„ALEXANDRU IOAN CUZA" UNIVERSITY OF IASI

FACULTY OF BIOLOGY

DOCTORAL SCHOOL OF BIOLOGY

SYNTHETIC FLAVONOIDS WITH HALOGEN SUBSTITUENTS – SOLUTIONS FOR FIGHTING ANTIBIOTIC RESISTANCE PHENOMENON

Summary of the doctoral thesis

Scientific advisor:

Prof. PhD habil. Marius STEFAN

PhD student:

Cristina-Veronica MOLDOVAN

Iasi, 2025

CONTENT

LIST OF ABBREVIATIONS

ADN – *deoxyribonucleic acid*

ARNm – *messenger ribonucleic acid*

ATCC – *American Type Culture Collection*

MBC – *minimum bactericidal concentration*

MIC – *minimum inhibitory concentration*

CSH – *cell surface hydrophobicity*

DHFR – *dihydrofolate reductase*

DHPS – *dihydropteroate synthase*

DMSO – *dimethyl sulfoxide*

EPS – *extracellular polymeric substances*

ESBL – *extended spectrum beta-lactamase*

ESKAPE – *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.

FIC – *fractional inhibitory concentration*

FICI – *fractional inhibitory concentration index*

HOME-BIO – *sHOtgun MEtagenomic analysis of BIOlogical entities*

IC_{50} – *half-maximal inhibitory concentration*

IL – *interleukin*

KPC – *Klebsiella pneumoniae carbapenemase*

MBL – *metallo-β-lactamase*

MDR – *multidrug-resistant bacteria*

MLS – *Macrolide-Lincosamide-Streptogramin antibiotics*

MRSA – *methicillin-resistant Staphylococcus aureus*

MSSA – *methicillin-susceptible Staphylococcus aureus*

MTT – *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide*

OMS – *World Health Organization*

PBP – *penicillin-binding proteins*

PDR – *pandrug resistance*

rsCDM – *rapid and simplified carbapenemase detection method*

SEAU – *wastewater treatment plants*

TNF- α – *tumour necrosis factor alpha*

UV – *ultraviolet*

XDR – *extensively drug-resistant*

INTRODUCTION

Infectious diseases caused by pathogenic microorganisms have been a major cause of mortality of human population in the pre-industrial era, as well as in the outbreaks of epidemics and pandemics that have marked human history. The discovery of antibiotics, the diversification of their chemical structure, and the widespread use of antimicrobial compounds allowed the treatment of many bacterial infections in the middle of the last century. However, the extensive use of antimicrobial substances quickly led to the emergence of antibiotic resistance.

With each new class and type of antibiotic developed, strains resistant to the compound used have emerged within one to two decades. Moreover, as the use of antibiotics has expanded, more and more clinically resistant relevant strains to different substances have been isolated, and subsequently multidrug-resistant (MDR), extensively drug-resistant (XDR) and even pandrug-resistant (PDR – strains resistant to all antibiotics available and approved for human use) strains have been identified.

This phenomenon has grown alarmingly in recent years, raising major concerns among medical practitioners, the scientific community, civil society and policy makers. The World Health Organization (WHO) considers antibiotic resistance a major public health problem, with the risk that major infectious diseases may become untreatable.

Flavonoids are compounds that naturally occur in plants and have an impressive variety of chemical structures. Modern studies on the chemical activity of some flavonoids have revealed remarkable antibacterial properties due to various ways of action. Important to mention is the synergistic effect that some flavonoids exhibit in combination with conventional antibiotics used in human therapy, which creates the premises for the development of effective treatment strategies. Many synthetic or semi-synthetic flavonoids exhibit more pronounced antibacterial activity against pathogenic germs than natural flavonoids due to changes in their chemical structure.

Knowing the consequences of antimicrobial resistance phenomenon, in the present study we focused on the investigating the presence of resistant bacteria in wastewater samples, analysing the mechanisms of resistance in isolated microorganisms and evaluating the antibacterial properties of synthetic tricyclic sulphur-containing flavonoids, against resistant bacterial strains. The investigation of the mechanisms of action of selected flavonoids, the evaluation of the synergistic effect produced by combinations formed by flavonoids with some conventional antibiotics, as well as the investigation of the cytotoxic and anti-inflammatory potential of a selected flavonoid (BrCl) were also considered. The data obtained suggest an important potential of the flavonoid BrCl as a promising alternative solution to combat some resistant pathogenic bacteria.

SCIENTIFIC OBJECTIVES

The main objective of this study was to evaluate the antibacterial activity of some synthetic tricyclic sulphur-containing flavonoids to identify effective solutions to fight antibiotic resistance phenomenon. To achieve the main objective, 7 secondary objectives were pursued, each of which presents a series of associated activities.

1. Characterization of antibiotic-resistant bacterial strains

Associated activities:

A.1.1. Isolation of bacterial strains from urban wastewater samples and pathological products

A.1.2. Analysis of macro- and micro-morphological characters of isolated strains

A.1.3. Determination of antibiotic resistance level

A.1.4. Taxonomic identification of isolated bacterial strains

2. Determination of antibiotic resistance genes in wastewater samples

Associated activities:

A.2.1. Isolation and quality assessment of metagenomic DNA

A.2.2. Analysis of genes involved in antibiotic resistance

3. Evaluation of antibiotic resistance mechanisms present in selected bacterial strains

Associated activities:

A.3.1. Assessment of the biofilm-forming capacity of some bacterial strains

A.3.2. Highlighting the production of β-lactamases

A.3.3. Highlighting the presence of efflux pumps

4. Evaluation of the antibacterial potential of synthetic tricyclic sulphur-containing flavonoids against resistant bacterial strains

Associated activities:

A.4.1. Determination of minimum inhibitory and bactericidal concentration

A.4.2. Evaluation of the influence on bacterial growth

A.4.3. Assessment of the effects on the viability of bacterial cells

5. Assessment of BrCl flavonoid mode of action

Associated activities:

A.5.1. Assessment of bacterial cell membrane integrity using fluorescence and scanning electron microscopy techniques

A.5.2. Determination of antibiofilm activity

6. Establishing the synergistic effect of BrCl flavonoid in combination with different antibiotics

Associated activities:

A.6.1. Determination of the synergistic effect using the checkerboard method

A.6.2. Evaluation of the synergistic effect of the flavonoid – antibiotic combinations on bacterial cells viability

7. Assessment of the cytotoxic and anti-inflammatory potential of BrCl flavonoid

Associated activities:

A.7.1. Testing the influence of the selected flavonoid on the viability of some human cells

A.7.2. Evaluation of the stimulatory effect of the flavonoid on the secretion of pro-inflammatory cytokines

Structure of the doctoral thesis

The doctoral thesis is structured in two parts:

Part I. Theoretical considerations, consisting of two chapters summarizing the specialized literature.

Part II. Personal contributions, consisting of six chapters, each divided into *Overview*, *Research Materials and Methods*, and *Results and Discussion*.

The thesis is finalized with *Conclusions*, *Prospects for further research*, *List of papers published by the author*, *List of published abstracts*, *Participation at international conferences*, *Participation at national conferences*, *Research projects*, *Patent proposal*, *Bibliography* and *Appendices*.

PART I – THEORETICAL CONSIDERATIONS

CHAPTER 1 – ANTIBIOTIC RESISTANCE

According to the World Health Organization (WHO), the resistance of micro-organisms to antimicrobial therapy is one of the biggest problems facing 21st century medicine. Antibiotic resistance, according to the European Centre for Disease Prevention and Control, is defined as the ability of microorganisms (viruses, bacteria, fungi) to resist the action of one or more antimicrobial-acting compounds (Moldovan *et al.*, 2022). Antimicrobial resistance occurs when microorganisms no longer respond to antimicrobial substances to which they were previously sensitive, and which were previously proven effective in treating infections caused by these pathogenic microorganisms (Mancuso *et al.*, 2021). The phenomenon of antibiotic resistance leads to a considerable decrease in the possibilities of treating many infectious diseases caused by pathogenic microorganisms. Excessive consumption along with inappropriate antibiotic prescription are important causes of the resistance phenomenon (Ventola, 2015).

1.1. MECHANISMS OF ANTIBIOTIC RESISTANCE

The mechanisms involved in antibiotic resistance are divided into two categories: natural and acquired resistance mechanisms (Uddin *et al.*, 2021).

Natural resistance can be either intrinsic (expressed at the species level) or induced (genes that confer resistance are naturally present in bacteria but are only expressed when the microorganism comes into contact with the antibiotic) (Uddin *et al.*, 2021).

Acquired resistance can occur through mutations in its own chromosomal DNA or due to the acquisition of genetic material through multiple pathways (horizontal gene transfer).

Some of the most important mechanisms of acquired resistance are enzymatic drug inactivation, drug efflux, drug uptake limitation and drug target modification (Uddin *et al.*, 2021).

1.1.1. Enzymatic drug inactivation

The main types of antibiotic-degrading enzymes are β-lactamases, acetyl transferases and aminoglycoside-degrading enzymes (Giedraitienė *et al.*, 2011).

1.1.2. Antibiotic efflux from the bacterial cell

Efflux pumps are a complex of proteins involved in the transport of various molecules (antibiotics, detergents, dyes) from inside the cells to the outside environment (Blair *et al.*, 2014). Efflux pumps with important medical implications belong to the RND family (Blair *et al.*, 2014; *Li et al.*, 2015).

1.1.3. Drug target modification

Modification of penicillin-binding proteins (PBPs) is a resistance mechanism found in Gram-positive bacteria, with the development of a mutation in PBPs leading to a decreased in affinity for β-lactam antibiotics. Macrolides, lincosamides and streptogramin A (MLS) block protein synthesis in Gram-negative bacteria by attaching to 50S ribosomal subunits. Glycopeptide antibiotics together with peptidoglycan precursors (D-alanyl-D-alanine) inhibit transpeptidation and transglycosylation in the bacterial cell wall synthesis process (Giedraitienė *et al.*, 2011; Kapoor *et al.*, 2017; Peterson and Kaur, 2018).

1.1.4. Drug uptake limitation

Bacteria have developed overtime mechanisms by which they prevent antibiotics from exerting their effect at intracellular or periplasmic level, by decreasing the uptake of molecules inside the cells due to the reduction in the porin number and/or their differential expression (Munita and Arias, 2016).

1.2. ANTIBIOTIC RESISTANCE OF SOME IMPORTANT BACTERIA

The main cause of nosocomial infections worldwide is a group of pathogenic microorganisms generically called ESKAPE - *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *Enterobacter* spp. which pose a real challenge for modern medicine, due to their increased ability to acquire resistance to antibiotics (Santajit and Indrawattana, 2016). For this reason, the WHO considers the mentioned bacterial strains to be a global threat to public health, for which there is an urgent need to identify new effective antimicrobial compounds (Ayobami *et al.*, 2022).

1.2.1. *Enterococcus* spp.

Enterococci are Gram-positive bacteria, frequently associated with urinary tract infections, infective endocarditis, meningitis, peritonitis, surgical wound infections, biliary

infections, liver abscesses, bacteremia (Panzaru, 2003; O'Driscoll and Crank, 2015). Enterococci exhibit increased resistance to β-lactam antibiotics, trimethoprim/sulfonamides, quinolones, aminoglycosides, glycopeptides and clindamycin (French, 2005; O'Driscoll and Crank, 2015).

1.2.2. *Staphylococcus aureus*

Staphylococcus aureus is an opportunistic pathogenic microorganism that colonizes the skin and nasal mucous membrane (Oliveira *et al.*, 2018). Staphylococci rapidly acquire resistance to erythromycin, ampicillin, tetracycline and vancomycin (Dorneanu and Vremera, 2011; Huemer *et al.*, 2020).

1.2.3. *Klebsiella pneumoniae*

K. pneumoniae is a Gram-negative encapsulated bacterium responsible for some nosocomial and community-acquired infections, such as urinary tract infections, pulmonary infections, liver abscess, catheter infections and sepsis (Moya and Maicas, 2020; Mancuso *et al.*, 2021). *K. pneumoniae* strains exhibit resistance to quinolones and fluoroquinolones, polymyxins and tigecycline.

1.2.4. *Acinetobacter baumannii*

A. baumannii strains are responsible for a variety of infections, including respiratory and urinary tract infections (Huemer *et al.*, 2020). According to Mancuso *et al.* (2021), *A. baumannii* strains can develop resistance to imipenem, meropenem, carbapenems and fluoroquinolones.

1.2.5. *Pseudomonas aeruginosa*

P. aeruginosa is an opportunistic pathogenic bacterium that cause increased mortality and morbidity among patients diagnosed with cystic fibrosis and immunocompromised individuals (Pang *et al.*, 2019). *P. aeruginosa* strains can exhibit resistance to aminoglycosides, quinolones and β-lactam antibiotics (Hancock and Speert, 2000).

1.2.6. *Enterobacter* spp.

Members of the *Enterobacter* genus are non-fastidious Gram-negative bacteria, sometimes encapsulated species that can cause infections in immunocompromised individuals similar to those caused by *K. pneumoniae* (Santajit and Indrawattana, 2016). *Enterobacter* strains

are resistant to most antibiotics used in therapy, except tigecycline and colistin (Santajit and Indrawattana, 2016).

1.3. ANTIBIOTIC RESISTANCE IN WASTEWATER TREATMENT PLANTS

Wastewater and wastewater treatment plants (WWTPs) are important reservoirs for the selection of antibiotic-resistant bacteria (Novo *et al.*, 2013), being considered among the most significant anthropogenic sources for dissemination of antibiotic resistance genes (Michael-Kordatou *et al.*, 2018).

1.3.1. Resistance to β-lactam antibiotics

β-lactam antibiotics are a class of broad-spectrum compounds that contain a β-lactam ring in their structure, responsible for antibacterial activity (Pazda *et al.*, 2019). Penicillins represent some of the most widely used antibacterial substances in the medical and veterinary sectors (Pazda *et al.*, 2019). Their presence in wastewater and activated sludge is rarely reported due to the instability and sensitivity to hydrolysis of the β-lactam ring (Hirsch *et al.*, 1999). The mechanisms by which bacteria acquire resistance to β-lactam antibiotics consist of decrease in the permeability of the outer membrane for antimicrobial compounds, a phenomenon due to a decreased porin number, PBP modification and antibiotic inactivation via β-lactamases (van Hoek *et al.*, 2011).

1.3.2. Resistance to aminoglycoside

Aminoglycosides are a class of natural or semi-synthetic compounds with activity on both Gram-positive and Gram-negative bacteria (Krause *et al.*, 2016). The use of aminoglycosides in medical practice is often restricted due to their adverse effects and toxic potential. Water contamination, despite to the low consumption of aminoglycosides, was detected in both influent and effluent of some treatment plants (Mutuku *et al.*, 2022). Resistance to aminoglycosides is due to the presence of overexpressed efflux pumps, decreased bacterial cell membrane permeability, ribosomal alteration, and inactivation of the antimicrobial compound by enzymes that degrade antibiotic molecules (van Hoek *et al.*, 2011).

1.3.3. Resistance to tetracyclines

Tetracyclines are broad-spectrum compounds that inhibit protein synthesis by blocking mRNA (Kaufman, 2011). Daghrir and Drogui (2013) identified the presence of

tetracyclines in both aquatic (wastewater, surface water, groundwater) and terrestrial (soil, sediment) environments. The mechanisms by which microorganisms acquire tetracycline resistance are represented by enzymatic inactivation, the presence of efflux pumps and ribosomal protein protection (van Hoek *et al.*, 2011).

1.3.4. Resistance to macrolides – lincosamides – streptogramin B

Macrolides, lincosamides and streptogramin B (MLS) affects the early stage of protein synthesis (translocation) (Kapoor *et al.*, 2017). Among these compounds, representatives of the macrolide class are the most detected in influent and effluent of sewage treatment plants, which is due to their increased consumption in the human and veterinary sector, as well as their high environmental stability (Mutuku *et al.*, 2022, Ngigi *et al.*, 2019). The mechanisms conferring resistance to antibiotics belonging to the MLS group are due to the presence of rRNA methylase encoded by the *erm* gene and overexpressed efflux pumps (van Hoek *et al.*, 2011).

1.3.5. Resistance to fluoroquinolone

Fluoroquinolones are synthetic, broad-spectrum antibiotics, that interfere with bacterial DNA synthesis by inhibiting two enzymes: topoisomerase II (DNA gyrase) and topoisomerase IV, essential enzymes involved in DNA replication (Dowling *et al.*, 2011). Due to the hydrophilic properties they exhibit, fluoroquinolones can easily spread in aquatic environments, the highest concentrations being reported in hospitals effluents, as well as in the influents of wastewater treatment plants (Mutuku *et al*., 2022). Fluoroquinolone resistance is due to the decrease of the permeability of the external membrane correlated with decreased porin number, overexpression of efflux pumps, and gyrase and topoisomerase IV mutations (van Hoek *et al.*, 2011).

1.3.6. Resistance to sulfonamides and diaminopyrimidines

Sulfonamides have an analogous structure with *p*-aminobenzoic acid, being involved in the inhibition of dihydropteroate synthase (DHPS), a protein with a role in the last stage of the folate biosynthesis required for thymine production and in the development of bacterial cells. According to Mutuku *et al.* (2022), sulfonamides are quite common in influent and effluent of wastewater treatment plants. The mechanism by which microorganisms acquire resistance to sulfonamides and trimethoprim is represented by mutations of *folP* and *folA* genes encoding the DHPS and DHFR enzymes (van Hoek *et al.*, 2011).

CHAPTER 2. SYNTHETIC FLAVONOIDS - PROMISING SOLUTIONS TO COMBAT ANTIBIOTIC RESISTANCE PHENOMENON

Flavonoids (latin word *flavus* = yellow) are a group of natural phenolic substances, with the core structure based upon a C6–C3–C6 skeleton, derivatives of 2-phenylbenzopyran (flavan) or 3-phenylbenzopyran (isoflavan) - Figure 2.1, which are found in fruits, vegetables, cereals, tree rhizomes, stems, flowers, tea and wine (Byalka *et al.*, 2004; Miscalencu *et al.*, 2008).

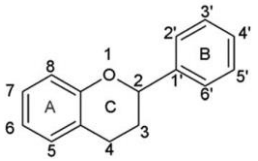


Figure 2.1. Basic structure of flavonoids (Panche *et al.*, 2016)

In addition to their important properties at different levels of various plant organs (they provide colour to flowers, protect leaves from pathogens as well as from UV-B radiation, are involved in the control of respiration, photosynthesis, morphogenesis etc.), flavonoids also exhibit a series of important medical functions, including promising antimicrobial activity (Sarbu *et al.*, 2019).

2.1. ANTIMICROBIAL ACTIVITY OF FLAVONOIDS

Flavonoids extracted from *Laurus nobilis* leaves (kaempferol 3-O-α-L-(2‴,4‴-di-*E*-*p*-coumaroyl)-ramnozide and kaempferol 3-*O*-α-L-(2‴-*Z*-*p*-coumaroyl-4‴-*E*-*p*-coumaroyl)-ramnozide) showed important activity against methicillin-resistant *S. aureus* strains; 2′-(OH)-chalcone, 2′,4′-$(OH)_2$-chalcone and 2′,4-$(OH)_2$-chalcone showed activity against *S. aureus* MSSA and MRSA strains; myricetin exhibits potent antimicrobial activity against *Burkholderia cepacia* strains, *K. pneumoniae* strains, vancomycin-resistant *Enterococcus* spp. and *P. aeruginosa* strains; tomentodiplacon and some prenylated flavonoids isolated from the roots of *Eriosema chinense* Vogel (eriosemaone A, lupinifolinol, flemichin D, dehydrolupinifolinol and lupinifolin) inhibit the growth of different *M. tuberculosis* strains; isoflavone, genistein, laburnetin, luteolin and epiafzelechin from *Ficus cordata* extracts show efficacy against *M. tuberculosis*, *S. aureus*, *B. cereus*, *Citrobacter freundii*, *E. cloacae*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. typhimurium* strains; some derivatives of chalcones (4′-carboxy-3-bromo-alkone, 4′-carboxy-3,5-dichloro-alkone, 4′-carboxy-2-chloro-alkone, carboxy-3-nitro-alkone etc.), 4-thioflavones and 4-iminoflavones exhibit important activity against *E. coli*, *B. subtilis*,

S. aureus, *Shigella flexneri*, *P. aeruginosa*, *S. typhimurium* and *S. enterica* strains (Alcarez *et al*., 2000; Tsuchiya and Iinuma, 2000; Xu and Lee, 2001; Otsuka *et al.*, 2008; Pistelli and Giorgi, 2012; Xie *et al.*, 2015; Shamsudin *et al.*, 2022).

2.2. FLAVONOIDS MECHANISMS OF ACTION

The antimicrobial activity of flavonoids can be attributed to 5 mechanisms: alteration of the cytoplasmic membrane (Ikigai *et al.*, 1993; Tsuchiya and Iinuma, 2000; Cushnie and Lamb, 2011), inhibition of nucleic acid synthesis (Bernard *et al.*, 1997; Plaper *et al.*, 2003; Navarro-Martínez *et al.*, 2005), inhibition of energy metabolism (Haraguchi *et al.*, 1998), blocking of cell wall synthesis (Wu *et al.*, 2008) and inhibition of cell membrane synthesis (Cushnie and Lamb, 2011).

In recent years, new mechanisms of action have been identified: inhibition of bacterial cell attachment to various supports and biofilm formation, modification of bacterial cell permeability, inhibition of quorum-sensing process, inhibition of efflux pumps, inhibition of fatty acid synthesis pathway, inhibition of bacterial motility, inhibition of bacterial toxins (Farhadi *et al.*, 2018; Biharee *et al.*, 2020).

2.3. SYNERGISTIC ACTION OF FLAVONOIDS WITH CONVENTIONAL ANTIBIOTICS

The specialized literature mentions several combinations formed by flavonoids, such as baicalein, apigenin or quercetin and various β-lactam antibiotics, tetracyclines or aminoglycosides, which lead to sensitization of various antibiotic-resistant strains of *S. aureus*, *E. coli*, *P. aeruginosa* (Fujita *et al.*, 2005; Eumkeb *et al.*, 2010; An *et al.*, 2011; Wang *et al.*, 2013; Mun *et al.*, 2015; Akilandeswari and Ruckmani, 2016).

PART II – PERSONAL CONTRIBUTIONS

CHAPTER 3 – ISOLATION OF ANTIBIOTIC-RESISTANT BACTERIA FROM WASTEWATER SAMPLES AND PATHOLOGICAL PRODUCTS

3.1. RESEARCH MATERIAL AND METHODS

3.1.1. Isolation of antibiotic-resistant bacterial strains

Isolation of antibiotic-resistant bacterial strains was performed on selective culture media, supplemented with antibiotics at concentrations that allow the growth of only antibiotic-resistant bacteria (Esiobu *et al.*, 2002).

3.1.2. Description of macro-morphological characters of isolated strains

Isolated strains were described macroscopically considering colony appearance, shape, margin, profile, adherence and colony colour (Dunca *et al.*, 2004).

3.1.3. Microscopic examination of Gram-stained smears

The micro-morphological description of the isolated strains was carried out on Gram-stained smears, observing the shape of the bacterial cells, their grouping, their dye affinity, as well as the presence or absence of spores under an optical microscope (Dunca *et al.*, 2004).

3.1.4. Taxonomic identification using MALDI-TOF mass spectrometry

The taxonomic identification of 79 resistant bacterial strains was carried out at the Charles Viollette Institute, University of Lille, France, as part of an Erasmus+ traineeship.

3.1.5. Determination of the minimum inhibitory concentration using the 2-fold dilution method

The MIC was determined using 2-fold dilutions in sterile microtiter plates. Resazurin was used to assess MIC (Sarker *et al.*, 2007).

3.1.6. Identification of antibiotic resistance genes

To identify antibiotic resistance genes, present in wastewater samples, an important step was to isolate metagenomic DNA and assess its quality. Subsequently, the extracted and

quantified DNA was sequenced using the services of Macrogen Europe BV (Amsterdam, Netherlands).

3.1.7. Bioinformatic analysis of genome sequences

The bioinformatic analysis of genomic sequences was performed using the HOME-BIO system (*sHOtgun MEtagenomic analysis of BIOlogical entities*).

3.2. RESULTS AND DISCUSSIONS

3.2.1. Macro- and micro-morphological characterization of strains isolated from the sewage samples

Following water sampling and inoculation on selective culture media, 190 resistant bacterial strains were isolated. In terms of macro-morphological appearance, the majority were S-type colonies, which presented a round/filamentous shape with regular (rarely wavy) edges, viscous consistency, variable colour, profile and size. Microscopic analysis revealed that the isolated bacteria were presented in the form of cocci, bacilli and coccobacilli. It was observed that bacterial cells were most often found either isolated or grouped in diplo-, less often in short chains or clusters. With few exceptions, most strains were non-sporulated.

3.2.2. Taxonomic identification of isolated bacterial strains

Of the total of 79 strains proposed for identification, 53 bacteria could be determined to the species level. Of these, *Aeromonas caviae* predominated among the identified isolates, followed by *E. coli*, *P. aeruginosa*, *Roultella ornithinolytica* and *Klebsiella oxytoca* - Figure 3.1.

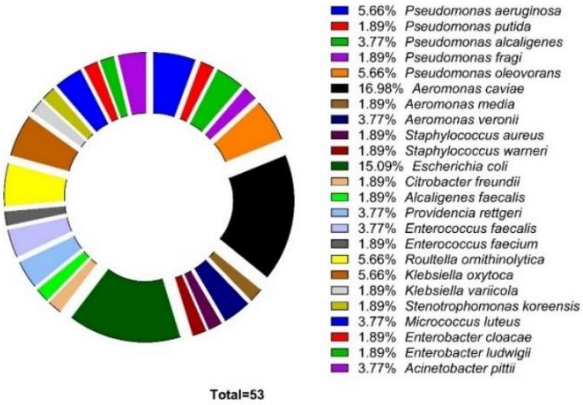


Figure 3.1. Taxonomic identification of the tested bacterial strains

3.2.3. Analysis of the taxonomic profile of the bacterial strains identified in the influent of Iasi WWTP

In the samples tested, the predominant domain was Bacteria (>98%). Of the 20 observed phyla, the majority were Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria and Verrucomicrobia - Figure 3.2.

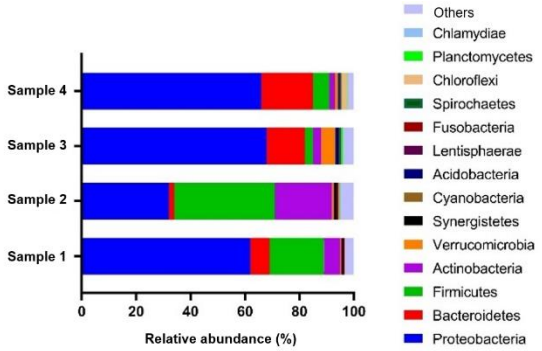


Figure 3.2. Percent relative abundance of dominant phyla identified in the influent of Iasi municipal WWTP. The relative abundance represents the no. of reads assigned to each bacterial taxon in relation to the total no. of reads assigned to the Bacteria domain.

The top 20 families with relative abundance equal to or greater than 1% are shown in Figure 3.3. Among these, the *Comamonadaceae* and *Pseudomonadaceae* families are the most

abundant. The *Caulobacteraceae*, *Flavobacteriaceae*, *Microbacteriaceae*, *Campylobacteraceae*, *Carnobacteriaceae*, *Bifidobacteriaceae* and *Eubacteriaceae* families were also well represented.

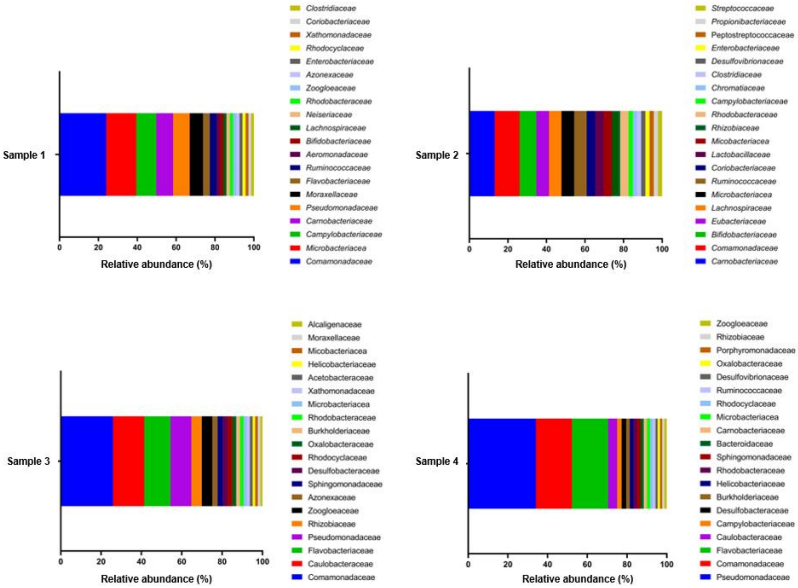


Figure 3.3. Bacterial families identified in the influent of Iasi municipal WWTP, with abundance ≥ 1%. The relative abundance represents the no. of reads assigned to each bacterial taxon in relation to the total no. of reads assigned to these families.

The priority pathogenic species identified in the present study (according to the World Health Organization report, 2024) presented a different frequency. For the analysed samples, the highest percentage was observed for *P. aeruginosa*, *E. coli*, *K. pneumoniae* and *A. baumannii* - Figure 3.4.

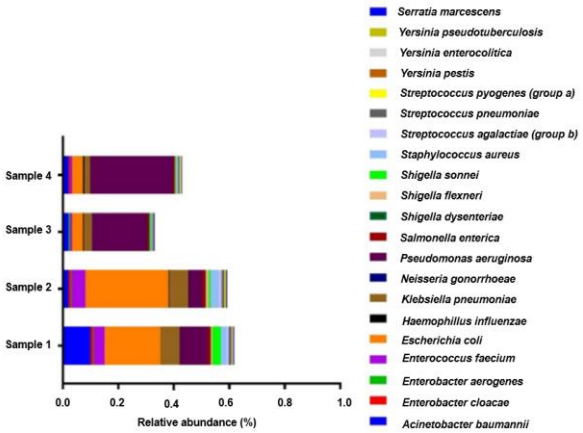


Figure 3.4. Presence of priority pathogenic bacteria (according to WHO, 2024) in the analysed water samples. The relative abundance represents the no. of reads assigned to each bacterial taxon in relation to the total no. of reads assigned to the Bacteria domain.

3.2.4. Assessment of antibiotic resistance levels

A. Assessment of the prevalence of resistance to certain antibiotics used for bacterial isolation

Based on the obtained data, we can state that the most numerous were the bacterial strains isolated on culture medium supplemented with tetracycline (51/190), chloramphenicol (41/190) and ampicillin (36/190), broad-spectrum antibiotics frequently administered in different sectors of human activity (Romero-Soto *et al.*, 2018; Popescu *et al.*, 2022).

B. Determination of minimum inhibitory concentrations for ampicillin, chloramphenicol and tetracycline

The MIC values varied considerably, depending on both the tested strains and the antibiotic used for isolation. The highest MIC values were noted for ampicillin, followed by chloramphenicol and gentamicin. Increased levels of resistance were detected for *E. coli* strains (ampicillin/chloramphenicol – 512 µg mL^{-1}, tetracycline – 32 µg mL^{-1}), medically important bacteria found in wastewater – Table 3.1.

Table 3.1. Minimum inhibitory concentration values of antibiotics used for resistant bacteria isolation from wastewater samples

Bacterial strain	Tested compound	Minimum inhibitory concentration (μg mL^{-1})
Cultivation temperature of 28 °C		
Raoultella ornithinolytica A_2 (3)	ampicillin	2048
Acinetobacter pittii Cl_2 (3)	chloramphenicol	256
Cultivation temperature of 37 °C		
Escherichia coli A_3 (2)	ampicillin	512
Escherichia coli A_5 (2)	ampicillin	64
Escherichia coli Cl_1 (3)	chloramphenicol	128
Escherichia coli T_2 (3)	tetracycline	32
Aeromonas caviae A_4 (2)	ampicillin	2048
Micrococcus luteus Cl_6 (3)	chloramphenicol	≤ 32

3.3.5. Highlighting the presence of resistance genes in wastewater samples

In all four water samples, a high proportion of genes conferring resistance to different types of antibiotics was observed (Figure 3.5), the most common being resistance genes for aminoglycosides, carbapenems + cephalosporins + penams (β-lactam antibiotics) and tetracyclines, and in a lower proportion for macrolides and phenicols. For other categories of genes (genes conferring resistance to glycopeptides, monobactams, sulphonamides etc.) low abundances were recorded. Most bacterial strains isolated from the wastewater samples showed resistance to ampicillin (β-lactam antibiotic), tetracyclines, chloramphenicol (phenicols), erythromycin (macrolides) and gentamicin (aminoglycosides), respectively to the same classes of antibiotics for which the highest relative abundances of resistance genes were highlighted.

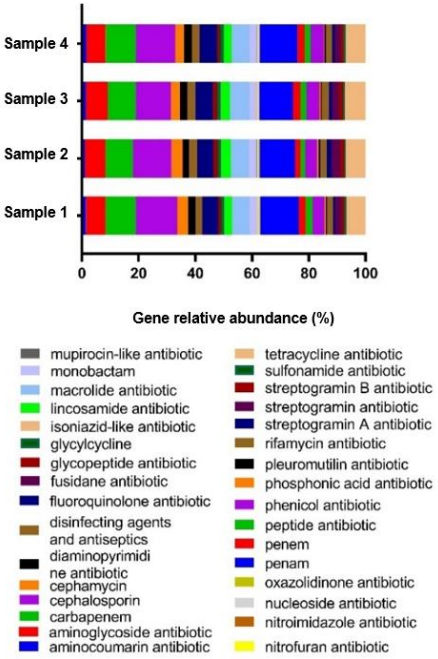


Figure 3.5. Proportional abundance of antibiotic resistance genes assigned to different drug classes. The relative abundance represents the no. of reads assigned to genes encoding resistance to a particular class of antibiotics in relation to the total no. of reads assigned to them.

In terms of the resistance mechanisms involved, the highest abundance was detected for genes encoding the synthesis of antibiotic degradation enzymes, efflux pumps and modification of the site of action, showing that these three mechanisms are the most common in the analysed wastewater samples - Figure 3.6.

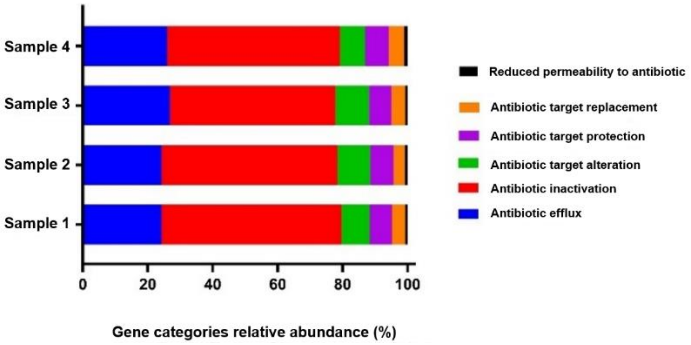


Figure 3.6. Proportional abundance of antibiotic resistance genes assigned to different resistance mechanisms. The relative abundance represents the no. of reads assigned to genes encoding antibiotic resistance through a particular mechanism in relation to the total no. of reads assigned to them.

CHAPTER 4. MECHANISMS INVOLVED IN ANTIBIOTIC RESISTANCE OF THE TESTED BACTERIAL STRAINS

4.1. RESEARCH MATERIAL AND METHODS

4.1.1. Assessment of the ability of resistant bacteria to adhere to different substrates

The ability of the tested strains to adhere to an abiotic support was assessed by quantification of the biofilm biomass formed in 96-well plates using crystal violet dye (Stepanović *et al.*, 2000). The ability of the bacterial strains to adhere to a biotic support was assessed by microscopic analysis of the attachment of prokaryotic cells (stained with Giemsa solution) to the surface of HeLa ECACC 93021013 epithelial cells (Kim and Lee, 2017).

4.1.2. Assessment of the production of antibiotic-degrading enzymes

A. Highlighting the secretion of extended-spectrum β-lactamases

The culture media used to evaluate the presence of extended-spectrum β-lactamases were supplemented with a mixture of chromogenic compounds that determine the different colouring of the developed bacterial colonies, depending on the secretory capacity of different enzymes.

B. Assessment of the presence of β-lactamases using the double-disk synergy test

To confirm the presence of the mentioned enzymes, antibiotic disks of recommended concentrations were used. These allows the phenotypic determination of extended-spectrum β-lactamases by observing synergism between third-generation cephalosporins and amoxacillin-clavulanic acid (Georgios *et al.*, 2014).

C. Determination of carbapenemase production

Carbapenemase production was determined by culturing the bacteria on special chromogenic media that inhibit the growth of non-carbapenem resistant strains.

D. Assessment of the presence of carbapenemases using the rsCDM assay

Determination and differentiation of carbapenemases produced by representatives of the *Enterobacterales* order were performed using imipenem or meropenem disks supplemented with three different β-lactamase inhibitors (Liao *et al.*, 2022).

4.1.3. Highlighting the presence of efflux pumps using ethidium bromide

Assessment of the presence of overexpressed efflux pumps was performed using ethidium bromide dye, a fluorogenic compound that penetrates the cells and binds to DNA molecules (Martins *et al.*, 2011).

4.2. RESULTS AND DISCUSSIONS

4.2.1. Assessment of biofilm-forming capacity

A. Assessment of adhesion capacity to an inert substrate

The strains isolated from the wastewater samples were tested for biofilm-forming capacity over a period of 24, 48 and 72 h. The obtained results showed that one selected strain was strongly adherent - *A. pittii* Cl_2 (3), while 2 strains - *E. coli* A_5 (2) and *E. coli* T_2 (3) were moderately, respectively weakly adherent - Table 4.1. An improvement of biofilm formation from 48 to 72 h was observed for *A. caviae* A_4 (2) and *M. luteus* Cl_6 (3) strains. For *E. coli* Cl_1 (3) strain a detachment of bacterial cells was observed at 72 h.

Table 4.1. Classification of bacterial strains isolated from wastewater samples according to their ability to form biofilms

Bacterial strain	Adherence		
	24 h	48 h	72 h
37 °C			
Escherichia coli A_3 (2)	+	++	++
Escherichia coli A_5 (2)	++	++	++
Escherichia coli T_2 (3)	+	+	+
Escherichia coli Cl_1 (3)	+	-	-
Aeromonas caviae A_4 (2)	++	++	+++
Micrococcus luteus Cl_6 (3)	-	-	+
28 °C			
Acinetobacter pittii Cl_2 (3)	+++	+++	+++
Roultella ornithinolytica A_2 (3)	++	+++	+++

„+++" – strong adherence, „++" – moderate adherence, „+" – weak adherence, „-" – non-adherence

Of the strains isolated from pathological products, *A. baumannii* medbio3-2013 strain showed the strongest adherence to the inert substrate that we used - Figure 4.1.

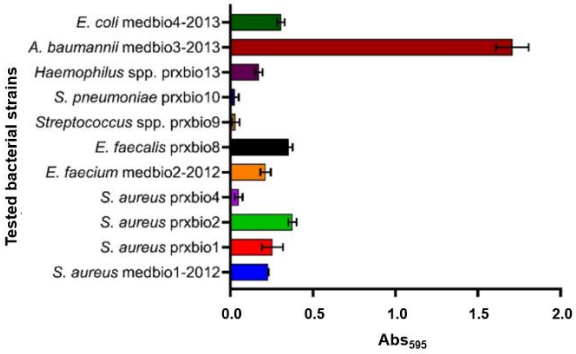


Figure 4.1. Quantification of biofilm biomass formed by clinically isolated bacterial strains. Bars represent the standard error of the mean.

B. Evaluation of the ability of the tested strains to attach to a cellular substrate

The results showed that *E. coli* A_5 (2) strain was able to attach to eukaryotic cells, the type of adhesion being aggregative (Figure 4.2). For *A. caviae* A_4 (2) and *E. coli* A_5 (2) cells, the type of adhesion was localized and aggregative, respectively.

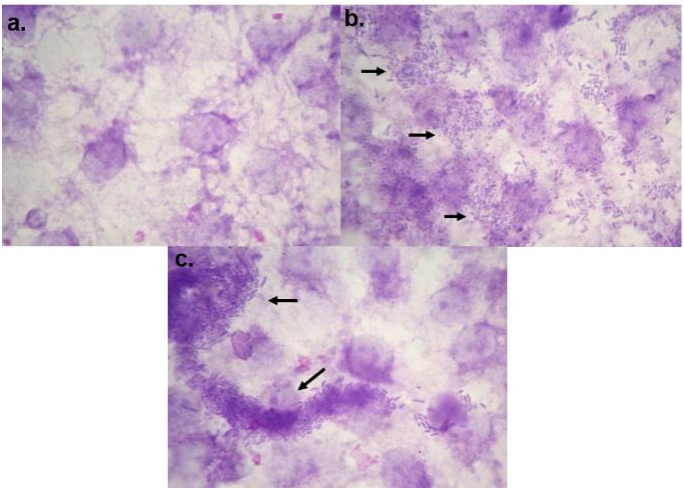


Figure. 4.2. Adherence pattern of selected resistant strains: a. non-adherent strain (*E. coli* A_3 (2), *E. coli* Cl_1 (3), *E. coli* T_2 (3)); b. adherent strain, localized-type adherence (*A. caviae* A_4 (2)); c. adherent strain, aggregative-type adherence (*E. coli* A_5 (2)). Arrows indicate the arrangement of bacterial cells to the cell monolayer used (magnification power 1000x).

4.2.2. Assessment of the presence of enzymes involved in antibiotic degradation

From the data presented in Table 4.2, of the tested strains, the majority were those producing extended-spectrum β-lactamases. Regarding the presence of carbapenemases, the results allowed to observe a much lower percentage of them at the evaluated strains (33%) compared to the production of ESBL (67%). MBL and KPL enzymes were observed only at *K. pneumoniae* medbio6-2013 strain.

Table 4.2. Assessment of the ability of selected strains to produce antibiotic-degrading enzymes

Bacterial strain	Type of enzymes		
	ESBL	Oxa_{48} / Porin modification	KPC + MBL
Escherichia coli medbio4-2013	+	-	-
Enterobacter cloacae medbio5-2013	-	-	-
Klebsiella pneumoniae medbio6-2013	-	-	+
Klebsiella pneumoniae prxbio11	-	-	-
Klebsiella pneumoniae prxbio12	+	-	-
Acinetobacter baumannii medbio3-2013	-	-	-
**Escherichia coli* A_3 (2)	+	-	-
**Escherichia coli* A_5 (2)	-	+	-
**Escherichia coli* Cl_1 (3)	+	-	-
**Escherichia coli* Cl_1 (2)	-	-	-

Bacterial strain	Type of enzymes		
	ESBL	Oxa$_{48}$ / Porin modification	KPC + MBL
**Escherichia coli* T_2 (3)	+	+	-
**Acinetobacter pittii* Cl_2 (3)	-	-	-
**Aeromonas caviae* A_4 (2)	+	-	-

„-“ – lack of enzymes that break down antibiotic molecules, „+“ – presence of enzymes that degrade antibiotic molecules, *bacterial strains isolated from wastewater samples

4.2.3. Efflux pumps identification

Following the cultivation of the selected strains on culture medium supplemented with ethidium bromide, the presence of fluorescence was revealed at a concentration of only 0.25 mg L^{-1}, the calculated efflux index suggesting low efflux pump activity of the selected compound - Figure 4.3.

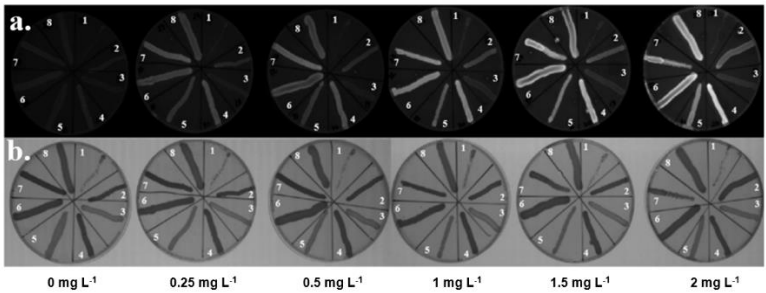


Figure 4.3. Highlighting the presence of efflux pumps at isolated bacterial strains: a. images obtained under UV light (1 - *Pseudomonas aeruginosa* ATCC 27853; 2-8 - tested bacterial strains - *Pseudomonas aeruginosa* medbio7-2013, *Acinetobacter pittii* Cl_2 (3), *Escherichia coli* A_3 (2), *Escherichia coli* A_5 (2), *Escherichia coli* T_2 (3), *Escherichia coli* Cl_1 (2), *Escherichia coli* Cl_1 (3)); b. image obtained without UV light to observe the development of bacterial strains.

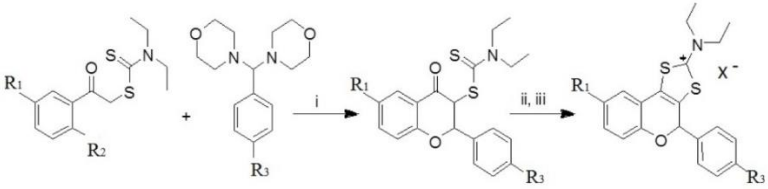
CHAPTER 5. EFFICACY OF SOME SYNTHETIC FLAVONOIDS AS ANTIMICROBIAL AGENTS

5.1. RESEARCH MATERIAL AND METHODS

5.1.1. Synthesis of tricyclic sulphur flavonoids

Tricyclic flavonoids with halogen substituents have been synthesized by the team led by Prof. PhD habil. Lucian Birsa, from the Faculty of Chemistry of the "Alexandru Ioan Cuza"

University of Iasi. The obtaining process took place in several steps (Figure 5.1). The structure and purity of the synthetic flavonoids (> 99%) were established by characteristic analyses, such as nuclear magnetic resonance, mass and infrared spectrometry, elemental analysis (Bahrin *et al.*, 2014).



i) EtOH, reflux 2 h; ii) H_2SO_4/AcOH, 80 °C; iii) X^- = ClO_4, BF_4

R_1	R_3
Br	F
	Br
	Cl
	I

Figure 5.1. General synthesis scheme of tricyclic flavonoids with halogen substituents (Bahrin *et al.*, 2014)

5.1.2. Determination of the minimum inhibitory concentration of synthetic tricyclic sulphur-containing flavonoids by the 2-fold dilution method

The MIC determination was performed according to section *3.1.5. Determination of the minimum inhibitory concentration using the 2-fold dilution method*.

5.1.3. Determination of the minimum bactericidal concentration

The MBC determination was carried out following the cultivation on solid media of bacterial cells that were exposed to different concentrations of the evaluated antibacterial agent.

5.1.4. Evolution of a bacterial population in an asynchronous batch culture

To evaluate the effect of synthetic flavonoids on the dynamics of bacterial multiplication, asynchronous batch cultures were performed.

5.1.5. Assessment of the viability of bacterial cells by colony counting on solid media

The method aims to assess the viability of bacterial cells treated with different antimicrobial compounds by counting colonies developed on a solid culture medium (Bag *et al.*, 2012).

5.2. RESULTS AND DISSCUSIONS

5.2.1. Antimicrobial activity of some synthetic tricyclic sulphur-containing flavonoids

The obtained results, presented in Table 5.1, indicate that the most important antibacterial activity was exhibited by BrCl flavonoid against most isolates from both the clinical environment and the influent of the Iasi municipal wastewater treatment plant. The MIC values for BrCl flavonoid varied for Gram-positive bacteria, with concentrations ranging from 0.24 to 31.25 μg mL^{-1}. The lowest MIC values recorded for Gram-negative bacteria were between 0.48 and 3.9 μg mL^{-1}.

The MIC values obtained for BrF flavonoid ranged from 15.62 to 62.5 μg mL^{-1} for Gram-positive bacteria and from 15.62 to 125 μg mL^{-1} for Gram-negative bacteria. For the other two synthetic flavonoids, the lowest MIC values recorded for Gram-positive bacteria were 0.97 μg mL^{-1} (BrBr flavonoid) and 0.48 μg mL^{-1} (BrI flavonoid). For Gram-negative bacteria, the MIC values determined ranged from 1.95 to 125 μg mL^{-1} for BrBr flavonoid and from 3.9 to 125 μg mL^{-1} for BrI flavonoid.

Table 5.1. Minimum inhibitory concentrations of synthetic flavonoids

Bacterial strains	Minimum inhibitory concentration (μg mL^{-1})					
	Flavonoids				DMSO	Reference antibiotic
	BrCl	BrF	BrBr	BrI		
Staphylococcus aureus medbio1-2012	0.48	15.62	0.97	0.48	> 125	7.8[c]
Staphylococcus aureus prxbio1	1.95	-	-	0.97	> 125	3.9[c]
Staphylococcus aureus prxbio2	7.81	-	-	-	250	7.8[c]
Staphylococcus aureus prxbio3	0.48	-	-	-	250	1.9[c]
Staphylococcus aureus prxbio4	0.24	-	-	-	250	3.9[c]
Staphylococcus aureus prxbio5	0.24	-	-	-	250	7.8[c]
Staphylococcus aureus prxbio6	7.81	-	-	-	250	7.8[c]

Bacterial strains	Minimum inhibitory concentration (μg mL^{-1})					
	Flavonoids				DMSO	Reference antibiotic
	BrCl	BrF	BrBr	BrI		
Staphylococcus aureus prxbio7	0.48	-	-	-	250	7.8[c]
Streptococcus spp. prxbio9	3.9	-	-	-	250	< 0.9[c]
Streptococcus pneumoniae prxbio10	0.48	-	-	-	250	3.9[c]
Enterococcus faecium medbio2-2012	7.81	62.5	31.25	62.5	> 125	< 0.9[c]
Enterococcus faecalis prxbio8	31.25	-	-	-	125	7.8[c]
Acinetobacter baumannii medbio3-2013	3.9	15.62	7.81	3.9	> 125	< 0.9[g]
Acinetobacter pittii Cl_2 (3)	15.62	125	7.81	31.25	125	-
Escherichia coli medbio4-2013	3.9	125	31.25	7.81	> 125	< 0.9[g]
Escherichia coli A_3 (2)	125	125	125	125	125	-
Escherichia. coli A_5 (2)	125	125	62.5	125	125	-
Escherichia coli T_2 (3)	62.5	125	62.5	62.5	125	-
Enterobacter cloacae medbio5-2013	125	-	-	125	125	< 0.9[g]
Klebsiella pneumoniae medbio6-2013	125	125	15.62	15.62	> 125	> 125[g]
Klebsiella pneumoniae prxbio11	125	-	-	-	125	< 0.9[g]
Klebsiella pneumoniae prxbio12	125	-	-	-	125	< 0.9[g]
Pseudomonas aeruginosa medbio7-2013	31.25	-	-	62.5	> 125	< 0.9[g]
Salmonella enterica medbio8-2013	125	-	-	62.5	> 125	< 0.9[g]
Haemophillus spp. prxbio13	0.48	-	-	-	250	0.9[g]
Staphylococcus aureus ATCC 43300	3.9	-	-	-	125	7.8[c]
Klebsiella pneumoniae ATCC BAA-1705	125	-	-	-	250	3.9[g]

[c]= chloramphenicol; [g]= gentamycin, „ - ”= absence of minimum inhibitory concentration testing

Concerning the minimum bactericidal concentrations presented in Table 5.2, the lowest values were recorded for Gram-positive bacteria for all synthetic flavonoids. For Gram-negative bacteria, the lowest MBC values ranged from 0.48 to 62.5 μg mL^{-1}- BrI and BrF flavonoids.

Table 5.2. Minimum bactericidal concentrations of synthetic flavonoids

Bacterial strains	Minimum bactericidal concentration (μg mL^{-1})				
	Flavonoids				Reference antibiotic
	BrCl	BrF	BrBr	BrI	
Staphylococcus aureus medbio1-2012	1.95	15.62	0.97	0.48	31.25[c]
Staphylococcus aureus prxbio1	7.81	-	-	0.97	125[c]
Staphylococcus aureus prxbio2	15.62	-	-	-	125[c]
Staphylococcus aureus prxbio3	0.97	-	-	-	62.5[c]
Staphylococcus aureus prxbio4	0.97	-	-	-	15.62[c]
Staphylococcus aureus prxbio5	0.48	-	-	-	62.5[c]
Staphylococcus aureus prxbio6	250	-	-	-	125[c]
Staphylococcus aureus prxbio7	0.97	-	-	-	62.5[c]
Streptococcus spp. prxbio9	7.81	-	-	-	> 125[c]
Streptococcus pneumoniae prxbio10	0.97	-	-	-	31.25[c]
Enterococcus faecium medbio2-2012	31.25	> 250	62.5	> 250	15.62[c]
Enterococcus faecalis prxbio8	62.5	-	-	-	> 125[c]
Acinetobacter baumannii medbio3-2013	15.62	125	31.25	125	< 0.9[g]
Acinetobacter pittii Cl_2 (3)	15.62	125	31.25	125	-
Escherichia coli medbio4-2013	15.62	250	125	62.5	< 0.9[g]
Escherichia coli A_3 (2)	125	125	125	125	-
Escherichia. coli A_5 (2)	125	125	125	125	-
Escherichia coli T_2 (3)	125	125	125	125	-
Enterobacter cloacae medbio5-2013	125	-	-	> 250	< 0.9[g]
Klebsiella pneumoniae medbio6-2013	125	250	62.5	125	> 125[g]
Klebsiella pneumoniae prxbio11	125	-	-	-	< 0.9[g]
Klebsiella pneumoniae prxbio12	125	-	-	-	< 0.9[g]
Pseudomonas aeruginosa medbio7-2013	31.25	-	-	250	< 0.9[g]
Salmonella enterica medbio8-2013	125	-	-	125	< 0.9[g]
Haemophillus spp. prxbio13	0.48	-	-	-	125[g]
Staphylococcus aureus ATCC 43300	3.9	-	-	-	62.5[c]
Klebsiella pneumoniae ATCC BAA-1705	125	-	-	-	15.62[g]

[c]= chloramphenicol; [g]= gentamycin, „-"= absence of minimum bactericidal concentration testing

5.2.2. Effect of synthetic sulphur-containing flavonoids on the growth of antibiotic-resistant bacterial strains

BrCl, BrBr and BrI flavonoids determined a dose- and time-dependent bacteriostatic effect for all isolated bacterial strains. For *S. aureus* medbio1-2012 strain, BrCl flavonoid produced a growth delay up to 6 h at concentrations equivalent to MIC and 2 × MIC - Figure 5.1 - a. BrBr and BrI flavonoids produced a bacteriostatic effect up to 21 h at a concentration

equivalent to 2 × MIC - Figure 5.1 - b, c, while a concentration equivalent to MIC determined a growth inhibition of up to 9 h.

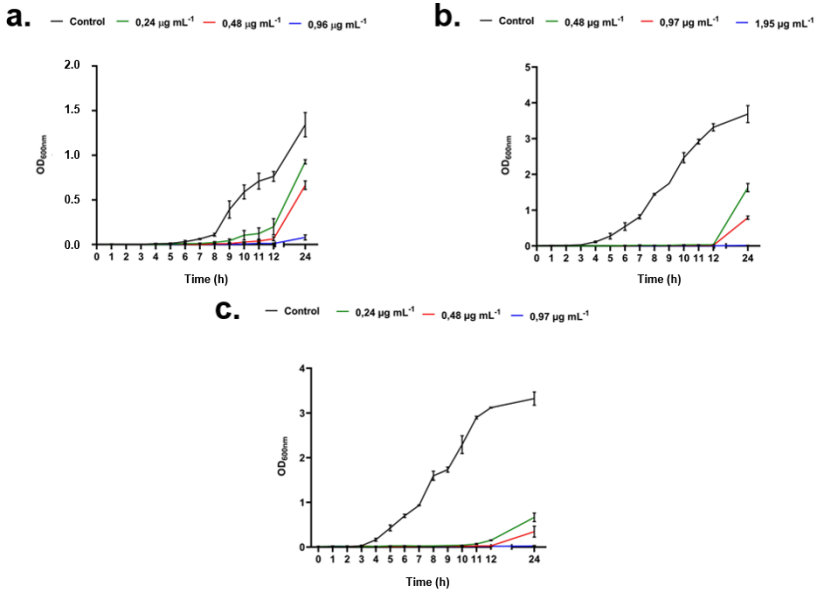


Figure 5.1. Growth dynamics of *S. aureus* medbio1-2012 strain in the presence of synthetic flavonoids: a. BrCl (MIC = 0.48 µg mL^{-1}); b. BrBr (MIC = 0.97 µg mL^{-1}); c. BrI (MIC = 0.48 µg mL^{-1}). Bars represent the standard error of the mean.

In the case of the *S. aureus* prxbio1 strain, BrCl flavonoid used in a concentration equivalent to MIC and 2 × MIC, determined a bacteriostatic effect for more than 20 h (Figure 5.2 - a). Tested against the *E. faecium* medbio2-2012 strain, the same flavonoid in concentrations equivalent to MIC and 2 × MIC, led to a prolongation of the lag phase of up to 4 h compared to the control, while a concentration equivalent to ½ MIC produced a growth inhibition of up to 1 h - Figure 5. 2 - b. For the *A. baumannii* medbio3-2013 strain, BrCl flavonoid determined the appearance of an inhibitory effect of up to 8 and 9 h, respectively, in the presence of concentrations equivalent to MIC ($p = 0.0009$) and 2 × MIC ($p = 0.008$) - Figure 5.2 - c.

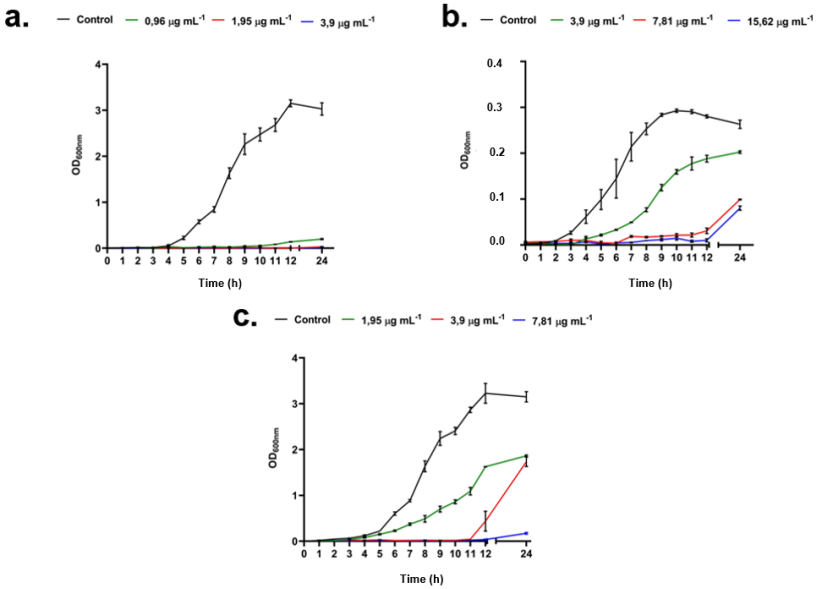


Figure 5.2. Bacterial growth dynamics in the presence of BrCl flavonoid: a. *S. aureus* prxbio1 (MIC = 1.95 µg mL^{-1}); b. *E. faecium* medbio2-2012 (MIC = 7.81 µg mL^{-1}); c. *A. baumannii* medbio3-2013 (MIC = 3.9 µg mL^{-1}). Bars represent the standard error of the mean.

5.2.3. Assessment of the effect produced by synthetic tricyclic sulphur-containing flavonoids on bacterial cell viability

A total kill effect was recorded for *S. aureus* medbio1-2012 (Figure 5. 3 - a) and *S. aureus* prxbio1 (Figure 5.3 - b) strains, after 3 hours of incubation in the presence of BrCl flavonoid at concentrations of 1.95 and 7.81 µg mL^{-1}, respectively after only one hour of incubation of *E. faecium* medbio2-2012 cells in the presence of BrCl flavonoid at a concentration of 31.25 µg mL^{-1} (Figure 5.3 - c).

Exposure of *A. baumannii* medbio3-2013 cells to BrCl flavonoid at a concentration equivalent to MBC (15.62 µg mL^{-1}) caused a significant loss of cell viability ($p < 0.0001$) after half an hour compared to control cells (Figure 5.4-a).

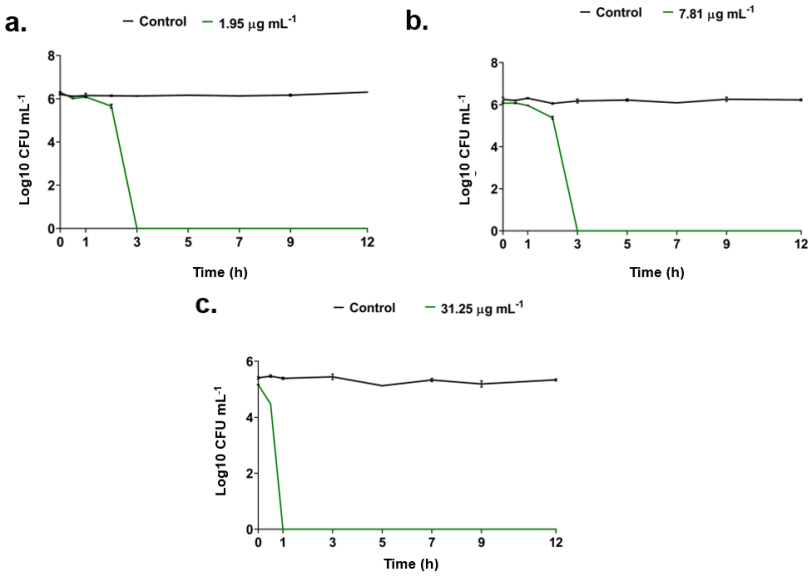


Figure 5.3. BrCl flavonoid affects cell viability of: a. *S. aureus* medbio1-2012 (MBC = 1.95 µg mL^{-1}); b. *S. aureus* prxbio1 (MBC = 7.81 µg mL^{-1}); c. *E. faecium* medbio2-2012 (MBC = 31.25 µg mL^{-1}). Bars represent the standard error of the mean.

In the case of *E. coli* medbio4-2013 cells, a pronounced bactericidal effect was recorded after only 2 hours of incubation. This effect was maintained for more than 24 h (Figure 5.4 - b).

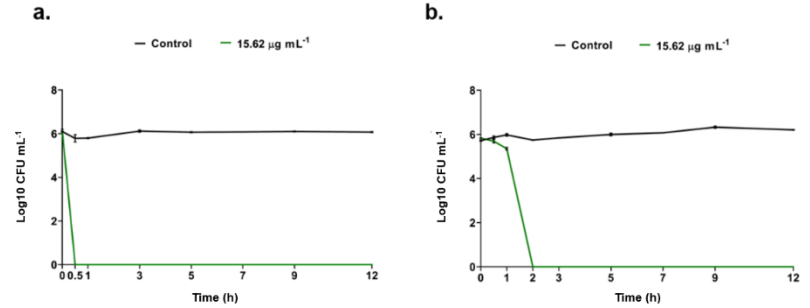


Figure 5.4. Assessment of cell viability in the presence of BrCl flavonoid: a. *A. baumannii* medbio3-2013 (MBC = 15.62 µg mL^{-1}) and b. *E. coli* medbio4-2013 (MBC = 15.62 µg mL^{-1}). Bars represent the standard error of the mean.

CHAPTER 6. BrCl FLAVONOID MODE OF ACTION

6.1. RESEARCH MATERIAL AND METHODS

6.1.1. Assessment of bacterial cell membrane permeability using fluorescence microscopy

Intact membranes of bacterial cells are impermeable to fluorochromes such ethidium bromide and propidium iodide, permeabilization being possible only when the membranes are damaged or destroyed. In the presence of these dyes, cells whose membranes are damaged or show changes in permeability allows fluorochromes to penetrate and attach to the nucleic acids, which causes the appearance of red fluorescence (Lambert *et al.*, 2001).

6.1.2. Assessment of bacterial cell integrity using scanning electron microscopy

Scanning electron microscopy was used to observe the morphology of bacterial cells treated with BrCl flavonoid.

6.1.3. Evaluation of BrCl flavonoid activity against bacterial cells adhesion

The method involves assessing the effect of BrCl flavonoid on biofilm cell adhesion by quantifying the biofilm biomass using crystal violet dye (Sandasi *et al.*, 2010).

6.1.4. Quantification of bacterial biofilm biomass formed in the presence of BrCl flavonoid

The method involves assessing the effect produced by BrCl flavonoid against biofilm formation by quantifying the biofilm biomass using crystal violet dye. The effect of BrCl flavonoid on the morphology of biofilm cells was assessed using scanning electron microscopy (Sandasi *et al.*, 2010).

6.1.5. Assessment of metabolic activity of biofilm-forming cells

Determination of the metabolic activity of biofilm-forming cells was performed using tetrazolium salt (MTT) (Li *et al.*, 2023).

6.1.6. Evaluation of BrCl flavonoid effect against mature biofilms

The effect of BrCl flavonoid against mature biofilms was determined using crystal violet staining (Sandasi *et al.*, 2010).

6.1.7. Assessment of BrCl flavonoid effect on hydrophobicity of *Acinetobacter* spp. cells

The effect of BrCl flavonoid on bacterial cell hydrophobicity was carried out using *p*-xylene as hydrocarbon (Zoueki *et al.*, 2010).

6.1.8. Effect of BrCl flavonoid on EPS production

The total carbohydrate content produced in the presence of BrCl flavonoid was determined using phenol-sulphuric acid method (Nielsen, 2017).

6.1.9. Assessment of BrCl flavonoid effect on bacterial cell motility

The effect of BrCl flavonoid on swarming and swimming motility was assessed by measuring the diameter of the migration distance of bacterial cells on solid medium. The effect of BrCl flavonoid on twitching motility was assessed by measuring the area coloured with crystal violet.

6.2. RESULTS AND DISCUSSIONS

6.2.1. The effect of BrCl flavonoid on bacterial cell membrane integrity

Incubation of *S. aureus* (Figure 6.1 - a) and *E. coli* (Figure 6.1 - b) cells in the presence of BrCl flavonoid at concentrations equivalent to MBC (1.95 µg mL^{-1} and 15.62 µg mL^{-1}, respectively), led to recording of a percentage between 75 and 90% fluorescent cells after only 30 min of exposure, while after 150 min, the percentage of fluorescent cells was 100%.

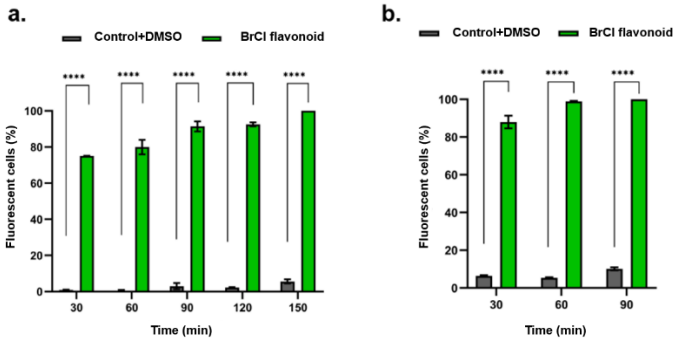


Figure 6.1. Effect of BrCl flavonoid on cell membranes: a. *S. aureus* medbio1-2012 (MBC = 1.95 µg mL^{-1}); b. *E. coli* medbio4-2013 (MBC = 15.62 µg mL^{-1}) ($p < 0.05$; **** = $p < 0.0001$). Bars represent standard error of the mean.

Regarding the fluorescence dynamics, the results revealed the appearance of the first red fluorescent cells after only 2 and 3 minutes of exposing *S. aureus* and *E. coli* strains, respectively, to BrCl flavonoid - Figure 6.2. The number of these cells became almost 100% after 10 minutes of exposure.

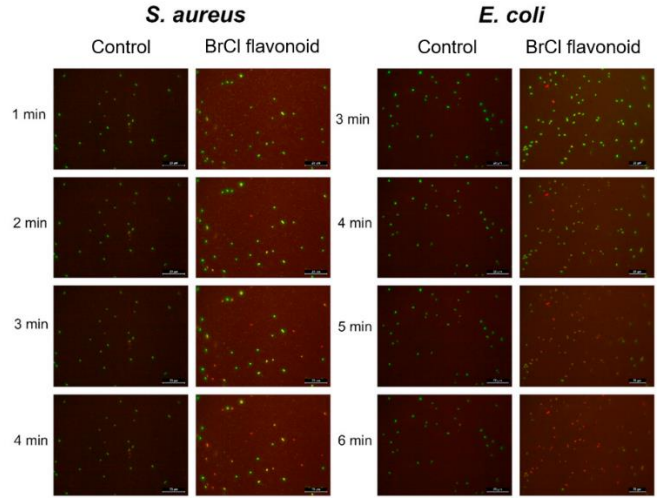


Figure 6.2. Membrane permeabilization dynamics of *S. aureus* medbio1-2012 (MBC = 1.95 µg mL^{-1}) and *E. coli* medbio4-2013 (MBC = 15.62 µg mL^{-1}) cells in the presence of BrCl flavonoid: red fluorescence - cells with damaged membrane; green fluorescence - live cells with intact membrane. For technical reasons, for *E. coli* medbio4-2013 strain it was impossible to obtain relevant images before 3 min.

6.2.2. BrCl flavonoid induce significant changes in *S. aureus* and *E. coli* cell morphology

SEM analysis showed that BrCl flavonoid, at concentrations equivalent to MBC, induces major morphological changes after 6 hours of exposure (cells with deformed, perforated and wrinkled surface) compared to control (intact cells with regular shape and smooth surface) - Figure 6. 3. The irreversible morphological changes revealed by scanning electron microscopy support the hypothesis by which BrCl flavonoid targets cell membranes, inducing changes in their structure, followed by cell lysis, the indicated mechanism of action being probably a primary membrane-type.

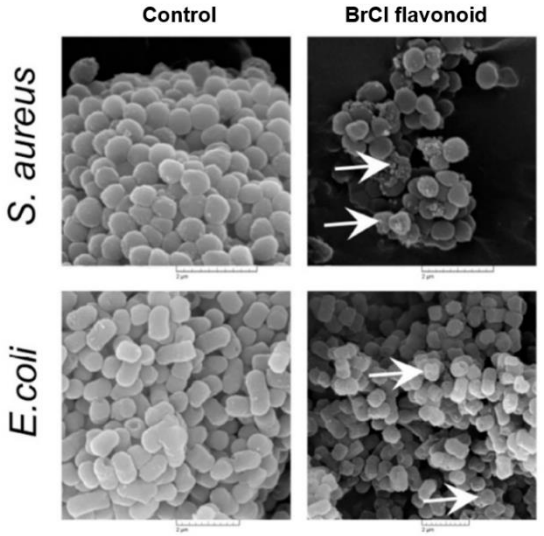


Figure 6.3. The effects of BrCl flavonoid on the morphology of *S. aureus* medbio1-2012 and *E. coli* medbio4-2013 cells exposed to concentrations equivalent with MBC. White arrows indicate morphological damages and cellular debris.

6.2.3. Adhesion of *Acinetobacter* spp. cells is influenced by BrCl flavonoid

The experiments showed that for both *A. baumannii* medbio3-2013 (Figure 6.4 - a) and *A. pittii* Cl_2 (3) (Figure 6.4 - b), the minimum time required for cell adhesion were 15 minutes.

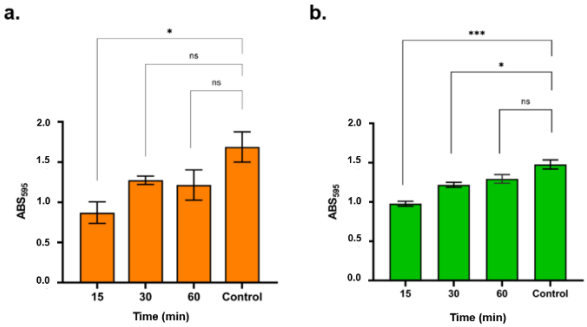


Figure 6.4. Determination of the minimum time required for bacterial cell to adhesion in the biofilm forming process: a. *A. baumannii* medbio3-2013 and b. *A. pittii* Cl_2 (3). Control cells were incubated for 24 h without changing the culture medium. Asterix represent significant difference between sample and control ($p < 0.05$; *** - $p = 0.0008$; * - $p > 0.0039$; ns - insignificant differences). Bars represent standard error of the mean.

A. baumannii medbio3-2013 cell adhesion was significantly inhibited at only three of the tested flavonoid concentration, compared to DMSO-supplemented control (Figure 6.5 - a). At concentrations equivalent to MIC (3.9 µg mL^{-1}) and ½ MIC (1.95 µg mL^{-1}), an inhibition of up to 94% was observed (Figure 6.5 - b).

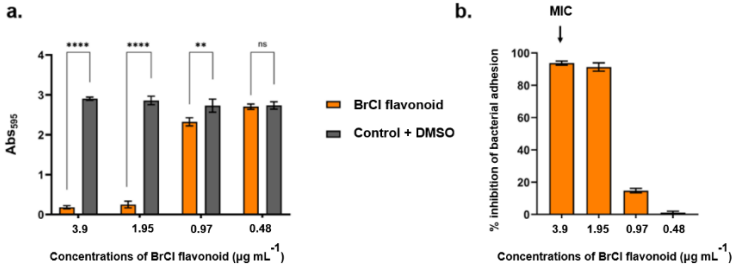


Figure 6.5. Inhibitory effect of BrCl flavonoid on *A. baumannii* medbio3-2013 cell adhesion: a. quantification of bacterial biofilm biomass using crystal violet staining (MIC = 3.9 µg mL^{-1}); b. percentage of adhesion inhibition. After 15 min of incubation, non-adhered cells were removed from the wells by washing. Biofilm biomass was determined after 24 h of incubation in MHB medium ($p < 0.05$; **** - $p < 0.0001$; ** - $p = 0.0021$; ns - non-significant differences). Bars represent standard error of the mean.

A dose-dependent anti-adhesion activity was also observed for *A. pittii* Cl_2 (3) strain (Figure 6.6-a). BrCl flavonoid, administered at a concentration of 15.62 µg mL^{-1} (equivalent to

MIC value), induced a significant inhibition of bacterial cell adhesion, the inhibition percentage recorded being approximately 78% (Figure 6.6 - b).

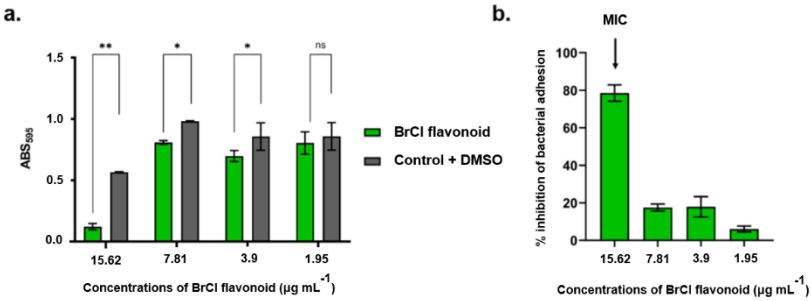


Figure 6.6. Inhibitory effect of BrCl flavonoid on *A. pittii* Cl_2 (3) cell adhesion: a. quantification of bacterial biofilm biomass by crystal violet staining; b. percentage of adhesion inhibition (MIC = 15.62 µg mL^{-1}). After 30 min of incubation, non-adhered cells were removed from the wells by washing. Biofilm biomass was determined after 24 h of incubation in MHB medium ($p < 0.05$; ** - $p = 0.0012$; * - $p > 0.0197$; ns - non-significant differences). Bars represent standard error of the mean.

6.2.4. Influence of BrCl flavonoid on the inhibition of biofilm produced by *Acinetobacter* species

BrCl flavonoid concentrations that led to a significant inhibitory effect ($p < 0.0001$) against *A. baumannii* medbio3-2013 biofilm ranged from 3.9 to 62.5 µg mL^{-1} (Figure 6.7 - a). A concentration equivalent to ½ MIC produced an inhibition of the bacterial biofilm at a percentage of only 30%, while for the other concentrations used, including MIC, the percentage of biofilm inhibition was greater than 90% (Figure 6.7 - b).

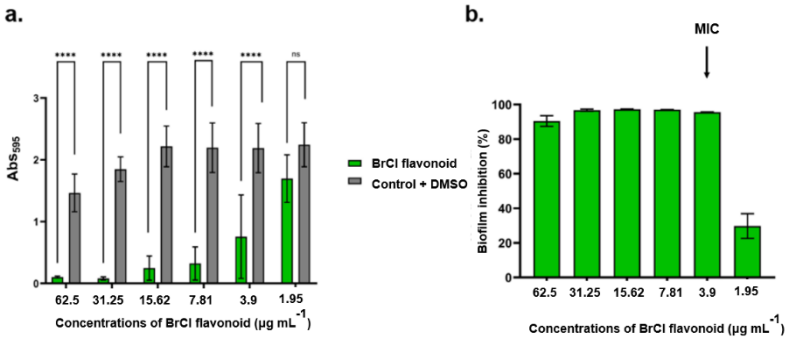


Figure 6.7. Antibiofilm activity of BrCl flavonoid on *A. baumannii* medbio3-2013 strain: a. quantification of bacterial biofilm biomass by crystal violet staining; b. percentage of biofilm inhibition (MIC = 3.9 µg mL^{-1}) ($p < 0.05$; **** - $p < 0.0001$; ns - insignificant differences). Bars represent standard error of the mean.

A much more pronounced inhibitory activity of synthetic flavonoid (Figure 6.8 - a) was observed for the second bacterial strain. In this case, as shown in Figure 6.8 - b, at sub-inhibitory concentrations of BrCl flavonoid, ranging from 7.81 to 1.95 µg mL^{-1}, a percentage of biofilm inhibition of up to 88% was observed.

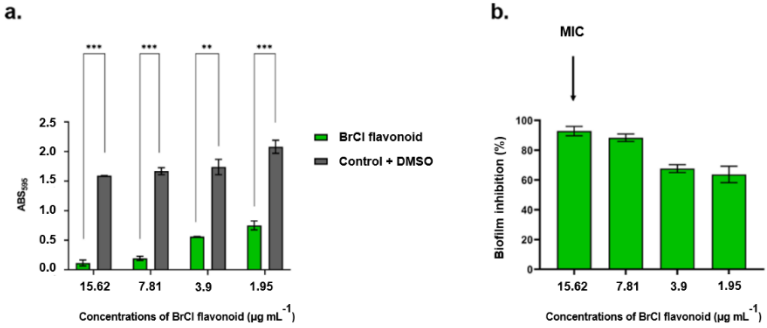


Figure 6.8. Antibiofilm activity of BrCl flavonoid on *A. pittii* Cl_2 (3) strain: a. quantification of bacterial biofilm biomass using crystal violet staining; b. percentage of biofilm inhibition (MIC = 15.62 µg mL^{-1}) ($p < 0.05$; ** - $p = 0.001$; *** - $p < 0.0005$). Bars represent standard error of the mean.

At sub-inhibitory concentrations, BrCl flavonoid exhibited an inhibitory effect up to 40% more pronounced compared to ciprofloxacin. The most significant effects produced by ciprofloxacin were observed at concentrations equivalent to MIC and ½ MIC (Figure 6.9 - a). In this case, bacterial biofilm formation was inhibited by up to 94% compared to control. For

concentrations equivalent to ¼ MIC and ⅛ MIC, the biofilm formed by *A. pittii* Cl_2 (3) strain was inhibited by up to 33% (Figure 6.9 - b).

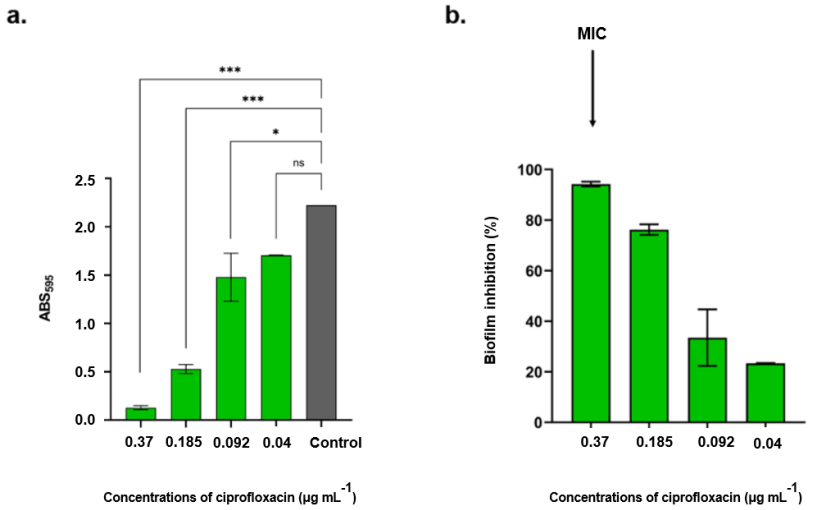


Figure 6.9. Antibiofilm activity of ciprofloxacin on *A. pittii* Cl_2 (3) strain: a. quantification of bacterial biofilm biomass using crystal violet staining; b. percentage of biofilm inhibition (MIC = 0.37 µg mL^{-1}) ($p < 0.05$; *** - $p < 0.0002$; * - $p = 0.016$; ns - insignificant differences). Bars represent standard error of the mean.

Using scanning electron microscopy, it was observed that BrCl flavonoid, at a concentration equivalent to ½ MIC (7.81 µg mL^{-1}), led to a significant decrease in the number of cells in the bacterial biofilm produced by *A. pittii* Cl_2 (3) strain compared to control (culture medium supplemented with DMSO) - Figure 6.10.

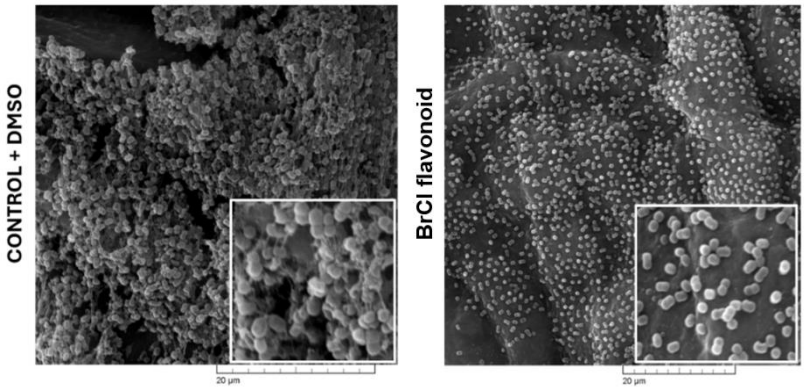


Figure 6.10. Electron microscopic images showing the effect of BrCl flavonoid on the biofilm produced by *A. pittii* Cl_2 (3) strain. Bacterial biofilms were formed for 48 h in the presence of of BrCl flavonoid at a concentration equivalent to ½ MIC (7.81 µg mL^{-1}).

6.2.5. BrCl flavonoid reduce the metabolic activity of *A. pittii* Cl_2 (3) biofilm-forming cells

Metabolic activity of bacterial cells was reduced by approximately 95% at concentrations of BrCl flavonoid equivalent to MIC and ½ MIC (15.62 and 7.81 µg mL^{-1}) compared to DMSO-supplemented control (Figure 6.11-a). At a concentration of 3.9 µg mL^{-1}, the metabolic activity of the cells was reduced by up to 28% (Figure 6.11-b).

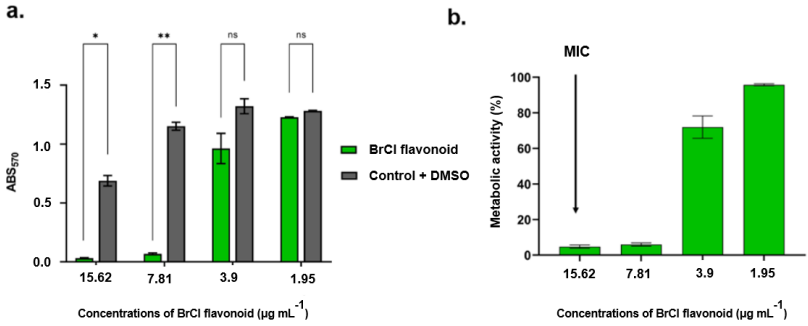


Figure 6.11. Metabolic activity of *A. pittii* Cl_2 (3) cells after 24 h of incubation in the presence of BrCl flavonoid: a. spectrophotometric quantification of formazan; b. percentage inhibition of metabolic activity (MIC = 15.62 µg mL^{-1}) ($p < 0.05$; ** - $p = 0.0059$; * - $p = 0.021$; ns - insignificant differences). Bars represent standard error of the mean.

A similar effect was observed for ciprofloxacin (Figure 6.12-a). This time, for all the concentrations that we used (MIC, ½, ¼ and ⅛ MIC) a significant reduction in the metabolic activity of the cells was recorded ($p < 0.05$).

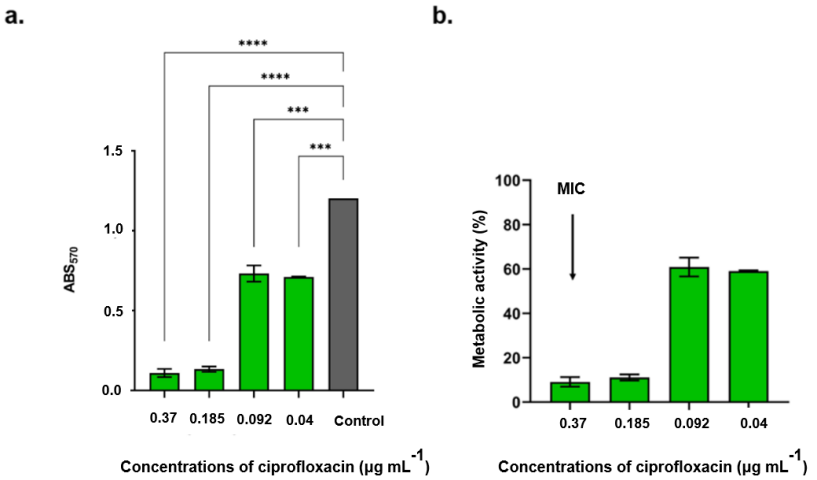


Figure 6.12. Metabolic activity of *A. pittii* Cl_2 (3) cells after 24 h of incubation in the presence of ciprofloxacin (MIC = 0.37 µg mL^{-1}): a. spectrophotometric quantification of formazan; b. percentage inhibition of metabolic activity ($p < 0,05$; *** - $p = 0,0002$; **** - $p < 0,0001$). Control cells were incubated for 24 h in the absence of ciprofloxacin. Bars represent standard error of the mean.

6.2.6. BrCl flavonoid disrupt *A. baumannii* and *A. pittii* mature biofilms

BrCl flavonoid disrupt mature biofilm formed by *A. baumannii* medbio3-2013 strain - Figure 6.13. The antibiofilm effect was recorded even at concentrations equivalent to MIC and ½ MIC, for which the percentage of destruction was up to 60% (Figure 6.3 - a). For the *A. pittii* Cl_2 (3) strain, the preformed biofilm was destroyed in a proportion of up to 32% - Figure 6.13 - b.

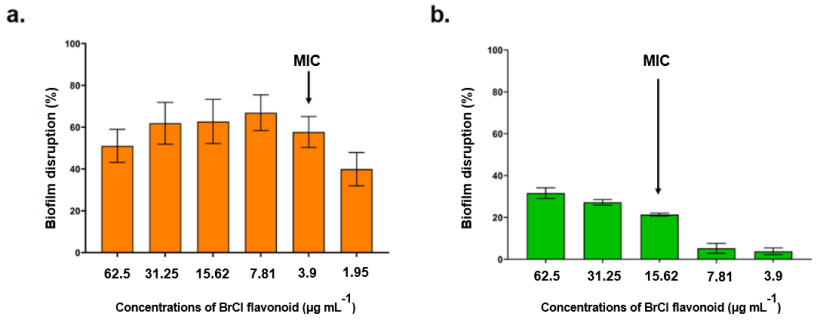


Figure 6.13. Effect of BrCl flavonoid on mature biofilm produced by: a. *A. baumannii* medbio3-2013 (MIC = 3.9 µg mL^{-1}); b. *A. pittii* Cl$_2$ (3) (MIC = 15.62 µg mL^{-1}). The biofilm was formed for 24 h in the absence of synthetic flavonoid. Bars represent standard error of the mean.

Similarly, ciprofloxacin showed a significant antibiofilm effect ($p > 0.03$) at concentrations between 6 and 1.5 µg mL^{-1} (Figure 6.14-a). Ciprofloxacin destroys *A. pittii* Cl$_2$ (3) biofilm in a percentage of up to 37%, which is relatively similar to the value recorded for BrCl flavonoid (31%) administered at a concentration equivalent to 4 × MIC (Figure 6.14 - b).

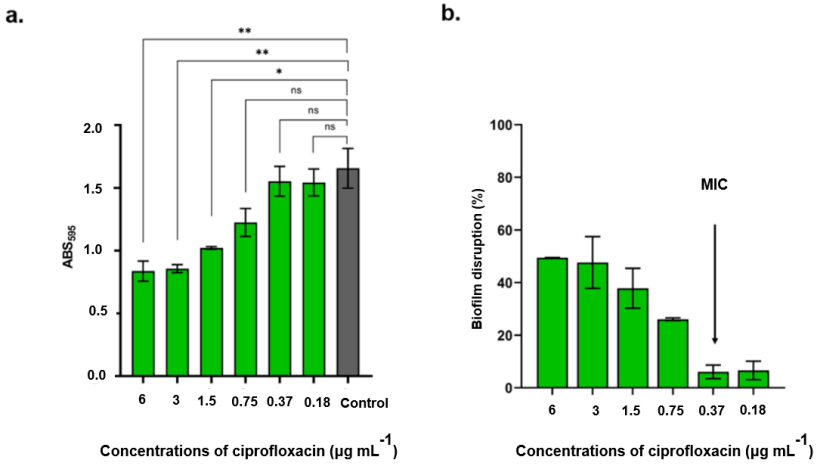


Figure 6.14. Effect of ciprofloxacin on mature biofilm produced by *A. pittii* Cl$_2$ (3) (MIC = 0.37 µg mL^{-1}): a. quantification of bacterial biofilm biomass using crystal violet staining; b. percentage of biofilm destruction ($p < 0,05$; ** - $p > 0,030$; * - $p = 0,012$; ns - insignificant differences). Biofilm was formed for 24 h in the absence of ciprofloxacin. Control cells were subsequently incubated in the absence of ciprofloxacin. Bars represent standard error of the mean.

6.2.7. BrCl flavonoid reduce the cell surface hydrophobicity of *A. pittii* Cl_2 (3) strain

A concentration equivalent to ½ MIC reduced CSH by up to 28.1% compared to DMSO-supplemented control cells - Figure 6.15.

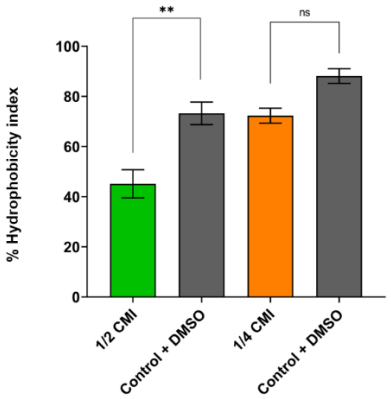


Figure 6.15. Assessment of *A. pittii* Cl_2 (3) cell surface hydrophobicity in the presence of BrCl flavonoid (½ MIC = 7.81 µg mL^{-1}; ¼ MIC = 3.9 µg mL^{-1}) ($p < 0.05$; ** - $p = 0.059$; ns - insignificant differences). Bars represent standard error of the mean.

6.2.8. Effect of BrCl flavonoid on EPS production

BrCl flavonoid, administered at a concentration equivalent to ½ MIC (7.81 µg mL^{-1}), reduce the total carbohydrate content of the sample by approximately 55% compared to the DMSO-supplemented control - Figure 6.16.

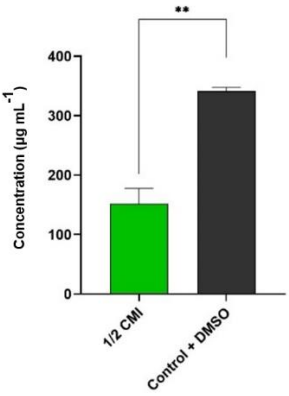


Figure 6.16. Effect of BrCl flavonoid (½ MIC = 7.81 µg mL^{-1}) on the total carbohydrate content produced by *A. pittii* Cl_2 (3) strain ($p < 0.005$; ** - $p = 0.0059$). Bars represent standard error of the mean.

6.2.9. BrCl flavonoid do not affect swarming, swimming and twitching motility

Tested against *P. aeruginosa* medbio7-2013 and *A. pittii* Cl_2 (3) strains, BrCl flavonoid, at concentrations equivalent to ½ (15.62 µg mL^{-1}, 7.81 µg mL^{-1} respectively) and ¼ MIC (7.81 µg mL^{-1}, 3.9 µg mL^{-1} respectively), do not manifest anti-swarming, anti-swimming and anti-twitching activity, the differences recorded between sample and control being insignificant.

CHAPTER 7. SYNERGISTIC EFFECT OF SYNTHETIC TRICYCLIC FLAVONOIDS AND ANTIBIOTICS

7.1. RESEARCH MATERIAL AND METHODS

7.1.1. Assessment of the synergistic effect produced by synthetic flavonoid – antibiotic combinations using the checkerboard method

The checkerboard method is based on testing the combinations of two antimicrobial compounds resulting from two serial dilutions of them at a rate of 2. The aim of this method is to discover the effect produced by the interaction between the two substances: synergistic, additive, indifferent and antagonistic (Hemaiswarya *et al.*, 2008).

7.2. RESULTS AND DISCUSSIONS

Effects of BrCl flavonoid – penicillin combinations

The results obtained (Table 7.1) revealed the presence of five synergistic effects (BrCl flavonoid in a concentration ranging from 0.12 - 0.007 μg mL^{-1} and penicillin in a concentration of 32 μg mL^{-1}).

Table 7.1. Fractional inhibitory concentration index recorded for the combination of BrCl flavonoid and penicillin against *S. aureus* medbio1-2012 strain

Bacterial strain	Minimum inhibitory concentration (μg mL^{-1})				FICI	Effect
	Alone		In combination			
	BrCl flavonoid	Penicillin	BrCl flavonoid	Penicillin		
Staphylococcus aureus medbio1-2012	0.48	128	0.96	8	2.06	Indifferent
			0.48	16	1.125	Indifferent
			0.24	32	0.75	Additive
			0.12	**32**	**0.5**	**Synergic**
			0.06	**32**	**0.375**	**Synergic**
			0.03	**32**	**0.312**	**Synergic**
			0.015	**32**	**0.281**	**Synergic**
			0.007	**32**	**0.264**	**Synergic**

*FICI = fractional inhibitory concentration index

Effects of BrCl flavonoid – ciprofloxacin combinations

The concentration range of BrCl flavonoid and ciprofloxacin was between 0.48 and 0.0002 μg mL^{-1}, respectively 156.25 and 1.22 μg mL^{-1} (Table 7.2). This time, the most numerous effects were the indifferent ones, followed by the additive effects. No synergistic effects were recorded for the tested combinations.

Table 7.2. Fractional inhibitory concentration index recorded for the combination of BrCl flavonoid and ciprofloxacin against *S. aureus* medbio1-2012 strain

Bacterial strain	Minimum inhibitory concentration (μg mL^{-1})				FICI	Effect
	Alone		In combination			
	BrCl flavonoid	Ciprofloxacin	BrCl flavonoid	Ciprofloxacin		
Staphylococcus aureus medbio1-2012	0.48	156.25	0.48	9.76	1.06	Indifferent
			0.48	4.88	1.03	Indifferent
			0.48	2.44	1.015	Indifferent
			0.48	1.22	1.007	Indifferent
			0.24	39.06	0.74	Additive
			0.24	19.53	0.62	Additive
			0.12	78.12	0.74	Additive
			0.06	78.12	0.615	Additive
			0.06	156.25	1.125	Indifferent
			0.03	156.25	1.06	Indifferent

Bacterial strain	Minimum inhibitory concentration (μg mL^{-1})				FICI	Effect
	Alone		In combination			
	BrCl flavonoid	Ciprofloxacin	BrCl flavonoid	Ciprofloxacin		
			0.015	156.25	1.031	Indifferent
			0.007	156.25	1.015	Indifferent
			0.003	156.25	1.007	Indifferent
			0.001	156.25	1.003	Indifferent
			0.0009	156.25	1.001	Indifferent
			0.0004	156.25	1.0009	Indifferent
			0.0002	156.25	1.0004	Indifferent

*FICI = fractional inhibitory concentration index

Effects of BrCl flavonoid – tetracycline combinations

In addition to the two antimicrobial compounds evaluated (penicillin, ciprofloxacin), the interaction between BrCl flavonoid and tetracycline, a broad-spectrum antibiotic that inhibits protein synthesis by blocking mRNA, was also investigated, but no synergistic effect could be observed - Table 7.3.

Table 7.3. Fractional inhibitory concentration index recorded for the combination of BrCl flavonoid and tetracycline against *S. aureus* medbio1-2012 strain

Bacterial strain	Minimum inhibitory concentration (μg mL^{-1})				FICI	Effect
	Alone		In combination			
	BrCl flavonoid	Tetracycline	BrCl flavonoid	Tetracycline		
Staphylococcus aureus medbio1-2012	0.48	19.531	0.48	4.882	1.025	Indifferent
			0.48	2.441	1.012	Indifferent
			0.48	1.222	1.06	Indifferent
			0.48	0.61	1.031	Indifferent
			0.48	0.305	1.015	Indifferent
			0.48	0.152	1.007	Indifferent
			0.24	9.76	0.999	Additive
			0.12	19.531	1.25	Indifferent
			0.06	19.531	1.125	Indifferent
			0.03	19.531	1.06	Indifferent
			0.015	19.531	1.031	Indifferent
			0.07	19.531	1.015	Indifferent
			0.003	19.531	1.007	Indifferent
			0.001	19.531	1.003	Indifferent
			0.0009	19.531	1.001	Indifferent
			0.0004	19.531	1.0009	Indifferent
			0.0002	19.531	1.0004	Indifferent

*FICI = fractional inhibitory concentration index

Effects of BrBr flavonoid – penicillin combinations

The antimicrobial effect of BrBr flavonoid was investigated in combination with penicillin against the same methicillin-resistant *S. aureus* strains. The results obtained, presented

in Table 7.4, revealed the absence of synergistic effects, the only recorded effects being additive (FICI = 0.503 – 0.747) and indifferent (FICI = 1.001 – 1.01).

Table 7.4. Fractional inhibitory concentration index recorded for the combination of BrBr flavonoid and penicillin against *S. aureus* medbio1-2012 strain

Bacterial strain	Minimum inhibitory concentration (μg mL^{-1})				FICI	Effect
	Alone		In combination			
	BrBr flavonoid	Penicillin	BrBr flavonoid	Penicillin		
Staphylococcus aureus medbio1-2012	0.97	128	0.015	64	0.515	Additive
			0.03	64	0.503	Additive
			0.06	64	0.561	Additive
			0.12	64	0.623	Additive
			0.24	64	0.747	Additive
			0.48	32	0.744	Additive
			0.48	16	0.619	Additive
			0.48	8	0.556	Additive
			0.48	4	0.525	Additive
			0.97	2	1.01	Indifferent
			0.97	1	1.007	Indifferent
			0.97	0.5	1.003	Indifferent
			0.97	0.25	1.002	Indifferent
			0.97	0.125	1.001	Indifferent

*FICI = fractional inhibitory concentration index

Effects of BrI flavonoid – penicillin combinations

In the case of the combination of BrI flavonoid (concentrations between 0.97 and 0.007 μg mL^{-1}) and penicillin (concentrations between 256 and 0.125 μg mL^{-1}) tested against the *S. aureus* medbio1-2012 strain, no enhancement of antibiotic activity was observed in the presence of the synthetic sulphur compound for any of the analysed variants - Table 7.5.

Table 7.5. Fractional inhibitory concentration index recorded for the combination of BrI flavonoid and penicillin against *S. aureus* medbio1-2012 strain

Bacterial strain	Minimum inhibitory concentration (μg mL^{-1})				FICI	Effect
	Alone		In combination			
	BrI flavonoid	Penicillin	BrI flavonoid	Penicillin		
Staphylococcus aureus medbio1-2012	0.48	128	0.007	64	0.514	Additive
			0.015	64	0.531	Additive
			0.03	64	0.506	Additive
			0.06	64	0.512	Additive
			0.12	64	0.75	Additive
			0.24	32	0.75	Additive
			0.48	16	1.125	Indifferent
			0.48	8	1.062	Indifferent
			0.97	4	2.031	Antagonistic
			0.97	2	2.015	Antagonistic
			0.97	1	2.007	Antagonistic

Bacterial strain	Minimum inhibitory concentration (μg mL^{-1})				FICI	Effect
	Alone		In combination			
	BrI flavonoid	Penicillin	BrI flavonoid	Penicillin		
			0.97	0.5	2.003	Antagonistic
			0.97	0.25	2.001	Antagonistic
			0.97	0.125	2.0009	Antagonistic

*FICI = fractional inhibitory concentration index

CHAPTER 8. DETERMINATION OF THE CYTOTOXIC AND ANTI-INFLAMATORY POTENTIAL OF BrCl FLAVONOID

8.1. RESEARCH MATERIAL AND METHODS

8.1.1. Evaluation of the effect produced by BrCl flavonoid on human cell viability

The method is based on the spectrophotometric determination of the activity of mitochondrial hydrogenases that reduce tetrazolium salt to formazan (orange colour) (Präbst *et al.*, 2017).

8.1.2. Evaluation of the pro- and anti-inflammatory effect determined by BrCl flavonoid

The secretion of TNF-α and IL10 cytokines is quantified spectrophotometrically using ELISA (Enzyme-Linked Immunosorbent Assay) method.

8.2. RESULTS AND DISCUSSIONS

8.2.1. Assessment of the cytotoxic effect of BrCl flavonoid

Macrophages showed a high sensitivity to BrCl flavonoid, especially at concentrations higher than 5 μg mL^{-1}, the recorded IC_{50} value being 5.3 μg mL^{-1} – Figure 8.1 – a.

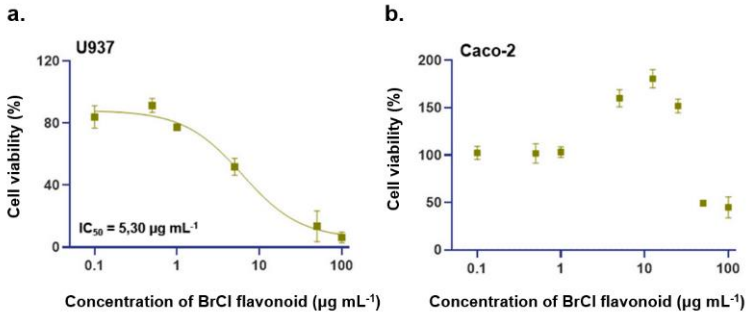


Figure 8.1. Effect of BrCl flavonoid on the viability of human cell lines: a) U937 and b) Caco-2. Cells were incubated for 24 h with BrCl flavonoid at increasing concentrations (0.1 to 100 µg mL^{-1}). The viability of the cells was evaluated by the measurement of mitochondrial hydrogenase activity assay with CCK8 reagent. Means are presented ± standard deviation (N = 2, n = 6). The molecule concentration required to cause 50% inhibition of the cell viability (IC_{50}) was determined using the nonlinear regression analysis function of GraphPad Prism.

8.2.2. Assessment of the pro- and anti-inflammatory effect determined by BrCl flavonoid

The tested flavonoid exerts a pro-inflammatory effect, due to an increase in TNF-α cytokine secretion (by approximately 44%), respectively a reduction in IL10 cytokine production (by approximately 59%), at all evaluated concentrations, but not in a dose-dependent manner - Figure 8.2.

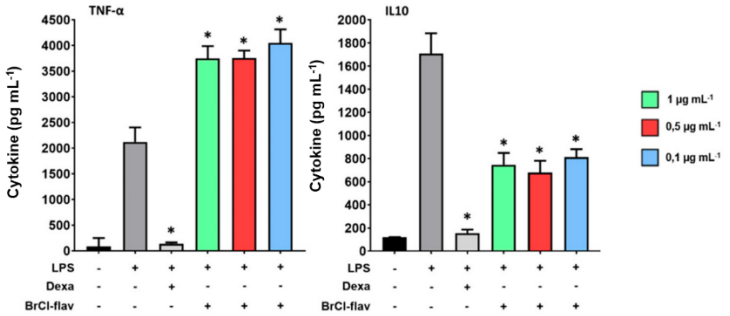


Figure 8.2. Effect of BrCl flavonoid on LPS-induced secretion of tumor necrosis factor-alpha (TNF-α) and interleukin 10 (IL10) in U937-macrophages. Cytokine production was measured after 4 h of incubation with culture medium (black), culture medium + LPS at 50 µg mL^{-1} (dark grey), or with a positive inflammation inhibition control (culture medium + LPS at 50 µg mL^{-1} + dexamethasone, 20 µM; light grey) or with LPS (50 µg mL^{-1}) + culture medium + different concentrations of BrCl flavonoid (1 µg mL^{-1} – green; 0,5 µg mL^{-1} – red and 0,1 µg mL^{-1} – blue) (* = $p < 0.05$). The bars represent the standard deviation of the mean.

CONCLUSIONS

1. Antibiotic resistance in pathogenic bacteria has become a major problem with global consequences for human health.
2. Wastewater and wastewater treatment plants are important reservoirs for the selection of antibiotic-resistant genes and antibiotic-resistant bacteria.
3. Natural and synthetic flavonoids exhibit important activity against resistant bacterial strains, being considered a viable alternative to traditional antibiotics.
4. The antimicrobial activity of flavonoids is due to several mechanisms of action, including cell membrane damage and inhibition of biofilm formation.
5. Taxonomic identification of the strains isolated from the wastewater samples revealed the presence of a high number of strains belonging to *Aeromonas caviae*, *Escherichia coli* and *Pseudomonas aeruginosa* species. A much lower proportion (2%) was observed for *Enterobacter cloacae*, *Enterococcus faecium* and *Staphylococcus aureus*.
6. The most well represented phyla were Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria. The high abundance of Proteobacteria in the analysed samples is directly correlated with their presence in feces, but also with their high survival capacity under characteristic conditions of wastewater.
7. Bacteria resistant to tetracycline, chloramphenicol and ampicillin (broad-spectrum antibiotics frequently administered in different sectors of human activity) were the most numerous in the collected water samples.
8. The highest minimum inhibitory concentration values recorded for the tested strains were obtained for ampicillin (2048 $\mu g\ mL^{-1}$), chloramphenicol (512 $\mu g\ mL^{-1}$) and gentamicin (78.12 $\mu g\ mL^{-1}$).
9. Among the resistance genes identified, the most frequent were those for aminoglycosides, β-lactam antibiotics, tetracyclines, macrolides and phenols. Furthermore, the most numerous isolated strains showed resistance to ampicillin (β-lactam antibiotic), tetracyclines, chloramphenicol (phenols), erythromycin (macrolides) and gentamicin (aminoglycosides). In this context, a link between the results obtained after resistant bacteria isolation on selective media and those obtained from sequencing could be established.
10. Resistance genes involved in the synthesis of β-lactamases, respectively in the activity of efflux pumps and modification of the antibiotic site of action were the most abundant, thus demonstrating that these three mechanisms involved in resistance are the most common in the analysed samples.
11. Among bacterial strains isolated from the wastewater samples, 2 of them (*Aeromonas caviae* A_4 (2) and *Acinetobacter pittii* Cl_2 (3)) showed a high adherence to the inert substrate that

we used. Among the bacterial strains isolated from the clinical environment, only *Acinetobacter baumannii* medbio3-2013 (a critical priority bacterium) produced the highest amount of biofilm (Abs_{595} = 1.7).

12. Regarding the ability of the selected resistant strains to adhere to HeLa cells (a cell line that serves as a model for adhesion assays involving various medically important bacteria), only *Aeromonas caviae* A_4 (2) and *Escherichia coli* A_5 (2) exhibited a localized or aggregative mode of attachment. The inability of the other evaluated strains to adhere to the epithelial cells may be correlated with both reduced adherence to an abiotic support and reduced affinity towards HeLa cells. Of the resistant strains evaluated, 6 were extended-spectrum β-lactamase (ESBL) producers, 2 were OXA_{48} producers, and one strain secreted both categories of enzymes – *Escherichia coli* T_2 (3). MBL and KPL enzymes were observed only at *Klebsiella pneumoniae* medbio6-2013 strain.
13. The phenotypic screening for the presence of efflux pumps in the tested bacterial strains resulted in a low activity of these channels towards ethidium bromide (a dye frequently used to highlight RND-type efflux pumps present especially at species from the *Enterobacteriaceae* family).
14. The antibacterial activity of synthetic tricyclic sulphur-containing flavonoids was evaluated against several species belonging to the genus *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Acinetobacter*, *Escherichia*, *Klebsiella*, *Pseudomonas*, *Salmonella* and *Haemophillus*. The results showed that BrCl flavonoid exhibited the most significant antibacterial properties, with MIC values of ≤ 7.81 μg mL^{-1} for Gram-positive bacteria and ≤ 125 μg mL^{-1} for Gram-negative bacteria.
15. BrCl flavonoid exhibited antibacterial activity up to 32-fold higher than chloramphenicol (reference antibiotic) for several strains of *Staphylococcus aureus*, as well as for *Streptococcus pneumoniae* prxbio10 and *Haemophillus* spp. prxbio13 strains. An activity comparable to gentamicin was recorded against *Klebsiella pneumoniae* medbio6-2013 strain.
16. BrCl, BrBr and BrI flavonoids induced a dose- and time-dependent bacteriostatic effect. At MIC values ranging from 0.48 to 7.81 μg mL^{-1}, the tested compounds led to a growth delay up to 20 h. Concentrations equivalent to 2 × MIC produced a bacteriostatic effect up to 21 h.
17. BrCl flavonoid, at concentrations of 1.95 and 7.81 μg mL^{-1}, exhibited a bactericidal effect against *Staphylococcus aureus* strains after 3 h of exposure, while a concentration of 15.62 μg mL^{-1} produced a similar effect after only 0.5 and 2 h against *Acinetobacter baumannii* medbio3-2013 and *Escherichia coli* medbio4-2013 strains. A total kill was observed after

only 1 h of incubation of *Enterococcus faecium* medbio2-2012 cells in the presence of 31.25 µg mL^{-1} flavonoid concentration.

18. Exposure of *Staphylococcus aureus* medbio1-2012 and *Escherichia coli* medbio4-2013 cells to an equivalent concentration of MBC induced changes in membrane permeability and integrity. These effects could be observed using fluorescence microscopy after only 30 minutes of contact with BrCl flavonoid.
19. Using scanning electron microscopy, it was observed that BrCl flavonoid caused major morphological alterations of bacterial cells. The irreversible morphological changes support the hypothesis that the mechanism of action of BrCl flavonoid involves targeting cell membranes, inducing changes in their structure followed by cell lysis.
20. Adhesion of *Acinetobacter baumannii* medbio3-2013 cells was inhibited up to 94% at concentrations of BrCl flavonoid equivalent to MIC (3.9 µg mL^{-1}) and ½ MIC (1.95 µg mL^{-1}). An anti-adhesion activity was also observed for *Acinetobacter pittii* Cl_2 (3) strain. This time, BrCl flavonoid used at a concentration of 15.62 µg mL^{-1} (MIC) induced a significant inhibition of bacterial cell adhesion, the recorded inhibition percentage being approximately 78%.
21. A biofilm inhibition percentage up to 97%, was observed at concentrations ranging from 62.5 to 0.12 µg mL^{-1} BrCl flavonoid for *Acinetobacter baumannnii* medbio3-2013 and *Acinetobacter pittii* Cl_2 (3) strains.
22. Compared to ciprofloxacin (a quinolone antibiotic used as a treatment option in infections caused by resistant strains of *Acinetobacter*), BrCl flavonoid administered at sub-inhibitory concentrations (½, ¼ and ⅛ MIC) showed an inhibitory effect up to 40%.
23. Using scanning electron microscopy, it was observed that the administration of BrCl flavonoid at a concentration equivalent to ½ MIC (7.81 µg mL^{-1}), led to a significant reduction of cells from the bacterial biofilm. However, at this sub-inhibitory concentration, BrCl flavonoid did not induce changes in cell morphology or cell size.
24. In the presence of concentrations equivalent to MIC and ½ MIC (15.62 and 7.81 µg mL^{-1}), BrCl flavonoid reduces the metabolic activity of biofilm cells by up to 95%. This activity is somehow comparable to that of ciprofloxacin, with only 11% difference between the percentages recorded for the two compounds.
25. BrCl flavonoid manifest a disruptive potential against mature biofilm produced by *Acinetobacter baumannnii* medbio3-2013 and *Acinetobacter pittii* Cl_2 (3) strains, of up to 67%, at concentrations ranging from 62.5 to 0.97 µg mL^{-1}.
26. BrCl flavonoid also reduce cellular hydrophobicity of up to 28% compared to the control used.

27. In addition, BrCl flavonoid administered at a concentration equivalent to ½ MIC (7.81 µg mL^{-1}) reduced the total carbohydrate content produced by *Acinetobacter pittii* Cl_2 (3) strain by approximately 55%. In this context, we hypothesize that the possible mechanism by which the inhibition of *Acinetobacter pittii* cell adhesion occurs could be related to the reduction of cell surface hydrophobicity, a determinant factor in surface adherence and colonization, respectively the inhibition of exopolysaccharide production.
28. BrCl flavonoid, administrated at concentrations equivalent to ½ and ¼ MIC (7.81 and 3.9 µg mL^{-1}), does not affect swarming, swimming and twitching motility of the tested strains.
29. In combination with penicillin, BrCl flavonoid induces several synergistic effects against *Staphylococcus aureus* medbio1-2012 strain, the minimum inhibitory concentrations for the 2 compounds registering a 4- to 68-fold decrease. No synergistic effects were recorded for the combinations of BrCl flavonoid and ciprofloxacin/tetracycline or BrBr/BrI flavonoid and penicillin.
30. The cytotoxic potential was evaluated on several human cell lines that could encounter the flavonoid BrCl after oral administration. Of these, monocytes differentiated into macrophages showed the highest sensitivity (IC_{50} = 5.3 µg mL^{-1}). For the intestinal epithelial cell line, the IC_{50} value could not be determine since BrCl flavonoid induced an increase in eukaryotic cell viability.
31. It was also observed that for some bacterial strains (*Staphylococcus aureus* MRSA, *Streptococcus pneumoniae* prxbio10, *Streptococcus* spp. prxbio9, *Haemophillus* spp. prxbio13, *Escherichia coli* medbio4-2013 and *Acinetobacter baumannii* medbio3-2013), the minimum inhibitory concentration value (MIC ≤ 3.9 µg mL^{-1}) was lower than the IC_{50} value determined for U937 line (IC_{50} = 5.30 µg mL^{-1}).
32. BrCl flavonoid exerts a pro-inflammatory effect due to increased TNF-α cytokine secretion and reduced IL10 cytokine production, but not in a dose-dependent manner.
33. The obtained results suggest that BrCl flavonoid has an important antibacterial potential against some antibiotic-resistant bacteria. In this regard, our compound can be successfully used in the development of new antimicrobial agents.

PROSPECTS FOR FURTHER RESEARCH

The results obtained so far on the antibacterial activity of synthetic tricyclic sulphur-containing flavonoids encourage us to continue research in the following directions:

1. Investigation of the synergistic effects of flavonoids - conventional antibiotics combinations on the inhibition and destruction of bacterial biofilms.
2. Observation, using confocal laser scanning microscopy, of the architecture of the bacterial biofilm formed in the presence of synthetic tricyclic sulphur-containing flavonoids.
3. Investigation of the potential of flavonoids to induce the appearance of reactive oxygen species.
4. Evaluation of the inhibitory effect of flavonoids on the enzymatic activity of β-lactamases.
5. Assessment of the effect of flavonoids on porin blocking mechanisms.
6. Testing the potential of tricyclic sulphur-containing flavonoids for inhibition of overexpressed efflux pumps (e.g. MexAB-OprM).
7. Investigation of flavonoids activity on bacterial cells adhered to a biotic support.
8. Evaluation of the ability of bacterial cells to acquire resistance to tricyclic sulphur-containing flavonoids.

DISSEMINATION OF RESULTS

Articles published *in extenso* in the field of the doctoral thesis

A. Articles published in WOS indexed journals

1. **Cristina-Veronica Moldovan**, Loredana-Elena Mantea, Mihaela Savu, Piter Jones, Laura Gabriela Sarbu, Marius Stefan, Mihail Lucian Birsa (2024) – *Novel tricyclic flavonoids as promising anti-MRSA agents*. Pharmaceuticals **17**(10): 1276, https://doi.org/10.3390/ph17101276 (**IF 4,3**).
2. Loredana-Elena Mantea, **Cristina-Veronica Moldovan**, Mihaela Savu, Laura Gabriela Sarbu, Marius Stefan, Mihail Lucian Birsa (2024) – *An eco-friendly method to synthesize potent antimicrobial tricyclic flavonoids.* Antibiotics **13**(9): 798, https://doi.org/10.3390/antibiotics13090798 (**IF 4,3**).
3. **Cristina-Veronica Moldovan**, Mihaela Savu, Elodie Dussert, Haïrati Aboubacar, Laura Gabriela Sarbu, Simona Matiut, Benoit Cudennec, François Krier, Rozenn Ravallec, Lucian Mihail Birsa, Marius Stefan (2022) – *Synthetic flavonoid BrCl-flav - an alternative solution*

to combat ESKAPE pathogens. Antibiotics **11**(10): 1389, https://doi.org/10.3390/antibiotics11101389 **(IF 4,8)**.

B. Articles published in BDI indexed journals

1. Awawou Manouore Njoya, Jean Samuel Eheth, Yves Poutoum Yogne, Claire Stéphane Metsopkeng, **Cristina-Veronica Moldovan**, Sylvie Chinche Belengfe, Laure Ngando, Marguerite Kamdem Simo, Paul Alain Nana, Edith Brunelle Mouafo Tamnou, Estelle Masseret, Télesphore Sime-Ngando and Moïse Nola (2022) – *Proteus bacteria species from hospital sewage and Mfoundi River in Yaounde (Cameroon, Central Africa): Comparison of the diversity, abundance and susceptibility against some β-lactams, quinolones and aminoglycosides antibiotics.* Journal of Advances in Microbiology Research **3**(2): 34-46, https://doi.org/10.22271/micro.2022.v3.i2a.47.

Articles published *in extenso* during doctoral studies

Articles published in WOS indexed journals

1. Gabriela Vochita, Anca Niculina Cadinoiu, Delia Mihaela Rata, Leonard Ionut Atanase, Marcel Popa, Athar Mahdieh, Cosmin-Teodor Mihai, Alexandru Stache, **Cristina-Veronica Moldovan**, Elena Simona Bacaita, Iustina Condriuc, Daniela Gherghel (2024) – *Comparative in vitro study between biocompatible chitosan-based magnetic nanocapsules and liposome formulations with potential application in anti-inflammatory therapy.* International Journal of Molecular Sciences **25**(15): 8454, https://doi.org/10.3390/ijms25158454 **(IF 4,9)**.

Abstracts

1. **Cristina-Veronica Moldovan**, Amada El-Sabeh, Marius Mihasan, Marius Stefan (2023) – *Identification of antibiotic resistance genes in sewage of Iasi municipal wastewater treatment plant using bioinformatic tools*. 4th International World of Microbiome Conference, p. 128, link to the volume of abstracts: https://microbiome.kenes.com/wp-content/uploads/sites/38/2023/10/WOM23-Abstracts.pdf.
2. **Cristina-Veronica Moldovan**, Lucian Mihail Birsa, Marius Stefan (2022) – *Antimicrobial activity of synthetic flavonoids against multidrug resistant bactaria*. FEMS Conference on Microbiology, p. 70, link to the volume of abstracts:

https://acrobat.adobe.com/link/track?uri=urn%3Aaaid%3Ascds%3AUS%3A8d1278c7-8f23-3e24-9ac9-8164b981a9b9.

3. **Cristina-Veronica Moldovan**, Marius Stefan (2022) – *Antibacterial activity of BrCl flavonoid against different antibiotic-resistant strains.* Annual Scientific Session of Naturalist Students, p. 18, link to the volume of abstracts: http://www.bio.uaic.ro/?page_id=12649.
4. **Cristina-Veronica Moldovan**, Lucian Mihail Birsa, Marius Stefan (2022) – *Antibacterial activity of synthetic BrCl-flavonoid*. J. Exp. Molec. Biol. **23**(2): 66, https://doi.org/10.47743/jemb-2022-84
5. **Cristina-Veronica Moldovan**, Marius Stefan (2021) – *Antimicrobial effects of a new synthetic flavonoid against different antibiotic-resistant bacterial strains.* Annual Scientific Session of Naturalist Students, pp. 12-13, link to the volume of abstracts: http://www.bio.uaic.ro/?page_id=6790.
6. Andreea Mihaela Mlesnita, **Cristina-Veronica Moldovan**, Fakhri Kallabi, Marius Stefan, Marius Mihasan (2021) – *Antibiotic residence of Paenarthrobacter nicotinovorans – an in-silico and in-vitro study*. J. Exp. Molec. Biol. **22**(2): 50, https://doi.org/10.47743/jemb-2021-58.
7. **Cristina-Veronica Moldovan**, Marius Stefan (2021) – *Study of the mechanisms of antibiotic resistance of bacteria isolated from the influent of a wastewater treatment plant*. J. Exp. Molec. Biol. **22**(2): 51, https://doi.org/10.47743/jemb-2021-58.
8. **Cristina-Veronica Moldovan**, Marius Stefan (2020) – *Antibacterial effects of a new synthetic flavonoid against a penicillin resistant strain of Staphylococcus aureus.* Annual Scientific Session of Naturalist Students, p. 8, link to the volume of abstracts: http://www.bio.uaic.ro/?page_id=3268.
9. **Cristina-Veronica Moldovan**, Marius Stefan (2020) – *Antibacterial effects of a synthetic flavonoid against penicillin-resistant strain of Staphylococcus aureus*. Victor Babes National Institute of Pathology, Annual Scientific Meeting & 13th National Pathology Symposium, link to the volume of abstracts: https://www.ivb.ro/wp-content/uploads/2022/09/VolumRezumate_IVB20201.pdf.

Participation at international conferences

1. **Cristina-Veronica Moldovan**, Amada El-Sabeh, Marius Mihasan, Marius Stefan – *Identification of antibiotic resistance genes in sewage of Iasi municipal wastewater treatment plant using bioinformatic tools*. 4th International World of Microbiome Conference, 26-28 October 2023, Sofia, Bulgaria.

2. **Cristina-Veronica Moldovan**, Lucian Mihail Birsa, Marius Stefan – *Antimicrobial activity of synthetic flavonoids against multidrug resistant bacteria*. FEMS Congress of European Microbiologists, 10th edition, 30 June – 2 July 2022, Belgrade, Serbia.
3. **Cristina-Veronica Moldovan**, Lucian Mihail Birsa, Marius Stefan – *Antibacterial effects of a synthetic flavonoid against antibiotic resistant bacteria.* FEMS Congress of European Microbiologists, 9th edition, 20-24 June 2021, Hamburg, Germany (Virtual).

Participation at national conferences

1. **Cristina-Veronica Moldovan**, Stefan-Mihaita Olaru, Lucian-Mihail Birsa, Marius Stefan – *Antibiofilm activity of BrCl-flav against Acinetobacter pittii.* New Trends in Biology: from molecules to complex systems, 25-26 October 2024, Iasi, Romania.
2. Mihaela Savu, **Cristina-Veronica Moldovan**, Loredana-Elena Mantea, Lucian-Mihail Birsa, Marius Stefan – *A new class of tricyclic flavonoids with antimicrobial activity*. New Trends in Biology: from molecules to complex systems, 25-26 October 2024, Iasi, Romania.
3. **Cristina-Veronica Moldovan**, Amada El-Sabeh, Marius Mihasan, Marius Stefan - *Identification of antibiotic resistance genes in the influent of Iasi municipal wastewater treatment plant using a bioinformatic approach*. 2023 RoBioInfo Conference, 11-13 May 2023, Bucharest, Romania.
4. **Cristina-Veronica Moldovan**, Lucian Mihail Birsa, Marius Stefan - *Antibacterial activity of synthetic BrCl-flavonoid*. New Trends in Biology: from molecules to complex systems, 27-28 October 2022, Iasi, Romania (Virtual).
5. **Cristina-Veronica Moldovan**, Lucian Mihail Birsa, Marius Stefan - *Antibacterial activity of BrCl-flav against multidrug resistant bacteria*. International Conference and XXXIX Scientific Session of the Romanian Society for Cell Biology, 21-23 October 2022, Cluj-Napoca, Romania (Virtual).
6. **Cristina-Veronica Moldovan**, Marius Stefan - *Antibacterial activity of BrCl flavonoid against different antibiotic-resistant strains.* Annual Scientific Session of Naturalist Students, 6th edition, 21 May 2022, Iasi, Romania (Virtual).
7. Andreea Mihaela Mlesnita, **Cristina-Veronica Moldovan**, Fakhri Kallabi, Marius Stefan, Marius Mihasan – *Antibiotic residence of Paenarthrobacter nicotinovorans – an in-silico and in-vitro study*. New Trends in Biology: from molecules to complex systems, 28-29 October 2021, Iasi, Romania (Virtual).
8. **Cristina-Veronica Moldovan**, Marius Stefan - *Study of the mechanisms of antibiotic resistance of bacteria isolated from the influent of a wastewater treatment plant*. New Trends

in Biology: from molecules to complex systems, 28-29 October 2021, Iasi, Romania (Virtual).

9. **Cristina-Veronica Moldovan**, Marius Stefan - *Antimicrobial effects of a new synthetic flavonoid against different antibiotic-resistant bacterial strains.* Annual Scientific Session of Naturalist Students, 5th edition, 22 May 2021, Iasi, Romania (Virtual), **First prize:** *Applied and Experimental Biology*.
10. **Cristina-Veronica Moldovan**, Marius Stefan - *Antibacterial effects of a synthetic flavonoid against penicillin-resistant strain of Staphylococcus aureus*. National Symposium on Pathology, Victor Babes National Research and Development Institute, 13th edition, 5 – 7 Novembre 2020, Bucharest, Romania (Virtual), **Second prize***: Short communication – Young Researchers Session*.
11. **Cristina-Veronica Moldovan**, Marius Stefan - *Antibacterial effects of a new synthetic flavonoid against a penicillin resistant strain of Staphylococcus aureus.* Annual Scientific Session of Naturalist Students, 4th edition, 31 October 2020, Iasi, Romania (Virtual).

Member of research project teams

1. Contract no. 550PED/2020. Experimental - demonstrative project: *Tricyclic 1,3-dithiolium flavonoids – New weapons to combat antibiotic resistance*. Acronym: TrisFlav. Project code: PN-III-P2-2.1-PED-2019-2235 (October 2020 – November 2022).
2. Contract no. 15/2020. Bilateral project (Romania - Norway): *Active targeted drug delivery systems based on peptide-functionalized magnetic nanoparticles for the treatment of inner ear diseases*. Acronym: TargEar. Project code: RO-NO-2019-0187 (January 2023 – May 2023).

Patent proposal

1. Title of the patent proposal: *Process for obtaining new synthetic flavonoids with antibacterial properties.* The patent application was registered at the State Office for Inventions and Trademarks (OSIM), with no. A/00697/31.10.2022.

BIBLIOGRAPHY

1. Akilandeswari K., Ruckmani K. (2016) - *Synergistic antibacterial effect of apigenin with β-lactam antibiotics and modulation of bacterial resistance by a possible membrane effect against methicillin resistant Staphylococcus aureus*. Cell. Mol. Biol. **62**(14): 74-82.
2. Alcaráz L.E., Blanco S.E., Puig O.N., Tomás F., Ferretti F.H. (2000) - *Antibacterial activity of flavonoids against methicillin-resistant Staphylococcus aureus strains.* J. Theor. Biol. **205**(2): 231-40.
3. An J., Zuo G.Y., Hao X.Y., Wang G.C., Li Z.S. (2011) - *Antibacterial and synergy of a flavanonol rhamnoside with antibiotics against clinical isolates of methicillin-resistant Staphylococcus aureus (MRSA).* Phytomedicine **18**: 990-993.
4. Ayobami O., Brinkwirth S., Eckmanns T., Markwart R. (2022) - *Antibiotic resistance in hospital-acquired ESKAPE-E infections in low- and lower-middle-income countries: a systematic review and meta-analysis.* Emerg. Microbes Infect. **11**(1): 443-451.
5. Bag A., Bhattacharyya S.K., Pal N.K., Chattopadhyay R.R. (2012) - *In vitro antibacterial potential of Eugenia jambolana seed extracts against multidrug-resistant human bacterial pathogens.* Microbiol. Res. **167**(6): 352 - 7.
6. Bahrin L.G., Apostu M.O., Birsa L.M., Stefan M. (2014) - *The antibacterial properties of sulphur containing flavonoids. Bioorg.* Med. Chem. Lett. **24**: 2315-2318.
7. Bernard F.X., Sablé S., Cameron B., Provost J., Desnottes J.F., Crouzet J., Blanche F. (1997) - *Glycosylated flavones as selective inhibitors of topoisomerase IV.* Antimicrob Agents Chemother. **41**(5): 992-8.
8. Biharee A., Sharma A., Kumar A., Jaitak V. (2020) - *Antimicrobial flavonoids as a potential substitute for overcoming antimicrobial resistance.* Fitoterapia **146**: 104720.
9. Blair J.M.A., Richmond G.E., Piddock L.J.V. (2014) - *Multidrug efflux pumps in Gram-negative bacteria and their role in antibiotic resistance.* Future Microbiol. **9**(10): 1165-1177.
10. Bylka W., Matlawska I., Pilewski N.A. (2004) - *Natural flavonoids as antimicrobial agents.* JANA **7**(2): 24-31.
11. Cushnie T.P.T., Lamb A. J. (2011) - *Recent advances in understanding the antibacterial properties of flavonoids.* Int. J. Antimicrob. Agents **33**: 99-107.
12. Daghrir R., Drogui P. (2013) - *Tetracycline antibiotics in the environment: a review.* Environ. Chem. Lett. **11**: 209-227.
13. Dorneanu O.S., Vremera T. (2011) - *Staphylococcus aureus: clasic versus modern*, Editura Gr. T. Popa, UMF, Iași, România, pp.: 1, 38, 40.

14. Dowling A., O'Dwyer J., Adley C.C. (2011) - *Antibiotics: mode of action and mechanisms of resistance.* Nurs. Stand. **25**(42):49-55.
15. Dunca S., Ailiesei O., Nimitan E., Stefan M. (2004) - *Microbiologie aplicată*. Editura Tehnopress, Iași, pp. 74-76; 210-213.
16. Esiobu N., Armenta L., Ike J. (2002) - *Antibiotic resistance in soil and water environments.* IJEHR **12**: 133-144.
17. Eumkeb G., Sakdarat S., Siriwong S. (2010) - *Reversing β-lactam antibiotic resistance of Staphylococcus aureus with galangin from Alpinia officinarum Hance and synergism with ceftazidime.* Phytomedicine **18**(1): 40-5.
18. Farhadi F., Khameneh B., Iranshahi M., Iranshahy M. (2018) - *Antibacterial activity of flavonoids and their structure-activity relationship: An update review.* Phytother. Res. **33**: 13-40.
19. French G.L. (2005) - *Clinical impact and relevance of antibiotic resistance.* Adv. Drug. Deliv. Rev. **57**(10): 1514-27.
20. Fujita M., Shiota S., Kuroda T., Hatano T., Yoshida T., Mizushima T., Tsuchiya T. (2005) - *Remarkable synergies between baicalein and tetracycline, and baicalein and beta-lactams against methicillin-resistant Staphylococcus aureus.* Microbiol. Immunol. **49**(4): 391-6.
21. Georgios M., Egki T., Effrosyni S. (2013) - *Phenotypic and molecular methods for the detection of antibiotic resistance mechanisms in gram negative nosocomial pathogens* în Saxena S.K., *Trends in Infectious Diseases*, Editura IntechOpen, New York, 139-162.
22. Giedraitienė A., Vitkauskienė A., Naginienė R., Pavilonis A. (2011) - *Antibiotic resistance mechanisms of clinically important bacteria.* Medicina (Kaunas) **47**(3): 137-146.
23. Hancock R.E., Speert D.P. (2000) - *Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and impact on treatment.* Drug Resist. Updat. **3**(4): 247-255.
24. Haraguchi H., Tanimoto K., Tamura Y., Mizutani K., Kinoshita T. (1998) - *Mode of antibacterial action of retrochalcones from Glycyrrhiza inflata.* Phytochemistry **48**(1): 125-9.
25. Hemaiswarya S., Kruthiventi A.K., Doble M. (2008) - *Synergism between natural products and antibiotics against infectious diseases.* Phytomedicine **15**: 639-652.
26. Hirsch R., Ternes T., Haberer K., Kratz K.L. (1999) - *Occurrence of antibiotics in the aquatic environment.* Sci. Total Environ. **225**(1-2): 109-118.
27. Huemer M., Mairpady Shambat S., Brugger S.D., Zinkernagel A.S. (2020) - *Antibiotic resistance and persistence - implications for human health and treatment perspectives.* EMBO Rep. **21**(12): e51034.

28. Ikigai H., Nakae T., Hara Y., Shimamura T. (1993) - *Bactericidal catechins damage the lipid bilayer.* BBA **1147**(1): 132-136.
29. Kapoor G., Saigal S., Elongavan A. (2017) - *Action and resistance mechanisms of antibiotics: A guide for clinicians.* J. Anaesthesiol. Clin. Pharmacol. **33**(3): 300-305.
30. Kaufman G. (2011) - *Antibiotics: mode of action and mechanisms of resistance.* Nurs. Stand. 25(42): 49-55.
31. Kim D.Y, Lee J.C. (2017) - *Adherence assay of uropathogenic Escherichia coli in vivo and in vitro.* Urogenic Tract Infect. **12**(3): 122-129.
32. Krause K.M., Serio A.W., Kane T.R., Connolly L.E. (2016) - *Aminoglycosides: an overview.* Cold. Spring. Harb. Perspect. Med. **6**(6): a027029.
33. Lambert R.J.W., Skandamis P.N., Coote P.J., Nychas G.-J.E. (2001) - *A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol.* J. Appl. Microbiol. **91**(3): 453-462.
34. Li H., Addo K., Yu Y., Xiao X. (2023) - *Cuminaldehyde inhibits biofilm formation by affecting the primary adhesion of Staphylococcus aureus.* S. Afr. J. Bot. **156**: 13-20.
35. Li X.Z., Plésiat P., Nikaido H. (2015) - *The challenge of efflux-mediated antibiotic resistance in gram-negative bacteria.* Clin. Microbiol. Rev. **28**(2): 337-418.
36. Mancuso G., Midiri A., Gerace E., Biondo C. (2021) - *Bacterial antibiotic resistance: the most critical pathogens.* Pathogens **10**(10): 1310.
37. Martins M., Viveiros M., Couto I., Costa S.S., Pacheco T., Fanning S., Pagès J.M., Amaral L. (2011) - *Identification of efflux pump-mediated multidrug-resistant bacteria by the ethidium bromide-agar cartwheel method.* In vivo **25**: 171-178.
38. Michael-Kordatou I., Karaolia P., Fatta-Kassinos D. (2018) - *The role of operating parameters and oxidative damage mechanisms of advanced chemical oxidation processes in the combat against antibiotic-resistant bacteria and resistance genes present in urban wastewater.* Water Res. **129**: 208-230.
39. Miscalencu D., Caraene G., Mailat F., Nichita C. (2008) - *Efectele biologice benefice ale flavonoidelor*, Editura Universității București, București, pp. 9-14.
40. Moldovan C.-V., Savu M., Dussert E., Aboubacar H., Sarbu L.G., Matiut S., Cudennec B., Krier F., Ravallec R., Birsa L.M., Stefan M. (2022) - *Synthetic flavonoid BrCl-Flav - An alternative solution to combat ESKAPE pathogens.* Antibiotics (Basel) **11**(10): 1389.
41. Moya C., Maicas S. (2020) - *Antimicrobial Resistance in Klebsiella pneumoniae Strains: Mechanisms and Outbreaks.* Proc. **66**(1): 11.
42. Mun S.H., Lee Y.S., Han S.H., Lee S.W., Cha S.W., Kim S.B., Seo Y.S., Kong R., Kang D.H., Shin D.W., Kang O.H., Kwon D.Y. (2015) - *In vitro potential effect of morin in the*

combination with β-lactam antibiotics against methicillin-resistant Staphylococcus aureus. Foodborne Pathog. Dis. **12**(6): 545-50.

43. Munita J.M., Arias C.A. (2016) - *Mechanisms of antibiotic resistance.* Microbiol. Spectr. **4**(2): VMBF-0016-2015.
44. Mutuku C., Gazdag Z., Melegh S. (2022) - *Occurrence of antibiotics and bacterial resistance genes in wastewater: resistance mechanisms and antimicrobial resistance control approaches.* World J. Microbiol. Biotechnol. **38**(9): 152.
45. Navarro-Martínez M.D., Navarro-Perán E., Cabezas-Herrera J., Ruiz-Gómez J., García-Cánovas F., Rodríguez-López J.N. (2005) - *Antifolate activity of epigallocatechin gallate against Stenotrophomonas maltophilia.* Antimicrob. Agents Chemother. **49**(7): 2914-20.
46. Ngigi A.N., Magu M.M., Muendo B.M. (2019) - *Occurrence of antibiotics residues in hospital wastewater, wastewater treatment plant, and in surface water in Nairobi County*, Kenya. Environ. Monit. Assess **192**: 18.
47. Nielsen S.S. (2017) - *Food Analysis Laboratory Manual*, 3rd edition, Editura Springer, Mason, Ohio, SUA, pp.:137-141.
48. Novo A., André S., Viana P., Nunes O.C., Manaia C.M. (2013) - *Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater.* Water Res. **47**: 1875-1887.
49. O'Driscoll T., Crank C.W. (2015) - *Vancomycin-resistant enterococcal infections: epidemiology, clinical manifestations, and optimal management.* Infect. Drug Resist. **8**: 217-30.
50. Oliveira D., Borges A., Simões M. (2018) - *Staphylococcus aureus toxins and their molecular activity in infectious diseases: Review.* Toxins (Basel) **10**(6): 252.
51. Otsuka N., Liu M.H., Shiota S., Ogawa W., Kuroda T., Hatano T., Tsuchiya T. (2008) - *Anti-methicillin resistant Staphylococcus aureus (MRSA) compounds isolated from Laurus nobilis.* Biol. Pharm. Bull. **31**(9): 1794-7.
52. Panche A.N., Diwan A.D., Chandra S.R. (2016) - *Flavonoids: an overview.* J. Nutr. Sci. **5**:e47.
53. Pang Z., Raudonis R., Glick B.R., Lin T.J., Cheng Z. (2019) - *Atibiotic resistance in Pseudomonas aeruginosa: mechanisms and alternative therapeutic strategies.* Biotechnol. Adv. **37**(1): 177-192.
54. Pazda M., Kumirska J., Stepnowski P., Mulkiewicz E. (2019) - *Antibiotic resistance genes identified in wastewater treatment plant systems - A review.* Sci. Total Environ. **697**: 134023.

55. Panzaru C. (2003) - *Streptococii și enterococii* în Buiuc D.T. - *Microbiologie medicală. Ghid pentru studiul și practica medicinei*, ediția a VI-a, Editura Gr. T. Popa, UMF, Iași, România, pp.: 161-173.
56. Peterson E., Kaur P. (2018) - *Antibiotic resistance mechanisms in bacteria: relationships between resistance determinants of antibiotic producers, environmental bacteria and clinical pathogens.* Front. Microbiol. **9**: 2928.
57. Pistelli L., Giorgi I. (2012) - *Antimicrobial properties of flavonoids* în Patra A. - *Dietary phytochemicals and microbes*, Editura Springer, Dordrecht, pp. 9-70.
58. Plaper A., Golob M., Hafner I., Oblak M., Solmajer T., Jerala R. (2003) - *Characterization of quercetin binding site on DNA gyrase.* Biochem. Biophys. Res. Commun. **306**(2): 530-6.
59. Popescu G.A., Serban R., Niculcea A., Leustean M., Pistol A. (2022) - *Consumul de antibiotice, rezistența microbiană și infecții asociate asistenței medicale în România* - 2020, Institutul Național de Sănătate Publică, România, pp. 11-17.
60. Präbst K., Engelhardt H., Ringgeler S., Hübner H. (2017) - *Basic colorimetric proliferation assays: MTT, WST, and resazurin.* Methods Mol. Biol. **1601**: 1-17.
61. Romero-Soto I.C., Dia O., Leyva-Soto L.A., Drogui P., Buelna G., Díaz-Tenorio L.M., Ulloa-Mercado R.G., Gortáres-Moroyoqui P. (2018) - *Degradation of chloramphenicol in synthetic and aquaculture wastewater using electrooxidation*. J. Environ. Qual. **47**(4): 805-811.
62. Sandasi M., Leonard C.M., Viljoen A.M. (2010) - *The in vitro antibiofilm activity of selected culinary herbs and medicinal plants against Listeria monocytogenes*. Lett. Appl. Microbiol. **50**(1): 30-35.
63. Santajit S., Indrawattana N. (2016) - *Mechanisms of antimicrobial resistance in ESKAPE pathogens.* Biomed. Res. Int. **2016**: 2475067.
64. Sarker S.D., Nahar L., Kumarasamy Y. (2007) - *Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals.* Methods **42**(4): 321-324.
65. Sarbu L.G., Bahrin L.G., Babii C., Stefan M., Birsa M.L. (2019) - *Synthetic flavonoids with antimicrobial activity: a review.* J. Appl. Microbiol. **127**: 1282-1290.
66. Shamsudin N.F., Ahmed Q.U., Mahmood S., Ali Shah S.A., Khatib A., Mukhtar S., Alsharif M.A., Parveen H., Zakaria Z.A. (2022) - *Antibacterial effects of flavonoids and their structure-activity relationship study: a comparative interpretation.* Molecules **27**(4): 1149.

67. Stepanović S., Vuković D., Dakić I., Savić B., Švabić-Vlahović M. (2000) - *A modified microtiter-plate test for quantification of staphylococcal biofilm formation.* J. Microbiol. Methods **40**: 175-179.

68. Tsuchiya H., Iinuma M. (2000) - *Reduction of membrane fluidity by antibacterial sophoraflavanone G isolated from Sophora exigua.* Phytomedicine **7**(2): 161-5.

69. Uddin T.M., Chakraborty A.J., Khusro A., Zidan R.M., Mitra S., Emran T.B., Dhama K., Ripon K.H., Gajdács M., Sahibzada M.U.K., Hossain J., Koirala N. (2021) - *Antibiotic resistance in microbes: History, mechanisms, therapeutic strategies and future prospects.* J. Infect. Public Health. **14**(12): 1750-1766.

70. van Hoek A.H.A.M., Mevius D., Guerra B., Mullany P., Roberts A.P., Aarts H.J.M. (2011) - *Acquired antibiotic resistance genes: an overview.* Front. Microbiol. **2**: 203.

71. Ventola C.L. (2015) - *The antibiotic resistance crisis: part 1: causes and threats*. P. T. **40**(4): 277-83.

72. Wang S.Y., Sun Z.L., Liu T., Gibbons S., Zhang W.J., Qing M. (2013) - *Flavonoids from Sophora moorcroftiana and their synergistic antibacterial effects on MRSA.* Phytother. Res. **28**(7): 1071-6.

73. World Health Organisation (2024) - *WHO bacterial priority pathogens list, 2024: bacterial pathogens of public health importance to guide research, development and strategies to prevent and control antimicrobial resistance.* Geneva: World Health Organization.

74. Wu D., Kong Y., Han C., Chen J., Hu L., Jiang H., Shen X. (2008) - *D-Alanine:D-alanine ligase as a new target for the flavonoids quercetin and apigenin.* Int. J. Antimicrob. Agents **32**(5): 421-6.

75. Xie J.H., Dong C.J., Nie S.P., Li F., Wang Z.J., Shen M.Y., Xie M.Y. (2015) - *Extraction, chemical composition and antioxidant activity of flavonoids from Cyclocarya paliurus (Batal.) Iljinskaja leaves.* Food Chem. **186**: 97-105.

76. Xu H.X., Lee S.F. (2001) - *Activity of plant flavonoids against antibiotic-resistant bacteria.* Phytother. Res. **15**(1): 39-43.

77. Zoueki C.W., Tufenkji N., Ghoshal S. (2010) - *A modified microbial adhesion to hydrocarbons assay to account for the presence of hydrocarbon droplets.* J. Colloid. Interface Sci. **344**(2): 492-496.