty of Pharmacy, University of Szeged), **Professor Leonard Amaral, Marta Martins** (Unit of Mycobacteriology, UPMM, Instituto de Higiene e Medicina Tropical, University of Lisbon), **Professor Vincenzo Fogliano** (Department of Food Sciences, University of Naples), **Professor Ernő Szegedi** (Corvinus University, Research Institute for Viticulture and Enology) for their help, support and constructive comments.

I am grateful to **Dr. Imre Ocsovszki** for performing the flow-cytometric measurements.

I heartfully thank **my parents**, and **my friends** for their support, love and patience through all these years.

This study has been supported by various organizations including **Foundation for Cancer Research Szeged** (Szegedi Rákkutatásért Alapítvány)**, Richter Gedeon Centenáriumi Alapítvány, COST B16 STSM** grant **and Federation of European Microbiological Societies**.

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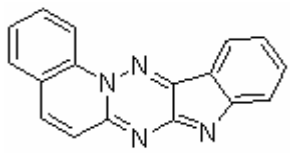
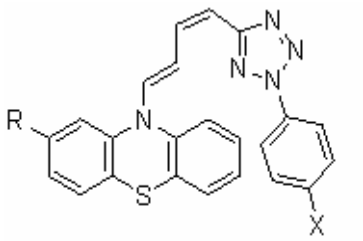
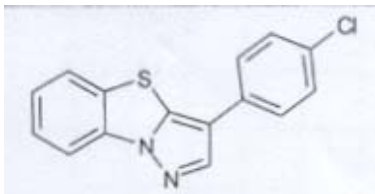
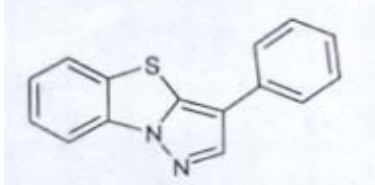
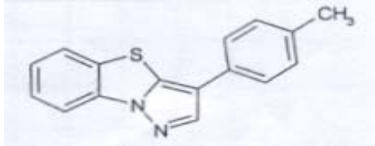
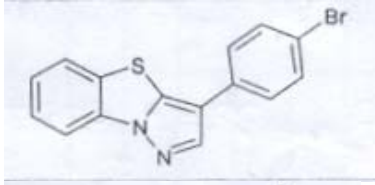
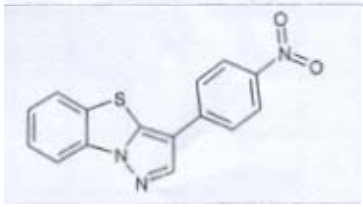
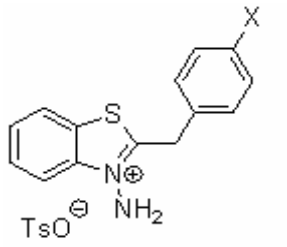
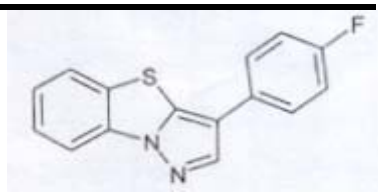
ANNEX

Annex 1. Molecular structure of Thioridazine derivatives

Compounds ID	Chemical name/Molecular formula	Formula
#1470	Indolo[2,3:5',6'][1,2,4]triazino[2,3-a]quinoline	
#1687	R = CF_3, X = OCH_3 3-Trifluoromethyl-10-{(1E,3Z)-4-[2-(4-anisyl)-2H-tetrazol-5-yl]buta-1,3-dien-1-yl}-10H-phenothiazine	
#1871	C15H9ClN2S	
#1872	C15H10N2S	
#1873	C16H12N2S	
#1874	C15H9BrN2S	
#1876	C15H9N3O2S	
#1875	X = NO_2	

	3-amino-2-(4-nitrophenyl)benzthiazolium tosylate (C21H19N3O5S)	
#1926	X = F 3-amino-2-(4-fluorophenyl)benzthiazolium tosylate (C21H19FN2O3S2)	
#1870	X = Cl 3-amino-2-(4-chlorophenyl)benzthiazolium tosylate (C21H19ClN2O3S2)	
#1868	X = CH_3 3-amino-2-(4-tolyl)benzthiazolium tosylate (C22H22N2O3S2)	
#1869	X = Br 3-amino-2-(4-bromophenyl)benzthiazolium tosylate (C21H19BrN2O3S2)	
#1929	C15H9FN2S	

Annex 2. LC-MS-MS characteristics of several phenols found in extracts of Anastasia Black.

Compound	Peak no.	Precursor ion $[MH]^-$ *m/z*	MS/MS ions	CE
Feruloyl glucopyranoside	1	355	193	-35
			134	-25
Sinapoyl glucopyranoside	2	385	223	-35
			208	-35
Quercetin rhamnopyranoside glucopyranoside	3	609	301	-49
			447	-35
Luteolin glucopyranoside arabinopyranoside	4	579	285	-45
			132.9	-44
Apigenin glucopyranoside arabinopyranoside	5	563	269	-35
			401	-35
Quercetin rhamnopyranoside	6	447	301	-32
			271	-45
Luteolin-(furanosyl-glucopyranosyl-malonyl)-glucopyranoside	7	827.5	285	-45
Hesperidine	8	609	315	-45
			301	-45
Feruyl alcohol-(methylhydroxypropionyl)-glucopyranoside	9	427.4	265.4	-35
Luteolin arabinopyranoside diglucopyranoside	10	741	579	-50
			285	-50
Luteolin glucuronide	11	461	285	-40
Ferulic acid	12	192.7	133.9	-25
			178	-15
Sinapinic acid	13	222.7	207.8	-20
			164	-19
Quercetin	14	300.7	150.9	-28
			179.1	-24
Luteolin	15	284.9	132.9	-44
			151	-32
Apigenin	16	269	151	-35

CE: collision energy

Annex 3. Comparison of the *Origanum vulgare* subsp. *hirtum* oils (2004). Percentage of essential oil components in the four lines.

Compounds[a]	% in samples				RI[b]	Identification
	A	B	C	D		
α-thujene	1,7	1,8	1,6	1,5	930	c
α-pinene	0,9	0,8	0,8	0,7	938	c, d
camphene	0,3	0,3	0,3	0,2	951	c
sabinene	0,5	0,4	0,1	0,3	974	c, d
β-pinene	2,1	2,1	2,1	1,4	980	c, d
α-phelladrene	0,1	0,2	0,2	0,1	1006	c
α-terpinene	0,9	1,0	2,1	0,4	1018	c, d
p-cymene	9,5	6,3	6,1	4,2	1025	c, d
limonene	0,4	0,4	0,4	0,3	1030	c, d
γ-terpinene	7,2	5,5	16,0	1,3	1060	c, d
cis-sabinen hydrate	0,5	0,5	0,4	0,2	1071	c
terpinolene	0,2	0,3	0,2	0,2	1087	c
borneol	0,3	0,5	0,5	0,2	1168	c, d
terpin-4-ol	0,3	0,4	0,2	0,3	1178	c
carvacrol methyl-eter	-	-	-	0,7	1242	c
thymol	0,3	0,3	3,1	0,3	1291	c, d
carvacrol	70,8	73,7	61,7	84,2	1297	c, d
β-caryophyllene	1,4	2,6	3,3	1,5	1420	c, d
germacrene-D	0,3	-	-	-	1480	c
Total	**97,7**	**97,1**	**99,1**	**98,0**		

[a]Compounds listed in order of elution from a DB-5 MS column.
[b]Kovats retention indices calculated agains C_9 to C_{24} n-alkanes on the DB-5 MS coloumn
[c]Comparison of mass spectra with MS libraries and retention indices
[d]Comparison with authentic compound