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Activity of Compounds Isolated from *Carpobrotus edulis* on Efflux Pumps of Bacteria and Cancer Cells

Ana Sofia Fernandes Martins

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SUPERVISOR:

Professor Doutor Leonard Amaral
Professor Catedrático Convidado e Director da Unidade de Micobactérias
Unidade de Micobactérias, UPMM
Instituto de Higiene e Medicina Tropical
Universidade Nova de Lisboa

TUTORIAL COMMISSION:

Professora Doutora Maria José Umbelino Ferreira
Professora Associada com Agregação
Medical Chemistry group
iMed.UL, Research Institute for Medicines and Pharmaceutical Sciences
Faculdade de Farmácia
Universidade de Lisboa

Professor Doutor Miguel Viveiros
Professor Auxiliar de Bacteriologia
Unidade de Micobactérias
Instituto de Higiene e Medicina Tropical
Universidade Nova de Lisboa

Professor Doutor Leonard Amaral
Professor Catedrático Convidado e Director da Unidade de Micobactérias
Unidade de Micobactérias, UPMM
Instituto de Higiene e Medicina Tropical
Universidade Nova de Lisboa

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Lisboa, 14 de Outubro de 2009

Ana Sofia Fernandes Martins

"The History of Medicine

- 2000 B.C. – Here, eat this root
- 1000 A.D. – That root is heathen. Here, say this prayer.
- 1850 A.D. – That prayer is superstition. Here, drink this potion.
- 1920 A.D. – That potion is snake oil. Here, swallow this pill.
- 1945 A.D. – That pill is ineffective. Here, take this penicillin.
- 1955 A.D. – Oops....bugs mutated. Here, take this tetracycline.
- 1960-1999 A.D. – 39 more "oops"...Here, take this more powerful antibiotic.
- 2000 A.D. – The bugs have won! Here, eat this root."

— Anonymous

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ABSTRACT

Introduction: Resistance to antibiotics and chemotherapy is a major health problem in Portugal and also globally. Nowadays, a significant proportion of clinical Gram-negative isolates are multi-drug resistant (MDR) and whenever studied, the MDR phenotype has been shown to be mediated by over-expressed efflux pumps (EPs). The over-expression of bacterial EPs is known to result from their exposure to one antibiotic that in some manner renders the bacterium with an MDR phenotype. Nevertheless, the process by which the development of an MDR phenotype that occurs during the period the patient is being treated with an antibiotic has yet to be completly demonstrated in the laboratory. Moreover, the degree of resistance of the Gram-negative clinical isolate is often-times many fold greater than the constant concentration used in therapy and reached in the patient plasma.

Among Enterobacteriaceae, the major EP belongs to the RND superfamily which is mainly driven by energy coming from the proton motive force (PMF). Environmental factors such as Calcium (Ca^{2+}), pH or glucose (energy source) have major influence in the mechanisms of retention or efflux of compounds by the cell. However, because the cell envelope is the first bacterial cell component to face changes in the environmental conditions such as hydrostatic pressure, osmolarity or antibiotic pressure, it is essential to have an over-view of all the processes involved in the acquisition of resistance. Therefore it is worthy to understand how such environmental conditions influence the outer-membrane composition of the cell and its mechanism of efflux.

The first part of this dissertation focuses on the effect of such environmental conditions, on the composition of the outer membrane and the cellular responses It was, then, studied the role of antibiotic-promoted stress via step-wise increasing concentrations of antibiotic or serial passages of the bacterial strain in the same concentration of antibiotic, simulating what happens in the patient when she/he is submitted to long periods of antibiotic therapy.

Efflux modulators can be used in therapy together with antibiotics for improvement of antibiotic action. Their use starts to be widely accepted as a new approach for the

therapy of multi-drug resistance. Therefore, the second part of this dissertation focuses on the purification and characterization of compounds purified from the plant *Carpobrotus edulis* whose methanolic extract had been previously shown to inhibit MDR EPs of bacteria. Because it was previously shown by others the relationship between EP in bacteria and cancer cells, the purified compounds were also studied for their inhibitory activity on one of the major efflux pump transporters of cancer cells (P-gp).

Methods: Methods of protein extraction and electrophoresis were employed to assess the composition of the outer membrane after the bacterial cells face two different kind of growth media: solid and liquid. The effect of the antibiotic pressure on the EP expression was studied by growing different bacterial strains under increasing concentration of antibiotic or maintaining them in the same concentration for longer periods of time. The progeny strains were then tested for their response to the antibiotics in the presence of EPI and for their EP expression by real time reverse transcription PCR (rtRT-PCR). The effect of efflux pump modulators such as CCCP, PAβN, verapamil, phenothiazines, and the modulating effects of calcium, pH and energy source were studied by the semi-automated ethidium bromide (EB) method that follows the accumulation or efflux of EB, on a real time manner, by the bacterial cells under the conditions applied to the media.

The assessment of *C. edulis* compounds for *in vitro* activity against wild type bacterial strains and their counterpart strains that over-produce given EPs was conducted by determination of the minimum inhibitory concentration (MIC) of the purified compounds as well as for other antibiotics of reference for each strain in the presence of the compounds to be tested. The activity of the compounds as efflux modulators were also tested by the semi-automated EB method, already mentioned. The compounds were also assessed for their capacity to increase the killing activity of macrophages infected with bacterial strains: *ex vivo* activity. Finally, the purified compounds were tested for their antiproliferative effect on cancer cell lines and their capacity to inhibit the P-gp responsible for the multi-drug resistance in those cell lines.

Results: During this study it was observed that in liquid medium a greater expression of a 55kDa protein takes place as opposed to Salmonella strains grown in solid medium. The simulation of the response of bacteria to the therapy with antibiotics through the two different adaptation processes showed that the bacterial response is dependent upon the method of adaptation to the antibiotic used.

The results of this dissertation also suggest that efflux and accumulation of EB by *E. coli* strains are dependent on pH and energy that influence the performance of the AcrAB pump. This EP is dependent upon protons present in the periplasm for its activation. The efflux response is independent of the pH of growth of the bacteria whereas it is dependent on the pH of the assay, suggesting that bacteria are able to adapt to different environmental conditions such as pH and presence of noxious agents. Due to its capacity for binding protons, CCCP was used at different pH, in order to understand the role of protons and PMF on the efflux. The use of CCCP together with variations in the pH helped to identify the main types of efflux transporters that respond to the different environmental conditions. However, PAβN modulates efflux of ethidium bromide by competing with it for the site of extrusion of the pump (a K_M was determined).

Oleanolic acid, β-amyrin, uvaol, catechin, epicatechin, MGDG and procyanidin B5 were the compounds isolated from the plant *C. edulis*. It was observed that the activity of some of these compounds was differed according to the mechanisms of resistance that characterizes the different strains against which their activity was studied. This is in agreement with the results obtained for the response of the bacterial cell adapted through different mechanisms to the use of efflux modulators. The results suggest that the triterpene uvaol was the most active compound as an efflux modulator in bacteria and cancer cells. It also has significant activity against intracellular *Staphylococcus aureus.*

Conclusion: A 55kDa protein was previously described as a virulence factor. The same protein had less expression when the bacteria were grown in presence of a phenothiazine, a compound described as an efflux modulator. Consequently, the action of these compounds as adjuvants may be due to their capacity to reduce the virulence of the strain. Therefore the results obtained for bacteria grown in solid and liquid media

are of extreme importance because they can be an evidence for the reasons by which these compounds are described as helper compounds. They can also indicate why infections by the same organism but through different food sources have different degrees of infection and virulence on the patient.

Adaptation caused by serial passages in the same concentration of antibiotic suggests the presence of "mutator" genes that allows the cell to survive under stress conditions and reduce energy consumption that would otherwise be higher with the over-expression of efflux systems as occurs when bacteria is exposed to step-wise increasing concentrations of antibiotic.

The results of this dissertation also suggest that the AcrAB mediated efflux is dependent upon protons present in the periplasm for their activation. Hence, when *E. coli* faces stress conditions caused by a noxious agent, its extrusion would be preferentially performed by an ABC type transporter at pH greater than 7. The efflux response is independent on the pH of growth of the bacteria but dependent on the pH of the assay suggesting that bacteria are able to adapt to different conditions such as environmental pH that it has to face during the infection process in the human body. Energy dependent efflux mechanisms vary upon the pH and the conjunction of pH and glucose is an important tool in the study and understanding of the physiology and mechanisms of efflux. Efflux pumps belonging to the ABC superfamily have an important role in efflux at pH 8; however, PMF is essential for RND family mediated efflux as per the results obtained at pH 5. The use of compounds that interfere with the PMF or directly affect the efflux systems has also a relevant role in the study of the efflux mechanisms and their physiology.

Based on the results obtained with compounds purified from *C. edulis*, this plant is a promising source for search of more effective antibacterial, antimycobacterial and anticancer compounds. It is worthy to mention that the extremely easy availability of this plant in the coast of Portugal makes it an outstanding raw material for large scale production of its constituents which is essential for the development of any products to be used in practical medicine.

RESUMO

Introdução: A resistência aos antibióticos é um grave problema de saúde quer em Portugal quer a nível mundial. Nos dias de hoje, uma grande percentagem dos isolados clínicos de bactérias Gram-negativas é multi-resistente (MR) e, sempre que estudado, o fenótipo MR é mediado pela sobre-expressão de bombas de efluxo (BE). A sobre-expressão de bombas de efluxo em bactérias resulta da exposição destas a um antibiótico que, por vários processos, lhes confere um fenótipo MR. Contudo, o processo pelo qual a estirpe bacteriana desenvolve resistência durante a terapia com determinado antibiótico, ainda não foi completamente demonstrado em laboratório. Frequentemente, o grau de resistência dos isolados clínicos é muitas vezes superior à dose constante de antibiótico usada na terapia e atingida no plasma do paciente.

Entre as Enterobacteriaceae, a principal BE pertence à família RND, na qual a energia necessária ao efluxo provém da força proto-motriz (PMF). Factores do meio envolvente, tais como, cálcio, pH ou glucose (fonte de energia), têm extrema influência nos mecanismos de retenção ou efluxo de compostos pela célula. Contudo, uma vez que o invólucro celular é o primeiro componente bacteriano a enfrentar alterações das condições do meio onde a bactéria se encontra, tais como alteração da pressão hidrostática, osmolaridade ou pressão antibiótica, é essencial compreender a generalidade dos processos envolvidos na aquisição de resistência. Assim, é urgente compreender como é que as condições do meio influenciam a composição da membrana celular e os seus mecanismos de efluxo.

A primeira parte desta dissertação estuda o efeito dessas condições na composição da membrana externa e nas respostas celulares. Foi então estudado o efeito do *stress* provocado quer por aumentos crescentes na concentração de antibiótico, quer por passagens sucessivas da estirpe bacteriana em concentrações constantes de antibiótico, simulando o que acontece no paciente quando submetido a longos períodos de terapia com antibiótico.

Moduladores do efluxo podem ser usados em terapia, conjuntamente com os antibióticos, de modo a aumentar o seu efeito terapêutico. A sua utilização começa a ser

aceite como uma nova abordagem terapêutica contra a multi-resistência. Deste modo a segunda parte desta tese foca a purificação e caracterização de compostos isolados da planta *Carpobrotus edulis*, cujo extracto metanólico mostrou, anteriormente, inibir BE bacterianas. Uma vez que foi já demonstrado existir uma relação entre BE de bactérias e de células cancerígenas, foi também estudado o efeito inibitório dos compostos purificados num dos transportadores com mais relevância em multi-resistência em células cancerígenas (P-gp).

Métodos: Foram utilizados métodos de extracção de proteínas e electroforese para estudar a composição da membrana externa de células bacterianas cujo crescimento ocorreu em dois meios diferentes: sólido e líquido. O efeito da pressão antibiótica na expressão de BEs foi estudada através do crescimento de estirpes bacterianas em concentrações crescentes de antibiótico ou mantendo-as em concentrações de antibiótico constantes por longos períodos de tempo. No final das sucessivas passagens foi estudada a resposta celular a diferentes antibióticos na presença de moduladores de efluxo bem como os níveis de expressão de BEs por RT-PCR em tempo real. O efeito de moduladores de efluxo tais como CCCP, PAβN, verapamil ou fenotiazinas e os efeitos do cálcio, pH e fontes de energia foram estudados pelo método semi-automático que segue a acumulação ou efluxo de brometo de etidium pelas células bacterianas, em tempo real, nas condições aplicadas ao meio do ensaio experimental.

O estudo das actividades *in vitro* dos compostos isolados de *C. edulis* em relação a estirpes de referência e outras multi-resistentes, que sobre-expressam determinadas BE, foi realizado por determinação das concentrações mínimas inibitórias dos compostos, bem como de outros antibióticos, aos quais as estirpes eram resistentes, na presença do composto. O método semi-automático atrás referido foi também utilizado no estudo destes compostos como moduladores de efluxo. A influência destes compostos na morte de estirpes bacterianas fagocitadas por macrófagos foi também estudada: ensaios *ex vivo*. Por fim, foi estudada a actividade antiproliferativa dos compostos isolados em linhas celulares cancerígenas bom como a sua capacidade de inibição da P-gp responsável pela multi-resistência nessas linhas celulares.

Resultados: Durante este estudo foi observado que em meio líquido há maior expressão de uma proteína com 55kDa em oposição ao que acontece quando a bactéria cresce em

meio sólido. A simulação da resposta bacteriana durante a terapia pelos dois processos descritos, mostrou que a resposta bacteriana é dependente do processo de adaptação seguido.

Os resultados desta dissertação sugerem, também, que o efluxo e a acumulação de EB por células de *E. coli* são dependentes do pH e de energia, os quais influenciam o desempenho da bomba de efluxo AcrAB. Esta BE depende da concentração periplasmática de protões para a sua activação. O efluxo é independente do pH do meio onde as células bacterianas cresceram, contudo, é dependente do pH do ensaio, o que sugere que a bactéria é capaz de se adaptar a diferentes condições do meio tais como pH ou agentes prejudiciais à sua sobrevivência. Devido à sua capacidade quelante, o composto CCCP foi usado a diferentes pH com o objectivo de compreender o papel da concentração protónica e da PMF no efluxo. O uso de CCCP juntamente com variações no pH, possibilitou a identificação dos principais tipos de sistemas de efluxo que respondem às diferentes condições do meio. Contudo, o composto PAβN interfere com o efluxo de EB, por competição com este, pelo sítio activo da bomba de efluxo (um K_M para esta competição foi determinado).

Os compostos isolados da planta *C. edulis* foram: ácido oleanólico, β-amirina, uvaol, catequina, epicatequina, MGDG e procianidina B5. Foi observado que estes compostos tinham diferentes actividades consoante o mecanismo de resistência característico de cada uma das estirpes em que a sua actividade foi estudada. Este facto está de acordo com os resultados obtidos para a resposta celular de bactérias, cuja multi-resistência foi obtida por diferentes mecanismos, perante o uso de moduladores. Os resultados obtidos sugerem que, de entre os compostos isolados, o composto uvaol foi o mais activo como modulador da actividade de efluxo, quer em células bacterianas quer em células cancerígenas. Também demonstrou uma actividade significativa contra *Staphylococcus aureus* intracelular.

Conclusão: Uma proteína de 55kDa foi anteriormente descrita como factor de virulência. A mesma proteína encontrava-se menos expressa em bactérias cultivadas na presença de uma fenotiazina, um composto descrito como modulador de efluxo. Deste modo a acção destes compostos como adjuvantes terapêuticos pode dever-se à sua capacidade de reduzir a virulência de determinada estirpe. Deste modo, os resultados

obtidos, quando células bacterianas cresceram em meios líquido e sólido, são extremamente importantes pois podem indicar o motivo pelo qual infecções pelo mesmo organismo, mas por via de diferentes origens alimentares, apresentam diferentes graus de infecção e virulência para o paciente.

A adaptação induzida por passagens sucessivas em meio com a mesma concentração de antibiótico sugere a presença de genes "mutantes" cuja actividade possibilita a sobrevivência celular em condições de "stress", reduzindo o consumo de energia. De outro modo este seria mais elevado devido à sobre-expressão dos sistemas de efluxo, tal como acontece quando a bactéria é sujeita a passagens em concentrações crescentes de antibiótico. Os resultados desta dissertação também sugerem que a activação do efluxo, mediado pela bomba de efluxo AcrAB, é dependente da concentração protónica no periplasma. Assim, quando células de *E. coli* experimentam condições adversas causadas por agentes tóxicos, o efluxo é efectuado preferencialmente por transportadores do tipo ABC se o pH for maior que 7. O facto de o efluxo ser uma resposta independente do pH a que a estirpe cresceu, mas dependente do pH do meio em que o ensaio está a decorrer, sugere que a bactéria é capaz de se adaptar a diferentes pH do meio, tais como os que encontra durante o processo de infecção. Os mecanismos de efluxo dependentes de energia também variam com o pH. Deste modo a conjugação destes dois factores é muito importante para o estudo e compreensão da fisiologia e dos mecanismos de efluxo. As BEs que pertencem à família ABC têm uma função importante a pH 8, contudo a PMF é fundamental para o efluxo por via dos transportadores da família RND, como observado nos ensaios a pH 5. O uso de compostos que interferem com a PMF ou afectam directamente os sistemas de efluxo tem também um papel relevante no estudo dos mecanismos de efluxo e sua fisiologia.

Os resultados obtidos com os compostos purificados da planta *C. edulis,* sugerem que esta planta contem compostos promissores com actividade antibacteriana e anticancerígena. É importante salientar que a abundância desta planta na orla marítima de Portugal faz com que a produção em larga escala dos seus constituintes seja fácil, o que é um factor essencial no desenvolvimento de quaisquer produtos a usar na prática clínica.

PUBLICATIONS

Publications resulted from this thesis are listed below and a copy of each is presented in Appendix.

1. Martins M, Santos B, **Martins A**, Viveiros M, Couto I, Cruz A, Pagès JM, Molnar J, Fanning S and Amaral L; Management Committee Members; of Cost B16; European Commission/European Science Foundation. **(2006)** *An instrument-free method for the demonstration of efflux pump activity of bacteria.* In Vivo. 20: 657-664;

2. **Martins A**, Couto I, Aagaard L, Martins M, Viveiros M, Kristiansen JE and Amaral L. **(2007)** *Prolonged exposure of MRSA COL is exposed to increasing concentrations of oxacillin result in an MDR phenotype*. Int. J. Antimicrob. Agents. 29(3):302-305;

3. Martins M, Schelz Zs, **Martins A**, Molnar J, Hajös G, Riedl Z, Viveiros M, Yalcin I, Aki-Sener E and Amaral L. **(2007)** *In vitro and ex vivo activity of thioridazine derivatives against Mycobacterium tuberculosis.* Int. J. Antimicrob. Agents. 29(3): 338-340;

4. Schelz Zs, Martins M, **Martins A**, Viveiros M, Molnar J and Amaral L. **(2007)** *Elimination of plasmids by SILA compounds that inhibit efflux pumps of bacteria and cancer cells.* In Vivo. 21(4): 635-639;

5. Viveiros M, **Martins A**, Paixão L, Rodrigues L, Martins M, Couto I, Fähnrich E, Kern WV and Amaral L. **(2008)** *Demonstration of intrinsic efflux activity of Escherichia coli K-12 AG100 by an automated ethidium bromide method*. Int. J. Antimicrob. Agents. 31(5):458-462;

6. Viveiros M, Martins M, Couto I, Rodrigues L, Spengler G, **Martins A**, Kristiansen JE, Molnar J and Amaral L. **(2008)** *New methods for the identification of efflux mediated MDR bacteria, genetic assessment of regulators and efflux pump*

constituents, characterization of efflux systems and screening for inhibitors of efflux pumps. Curr. Drug Targets. 9(9):760-778;

7. Spengler G, **Martins A**, Rodrigues L, Aagaard L, Martins M, Costa SS, Couto I, Viveiros M, Fanning S, Kristiansen JE, Molnar J and Amaral L. **(2009)** *Characterization of intrinsic efflux activity of Enterococcus faecalis ATCC29212 by a semi-automated ethidium bromide method*. In Vivo. 23(1): 81-87;

8. Spengler G, Viveiros M, Martins M, Rodrigues L, **Martins A**, Molnar J, Couto I and Amaral L. **(2009)** *Demonstration of the activity of the P-glycoprotein by a semi-automated fluorometric method*. Anticancer Research. 29(6): 2173-2177;

9. **Martins A**, Spengler G, Rodrigues L, Viveiros M, Ramos J, Martins M, Couto I, Fanning S, Pagès JM, Bolla JM, Molnar J and Amaral L. **(2009)** *pH modulation of efflux pump activity of multi-drug resistant E. coli: Protection during its passage and eventual colonization of the colon*. PloS One 4(8) e6656;

10. Spengler G, Ramalhete C, Martins M, **Martins A**, Serly J, Viveiros M, Molnar J, Duarte N, Mulhovo S, Ferreira MJU and Amaral L. **(2009)** *Evaluation of Cucurbitane-type Triterpenoids from Momordica balsamina on P Glycoprotein (ABCB1) by Flow cytometry and Real-time Fluorometry*. Anticancer Research.29: 3989-3994;

11. **Martins A,** Iversen C, Rodrigues L, Spengler G, Ramos J, Kern WV, Couto I, Viveiros M, Fanning S, Pages JM and Amaral L. **(2009)** *An AcrAB-mediated multidrug-resistant phenotype is maintained following restoration of wild-type activities by efflux pump genes and their regulators*. IJAA, *in press;*

12. **Martins A,** Vasas A, Schelz Zs, Viveiros M, Molnár J, Hohmann J and Amaral L. **(2009)** *Constituents of Carpobrotus edulis Inhibit the P-glycoprotein of mdr1 Transfected Mouse Lymphoma Cells*. Anticancer Research, *submitted*.

Bellow, **Presentations** that resulted from the work of this thesis are also listed:

1. **Martins A**, Aagaard L, Couto I, Martins M, Viveiros M, Kristiansen JE and Amaral L, "Prolonged Exposure of MRSA COL to Increasing Concentrations of Oxacillin Result in an MDR Phenotype", 8th European Congress on Chemotherapy and Infection (FESCI 8), Budapest, Hungary, 25th to 28th of October 2006;

2. **Martins A**, Ferreira MJU, Viveiros M, Amaral L, "Activity of Carpobrotus edulis extracts against multi-drug resistant bacteria", MICRO-BIOTEC 08, Lisboa, Portugal, 30th of November to 2nd of December 2007.

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LIST OF ABBREVIATIONS

ABC	ATP Binding Cassette
ABCB	ATP Binding Cassette Superfamily type B
ABCC	ATP Binding Cassette Superfamily type C
ABCG	ATP Binding Cassette Superfamily type G
ADP	Adenosine di-Phosphate
AIDS	Acquired Immune Deficiency Syndrome
AMC	Amikacin
AMP	Antimicrobial Peptide
ATCC	American Type Culture Collection
ATP	Adenosine tri-Phosphate
BCG	Bacillus Calmette-Guérin
BCRP	Breast Cancer Resistance Protein
Ca	Calcium ion – the same as Ca^{2+}
CCCP	m-chlorophenylhydrazone
CFU	Colony Forming Units
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CPZ	Chlorpromazine
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EB	Ethidium Bromide
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic Acid
EMB	Ethambutol
EP	Efflux Pump
EPI	Efflux Pump Inhibitor
ERY	Erythromycin
ETC	Electron Transport Chain
ETS	Electron Transport Systems
FAD	Flavine Adenine Dinucleotide
FAR	Fluorescence Activity Ratio

FIX	Fractional Inhibitory Concentration Index
Fl	Fluorescence
GI	Growth Index
GSH	Glutathione
HBSS	Hank's Balanced Salts Solution
HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple Bond Correlation
H-NMR	Proton Nuclear Magnetic Resonance
ESI-MS	Electronspray-Impact Mass Spectrometry
HSQC	Heteronuclear Single Quantum Coherence
IC_{50}	Concentration of a compound that inhibits cell proliferation in 50%
IFN	Interferon
IL	Interleucin
IM	Inner Membrane
INH	Isoniazid
IUPAC	International Union of Pure and Applied Chemistry
JMOD	J-modulated spin-echo
KAN	Kanamycin
Kd	Dissociation Constant
K_M	Michaelis-Menten Constant
LA	Luria Agar
LAM	Lipoarabinomannans
LB	Luria Broth
LM	Lipomannans
LPS	Lipopolysaccharide
LRP	Lung Resistance-related Protein
MATE	Multi-Antimicrobial and Toxin Extrusion
MBC	Minimum Bactericidal Concentration
MDR	Multi-Drug Resistance
MDRTB	Multi-drug Resistant Tuberculosis
MFP	Membrane Fusion Protein
MFS	Major Facilitator Superfamily
MGDG	Monogalactosyldiacylglycerol

MHB	Muller-Hinton broth
MIC	Minimum Inhibitory Concentration
mRNA	Messenger Ribonucleic Acid
MRP	Multi-drug Resistance Associated Protein
MRSA	Methicillin Resistant *Staphylococcus aureus*
MS	Mass Spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular Weight
NADH	Nicotinamide Adenine Dinucleotide (reduced form)
NBD	Nucleotide Binding Domain
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
NOR	Norfloxacin
NP-TLC	Normal Phase – Thin Layer Chromatography
OD	Optical Density
OFX	Ofloxacin
OM	Outer Membrane
OMP	Outer Membrane Protein
OXA	Oxacillin
PAβN	Phe-Arg-β-naphtylamide
PAGE	Polyacrylamide Gel Electrophoresis
PAN	the same as PAβN (use for legend of figures)
PANTA	Antimicrobial mixture that contain Polymixin B, Amphotericin B, Nalidixic acid, Trimethropim and Azlocillin
PAR	Parental mouse lymphoma cell line
PAS	Para-aminosalicylic acid
PBP	Penicillin-Binding Protein
PBS	Phosphate Buffer Saline
PEP	Phosphoenol Pyruvate
P-gp	P-glycoprotein
PIM	Phosphatidylinositol mannosides
PM	Plasma Membrane
PMF	Proton Motive Force

PTS	Phosphotransferase System
PZA	Pyrazinamide
QSAR	Quantitative Structure/Activity Relationship
RES	Reserpine
RFF	Relative Final Fluorescence
RIF	Rifampin
RNA	Ribonucleic Acid
RND	Resistance Nodulation Division
RPC	Rotation Planar Chromatography
RPM	Rotations per minute
RPMI	Roswell Park Memorial Institute Medium
RP-TLC	Reverse Phase – Thin Layer Chromatography
rRNA	Ribossomal Ribonucleic Acid
rtRT-PCR	real time Reverse Transcription Polymerase Chain Reaction
SDR	Single-drug Resistance
SDS	Sodium dodecyl sulfate
SM	Sulfonamide
SMR	Small Multi-drug Resistance
Spp	Species
TB	Tuberculosis
TCA	Tricarboxylic Acid
TDRU	Tetrazolium Dye Reduction Units
TET	Tetracycline
TLC	Thin Layer Chromatography
TMD	Transmembrane Domain
TNF	Tumor Necrosis Factor
TSA	Tryptone Soya agar
TSB	Tryptone Soya broth
TZ	Thioridrazine
v	Volume
VER	Verapamil
WHO	World Health Organization
XDRTB	Extensively Drug Resistant Tuberculosis

I. INTRODUCTION

1. Chemotherapeutics and resistance

1.1 The new Era of the antibiotics

Antibacterial agents are derived either from natural sources (the antibiotics) or from total chemical synthesis. Antibiotics that are sufficiently nontoxic to the host are used as chemotherapeutic agents. Some antibacterial agents act as bactericidal, destroying bacteria, while others are bacteriostatic, inhibiting the growth of bacteria without destruction (1;2).

Since 1928, when Alexander Fleming discovered penicillin (3), and mainly after its commercialization in 1941 (4), the number of antibiotics used in medicine had increased as fast as it could not be imagined at that time. The discovery of penicillin was considered as a miracle of science by many people, and, in fact, it saved thousands of lives. After the success of penicillin other antibiotics, mainly β-lactams, were developed: streptomycin (discovered in 1944 (5)), tetracycline (derived from aureomycin (6) and patented in 1955 (7)) or chloramphenicol (first isolated in 1947 (8)). Within a few years resistance to an antibiotic was observed in the laboratory as well as clinically (3). A few years later the first cases of multi-drug resistance (MDR) were published (9). Due to mono-resistance and multi-drug resistance the need for additional effective antibiotics was evident and resulted in the creation of new antimicrobials derived from the few natural antibiotics known at that time. The “golden age of antibiotics” was born! However, it is amazing that, after thirty years of success in the search for new antibacterials, only three classes of antibiotics have entered the market since 1970 (10).

Nowadays, the wide range of antibiotics is grouped into different classes according to their mode of action, spectrum of activity or similarities in the chemical structure. The latter classification is the most common.

In general, antibiotics are grouped as (8):

- **Aminoglycosides**: such as amikacin, gentamicin, kanamycin or streptomycin. This class of antibiotics derived from bacteria (order of Actinomycetales) interferes with protein synthesis by binding to the 30S component of the bacterial ribosome;

- **Antimycobacterial agents**: This group includes a wide range of antibacterials used against *Mycobacterium* spp. (tuberculosis, leprosy, etc) and includes, for example, rifamycin, isoniazid, pyrazinamide, ethambutol, streptomycin, amino salicylic acid, sulphones;

- **Cephalosporins and related β-lactams**: Cephalosporins are synthetic compounds derived for the natural antibiotic cephalosporin C. As the penicillins, members of this group inhibit bacterial cell wall synthesis. The first generation cephalosporins are active against Gram-positive bacteria but are not active against methicillin-resistant staphylococci. The second generation is highly resistant to β-lactamases and is active against Gram-negative bacteria. Third and fourth generations are broad range antibiotics because they are active against many Gram-positive and Gram-negative bacteria.

- **Chloramphenicol**: The first broad spectrum antibiotic whose activity is mainly bacteriostatic by interfering with protein synthesis.

- **Glycopeptides**: An example of this group is vancomycin that interferes with the cell wall synthesis. It is active against Gram-positive cocci.

- **Macrolides**: Large group of antibiotics that have a common macrocyclic lactone ring to which one or more sugars are attached. These antibiotics can be bacteriostatic or bactericidal, depending on the organism, and interfere with the protein synthesis. An example of this group is erythromycin.

- **Penicillins**: This group inhibits the cell wall synthesis and its action is, in general, bactericidal. Although penicillin is still in use, derivatives of this antibiotic, such as ampicillin, have been more widely used.

- **Quinolones**: Quinolones are synthetic antibiotics structurally related to nalidixic acid. Modifications of the structure of nalidixic acid yielded fluoroquinolones which include, for example, ciprofloxacin, ofloxacin or norfloxacin.

- **Sulfonamides**: They are usually bacteriostatic and interfere with the folic acid synthesis. Its use was greatly reduced because of the development of resistance. An example of a sulfonamide is the Prontosil.

- **Tetracyclines**: The compounds of this group are usually bacteriostatic and their mechanism of action is similar to that of aminoglycosides. Tetracyclines have a broad spectrum of activity. The increase of resistant strains and the adverse effects have reduced their use. However, they are the most commonly used antibiotics in animal husbandry.

The problem of resistance has been detected for all the classes of antibiotics described above (8). Development of antibiotic resistance results from the selection of bacterial populations whose antibiotic target has mutated during the time the population has been exposed to the antibiotic (antibiotic pressure). The frequency of resistance of a bacterial species to a given antibiotic is a product of antibiotic misuse and ineffective therapy. In economically disadvantaged countries, inadequate access to drugs contributes heavily to the frequency of antibiotic resistance (11). The problem of antibiotic resistance and its causes, are of extreme importance with respect to infections such as tuberculosis, and are the major motive for the work conducted in this thesis.

Antibiotic resistance is not restricted to bacteria. It is found in all infectious non-bacterial agents as well as in cells of an animal that is treated with a chemotherapeutic agent. Chemotherapeutically treated cancer that becomes refractory to the agent used for therapy and to any other chemotherapeutic agent, is an example of resistance that has been extensively described in the literature (12-15). A general overview of cancer, its therapy and resistance mechanisms will be discussed later on this thesis.

1.2 Fight against resistance

It is widely accepted that the problem of drug resistance, even in prokaryotic or eukaryotic cells, does not yet have solution (16-19).

Although, predictably, new resistance will emerge with time, there are ways to frustrate this possibility. Quality in education of health workers, easier access to a wide range of effective antibiotics, in economically disadvantaged countries, coupled to education of the patient population, the selection of appropriate therapy and patient compliance are the most important points according to this question.

Albeit, new drugs do not give the ultimate answer to this question, the discovery of new families of antibiotics, with new characteristics, still has high prospects.

On the other hand, to find new compounds that are able to potentiate or restore the decreased activity of existing antibiotics is, nowadays, commonly accepted as one of the best approaches to fight against multi-drug resistance (20-22).

The concept of adjuvant therapy has emerged (discussed in chapter 6 of this Introduction). In order to achieve practical benefits from this strategy, many studies have been performed leading to a better understanding of the mechanisms of resistance acquired by bacteria.

1.3 Mechanisms of resistance

As already mentioned, the understanding of the resistance mechanisms is one of the first steps leading to a possible solution against multi-drug resistance. Bacteria develop resistance against certain antibiotics or groups of antibiotics by several mechanisms. For the bacterial cells, these mechanisms can be intrinsic or acquired:

- **Intrinsic resistance**: some bacteria have low permeability to different classes of antibiotics/compounds (23). For example, Gram-negative bacteria and mycobacteria have thick and highly hydrophobic outer membranes, which act as a permeability barriers to hydrophilic compounds, such as macrolides (ex. erythromycin) (24).
- **Acquired resistance**: through mutation, acquisition of new genetic material (plasmids encoding for resistance mechanisms; foreign genetic elements; etc. (25;26)) affords survival under antibiotic pressure (19) - Figure 1.

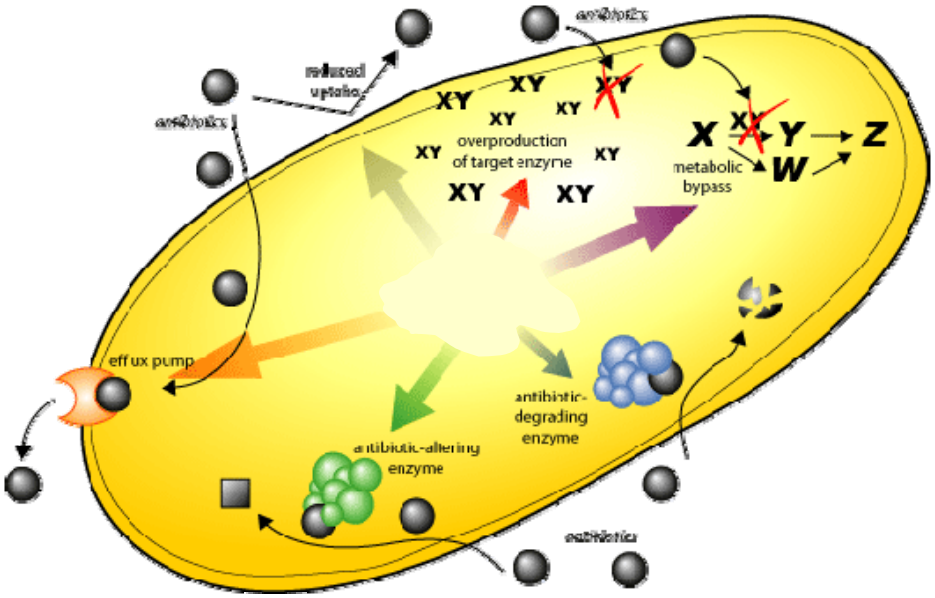


Figure 1 - Schematic representation of cell mechanisms of resistance.
Some mechanisms can lead to resistance through different approaches, as for example the different ways by which the uptake of the antibiotic can be decreased or even forbidden. These differences are described in the text. Adapted from (27).

The most typical mechanisms of resistance, as presented by Figure 1, are:

- **Modification of the antibiotic target / site of action**, so that the antibiotic cannot bind to it and render it inactive (resistance to macrolides, vancomycin, β-lactams, fluoroquinolones and aminoglycosides) (28);
- **Enzymatic inactivation of the antibiotic** by secretions of enzymes that degrade the antibiotic (ex. β-lactamases) or modify the antibiotic so that it is ineffective (ex. the resistance to chloramphenicol (CHL) - In resistant strains,

CHL is acetylated at the C3 hydroxyl group, by a cytoplasmic enzyme, CHL acetyltransferase. CHL acetyltransferase enzymes can be plasmid or chromosomally encoded.) (29);

- **Overproduction of the target** such that the amount of antibiotic is not compatible with the amount of target (30) (ex. Resistance to vancomycin in staphylococci. The strain over-expresses the targets sites of vancomycin far from the key places for the cell survival, cell wall synthesis, so that it acts at that place without block the critical target sites) (31);
- The target to be inhibited is part of a multi-chemical pathway that has alternative pathways which lead to the needed product **(Bypass of inhibitory steps)** (ex. Sulfonamide resistance mediated by different plasmid encoded enzymes with the same activity as the antibiotic target but structurally different and not affected by the antibiotic) (32);
- Developing **mechanisms that do not allow the accumulation of the antibiotic** within the cell, preventing it from reaching the necessary concentration to be active (19):

    - **Decreasing the permeability of the cell wall** to an antibiotic. For instance by decreasing the expression of porins (for example OmpF and OmpX which are involved in the control of the penetration of antibiotics such as β-lactams and fluoroquinolones through the enterobacterial outer membrane (33)) or reducing the internal size of the pore through mutation of some amino acids (34);
    - **Increasing efflux activity**, which by extrusion of the antibiotic decreases its cellular concentration (19;35;36). Ex. resistance of *Escherichia coli* to tetracycline (8;35;37).

The combination of these two last systems leads to high level of resistance mainly in Gram-negative bacteria (38).

With respect to eukaryotic cells and, especially cancer, the main mechanisms of resistance are yet to be completely understood. However, overproduction and

modification of enzymatic targets, over-expressed efflux mechanisms, ability of the cells to avoid apoptosis, are well studied mechanisms (39). Resistance to anticancer drugs also results from other factors including individual variation among patients and somatic cell genetic differences in tumors, even those from the same tissue of origin. Frequently, resistance is intrinsic to the cancer, but with prolonged or ineffective therapy, acquired resistance is common. The most frequent cause for acquisition of resistance to a broad range of anticancer drugs is the over-expression of one or more energy-dependent transporters – efflux mechanisms. However, insensitivity to drug-induced apoptosis and induction of drug-detoxifying mechanisms probably play an important role in acquired anticancer drug resistance (12;15;39-41).

The next two chapters will focus on the mechanisms of resistance that are related with the bacterial cell envelope.

2. Cell envelope and resistance

The structure and ensuing characteristics of the cell envelope have an important role in the resistance of bacterial cells to antibiotics. The cell envelope of Gram-negative and Gram-positive bacteria, mycobacteria and the plasma membrane of eukaryotic cells will be described in this chapter.

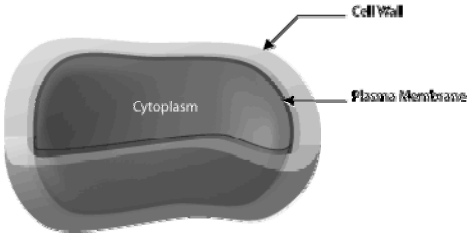


Figure 2 – Schematic representation of the cell envelope of a bacterial cell.
The thickness and composition of the cell wall change between the Gram-positive and Gram-negative cells. Source: http://www.scq.ubc.ca/

2.1 Cell envelope

The cytoplasm of prokaryotes and eukaryotes is surrounded by a unit - the plasma membrane (PM). The plasma membrane is itself, a very important organelle of the cell that controls input and output of compounds. With respect to bacteria, an additional membrane is external to the plasma membrane and provides supplementary properties that contribute to its survival in the environment it normally inhabits. This outer membrane (OM) differs between Gram-negative and Gram-positive bacteria; the former having a more complex structure than that of the latter. Whereas intrinsic or acquired resistance of eukaryotes is determined by the characteristics of its plasma membrane, intrinsic and acquired resistance of prokaryotes is due to the structure of the cell wall and properties of the plasma membrane.

The cell envelope consists of different structures:

1) **Plasma membrane**

Electron microscopic examinations of cell membranes have led to the development of a lipid bilayer model of the PM. The bi-molecular leaflet of phospholipids is arranged as shown by Figure 3.

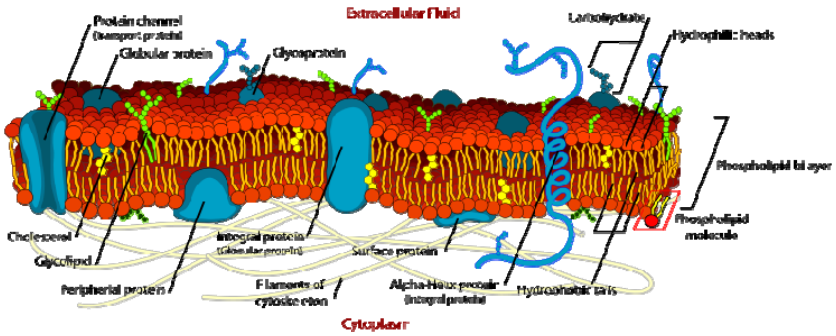


Figure 3 - General structure of the cell membrane.
Source: http://cellbiology.med.unsw.edu.au/units/images/cell_membrane.png

In general, membranes are highly conserved structures, similar for all living things (with exception of the virus). The bi-layer structure of the PM is stabilized by hydrophobic interactions - Wandervals forces, ionized groups of amino acids, lipid interactions. Membranes are very organized but asymmetric organelles, due to the difference of environments in both of their sides. Even if well organized organelles, membranes are dynamic in order to an easily adaptation to possible changes of the environmental conditions (42). Lipids and proteins are its main composition.

Attached to the surface and cytoplasmic side as well as within the leaflet layer of the PM are proteins. The majority of membrane proteins are responsible for structural functions, the moving of small molecules across the membrane

(transporters, ATPases, kinases, etc) or involved in energy generation (ATP synthase, enzymes involved in final steps of glycolysis, etc).

The lipids form a bilayer, with their hydrophilic portion facing the aqueous environment, and the hydrophobic parts clustering together inside the membrane. The majority of the lipid contents are phospholipids. The phospholipid bi-molecular leaflet with its functional transport units, bestow a semi-permeable barrier-like characteristic to the PM, allowing some molecules to enter under control and others are kept outside in a selective interaction manner (23). The fluidity of the lipid bilayer affects its permeability in an inversely proportional way. About 50% of the bacterial species also contain hopanoids (Figure 4), molecules with similar structure to sterols found in eukaryotic membranes that help to the stabilization of the membrane.

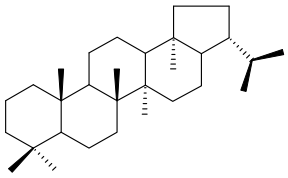


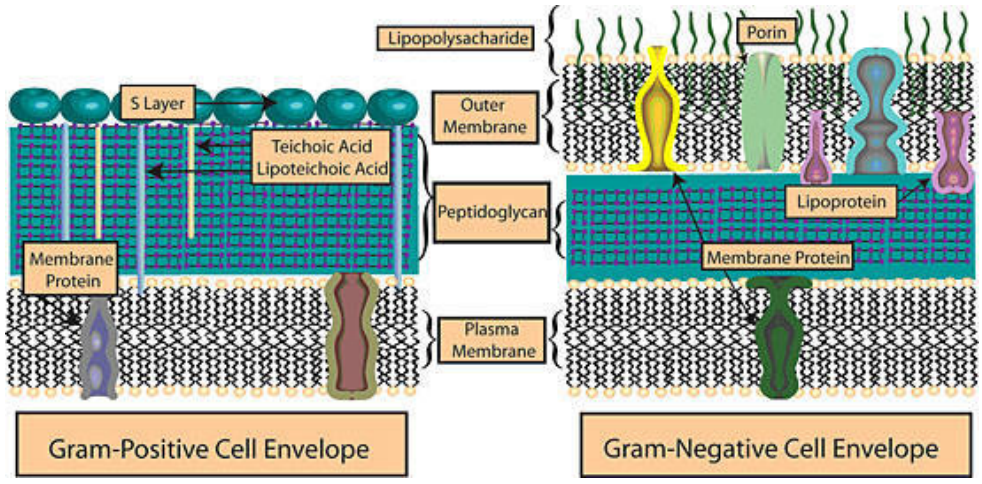
Figure 4 - Chemical structure of Hopanoid.
Hopanoid is a sterol molecule found in eukaryotic membranes that helps in its stabilization.

2) **Cell wall**

The cell wall is located outside the plasma membrane and protects the cell from osmotic lysis. It does not exist in all organisms. Some bacteria have cell walls containing peptidoglycan.

The structure of the cell envelope of Gram-negative and Gram-positive bacteria, as well as of mycobacteria, is different and provides them special characteristics and different levels of intrinsic resistance. The organization of their bacterial envelope is represented in Figure 5.

A



B

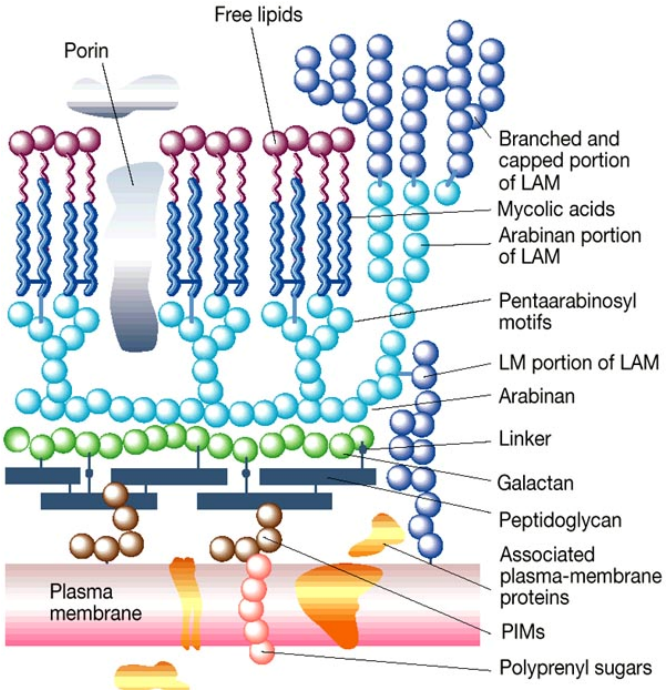


Figure 5 - Organization of the bacterial cell envelope.

A – Gram-negative and Gram-positive bacteria. Source: (43); **B** – Mycobacteria. Source: (44). LAM, lipoarabinomannans; LM, lipomannans; PIM, phosphatidylinositol mannosides

2.1.1 Gram-positive

In Gram-positive bacteria, the cytoplasmic membrane, that immediately surrounds the inside of the cell, is a thin structure with about 8 nm thick (Figure 5A). This structure is followed by a cell wall (15-80 nm thick), consisting of several layers of peptidoglycan complexed with teichoic acids. Some cell wall components protect against phagocytic engulfment or digestion. Examples of Gram-positive bacteria are the species belonging to the Staphylococcus genus. *Staphylococcus aureus* is an opportunistic pathogen and the major causative agent of numerous hospital and community acquired infections. The genome of *S. aureus* strains had been already sequenced (45) and became possible to identify many components of its cell envelope that interfere with its virulence, with its intrinsic resistance and components that are responsible to the acquisition of multi-drug resistance (discuss bellow on this section) (45;46).

2.1.2 Gram-negative

The cytoplasmic membrane is relatively fluid in order to allow the proper functioning of the cell membrane components (so then also permeable) and so, many bacteria developed other structures to protect themselves. Those include peptidoglycan cell wall, an outer membrane or layers of lipopolysaccharides (LPS) in which the lipid A possesses endotoxin activity (47).

The LPS, which are organized in several structures, according to the species, are, probably, the explanation of the unusual slow influx of lipophilic solutes through the outer membrane (23). The external membrane provides a very impermeable barrier and, so, bacteria developed other mechanisms that provide the influx of nutrients from the external medium. These uptake is done through proteins, called porins, which allow the influx of nutrients by providing non-specific and specific channels across the membrane (47). The hydrophobic (lipid) pathway, which is based on the interaction between the

membrane lipids is other option (38). The most important function for the outer membrane, in Gram-negative bacteria, is to serve as a selective permeation barrier (23). The need of a response to environmental changes could lead to modifications in the LPS structure or in the porin constitution (23;38;48). This LPS structure will be described below, in detail, as well as its contribution to the intrinsic and emerging of resistance to antibiotics among bacteria. Escherichia spp. or Salmonella spp. are examples of bacteria with this kind of cell envelope (Figure 5A).

2.1.3 Mycobacteria

Mycobacterium is an example of how important is the constitution and organization of the cell envelope in the permeability of the cell and how it can influence resistance to antibiotics. The most important characteristic of the cell envelope of mycobacteria is its constitution (Figure 5B): a cell membrane followed by a cell wall kind skeleton. This "cell wall" is composed by mycolic acids in a unique arrangement of these long chain fatty acids (up to 90 carbons) covalently bound to the arabinogalactan, a single polymeric head group (49). This characteristic confers to the barrier much less permeability than the outer membrane of Gram-negative bacteria (38). For example, its lipid rich cell wall also makes it resistant to Gram staining used to identify Gram-negative and Gram-positive strains. Acid-fast techniques are used instead. Ziehl-Neelsen staining is the most common technique in which the mycobacteria stain a bright red, which stands out clearly against the background.

2.2 Lipopolysaccharides composition

Resistance to antibiotics can be achieved by changes in the permeability of the cell membrane of the bacteria. Acquired resistance to active antibiotics through a decrease

in the permeability of the cell membrane requires major changes in membrane organization (50). However, due to the composition of the cell barrier, there is a difference in the susceptibility to hydrophobic and hydrophilic compounds (51).

Gram-negative bacteria have an extra "protection" given by the outer membrane. For this reason some antibiotics that are active against Gram-positives are not active against Gram-negatives. Its LPS composition increases the asymmetry in the membrane architecture and the cross binding between LPS and divalent cations decrease the permeability (23;50).

An increase in the permeability can be achieved by disruption of the cross bindings by compounds that act as metal ion chelators such as EDTA, certain cationic antimicrobial peptides (AMP) and polyamines(50). LPS (Figure 6) is typically composed of lipid A, a short core oligosaccharide, and an O antigen that may be a long polysaccharide, and is a very effective barrier for spontaneous diffusion of lipophilic compounds through the outer membrane (23).

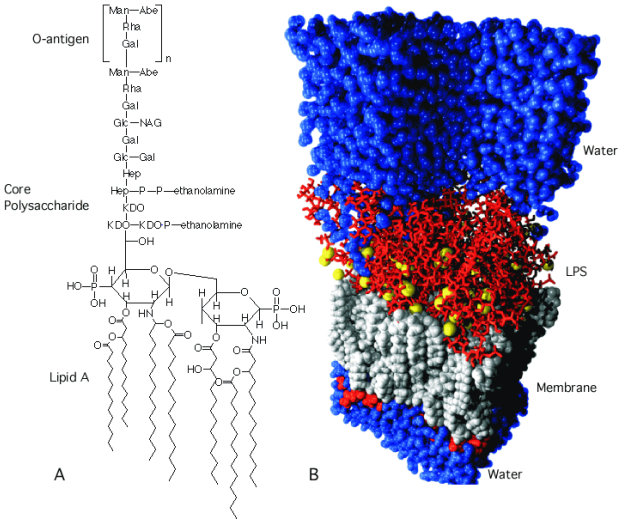


Figure 6 - Lipopolysaccharide (LPS) composition

A – The chemical structure of LPS; B – Molecular model of the membrane from *Pseudomonas aeruginosa*. Source: (42)

This effective barrier to hydrophilic compounds of the LPS leaflet is mainly due to 1) the low fluidity of the LPS hydrocarbon domain, 2) the strong lateral interactions between the LPS molecules, 3) conformation of the LPS in bilayers and 4) the conformation of LPS in a complex with the protein FhuA (23).

Salmonella spp is an example of the LPS importance in virulence as well as intrinsic and acquired resistance to antibiotics (23;52). LPS modifications are the end result of activation of the two-step PmrA/B regulon. This activation involves the phosphorylation of plasma membrane embedded sensor PmrB, which in turn phosphorylates PmrA (Figure 7). The latter activates PmrD which de-represses an operon that consists of 9 genes. Each gene codes for an enzyme in the lipid synthesis that is eventually introduced into the nascent LPS by the flipase MsbA protein. These modifications include both Ara4N and phosphoethanolamine additions to the lipid A and phosphoethanolamine addition to the LPS core. They mask the phosphate groups with positively charged moieties, affecting the electrostatic interaction of certain cationic antimicrobial peptides with the bacterial cell surface after its phagocytosis by the neutrophil (23;52). These mechanisms are part of two-component systems responsible for the response of the bacterial strains to their environment. Many of these systems activate virulence-factor expression. They are regulated by host-derived signals that control gene expression at the key time and place for optimal establishment and maintenance of infection. This two-component response of the bacterium strains allows them to survive within the neutrophil. The PmrA/B response activates virulence-factor expression. The PmrA/B response is regulated by host-derived signals that control gene expression at the key time and place for successful infection. (52).

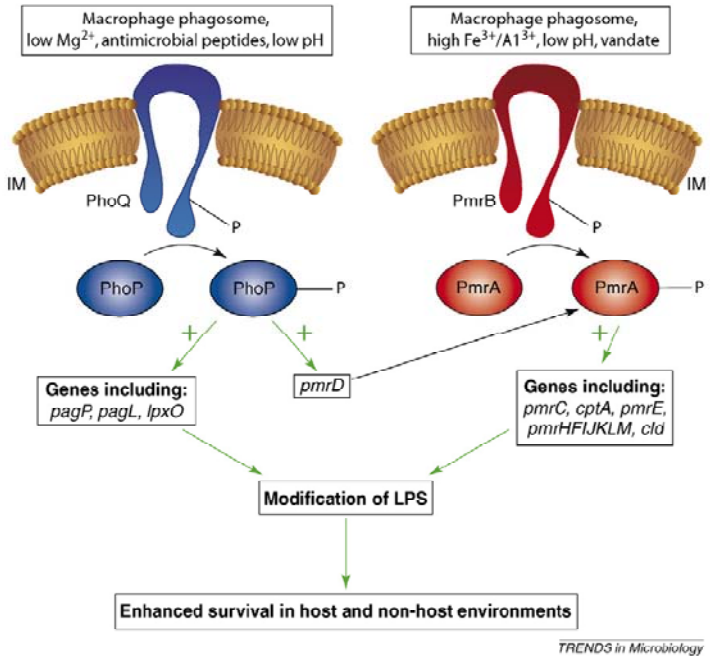


Figure 7 - Model of the activation and interaction of the PhoP/Q and PmrA/B two-component system in Salmonella spp.
PmrA/B can be activated in an indirect manner through PhoP/Q and PmrD or in a direct manner, involving activation and autophosphorylation of PmrB and subsequent phosphotransfer to PmrA, which enhances PmrA binding to regulated promoters. On sensing activating environmental signals, PhoP/Q enhances the transcription of several genes including *pmrD* (green arrow with '+'), whose product binds to and stabilizes PmrA in its phosphorylated state. This loop can be deactivated by the transcriptional repression of *pmrD* by PmrA. PmrB can sense environmental stimuli directly and can initiate a cascade to phosphorylate and activate PmrA. LPS modifications mediated by both systems aid in survival in host cells and in the environment. Source: (52)

Three main proteins are involved in the PmrA/B complex: phosphoethanolamine phosphotransferase (PmrC), a response regulator (PmrA) and a sensor kinase (PmrB), which regulates the expression of other genes in bacteria such as the ones involved in the modification of the LPS (52).

The PhoP/Q system is an example of another two-component system which affords either the indirect regulation of the PmrA/B or directly activates the operon that regulates Lipid A synthesis (Figure 7**).** Activation of PmrA/B *in vitro* takes place by acidic pH and certain antimicrobial peptides and is repressed by millimolar (mM)

concentrations of magnesium and calcium. This is an example of an indirect activation of the PmrA/B system (52;53).

All of the regulatory systems that preserve the integrity of the cell envelope have physiological significance in pathology. The response of bacteria to cations may have significant importance in an environment such as the wild aqueous environment, or, when bacteria are internalized by phagosomes, where the divalent cations concentration can achieve 1mM (53). However, Murata and co-authors showed that in the presence of 1mM of Mg^{2+} cells with a modified outer membrane were more resistant to the permeation of exogenous compounds than cells without modification. This can be an advantage to the bacterium because it slows the influx of antimicrobial compounds generated by the host cells, such as cationic, lipophilic antimicrobial peptides and nitric oxide (NO). The permeability properties of the bilayer are also indicators of the robustness of the membrane structure, because permeation of solutes involves local stretching and rearranging of the bilayer structure as solute molecules pass through it. In lipid A, the acidic phosphate and pyrophosphate residues of the neighbouring molecules are bound together by the bridging action of divalent cations. When some of these cations are removed, or their concentration is too low, or competitively used by the host cell itself, the membrane structure becomes unstable - the survival of the bacterium is compromised (53).

2.3 Other important functions of membranes

Another function associated with membranes is the generation of energy by the electron transport chain (42). The protons generated are translocated to the surface of the bacterium via channels where they are attracted to proton loving components of the LPS creating an electrochemical gradient. This process creates a proton gradient with the higher concentration of protons on the surface and the lowest concentration in the medial side of the plasma membrane (54). The distribution of protons along the surface of the cell creates a pH of the medium immediately surrounding the cell that is at least

two to three units lower (55). The difference in proton concentration between the surface and medial side of the plasma membrane creates the proton motive force (PMF). Protons present in the periplasm of the Gram-negative are used by PMF dependent transport systems, and energise the transporter component (52). After this step, they are released to the cytoplasm of the cell. Used protons of the periplasm are replaced from surface bound protons. The protons released to the cytoplasm decrease the local pH thereby shifting the equilibrium of ATP synthase towards the synthesis of ATP. This ATP may be used by ABC transporters which bind and hydrolyse ATP to ADP and two protons. These protons serve as the energy source for the membrane bound ABC transporter (56).

2.3.1 Plasma membrane bound enzymes.

The synthesis of the cell wall of bacteria is performed by a collection of membrane bound enzymes. Their location on the membrane is highly specific. Because these enzymes bind penicillin, they have been named “penicillin binding proteins” or PBPs. When penicillin or other β-lactam antibiotics bind to the PBPs the enzymatic activity of the enzymes is obviated, the cell wall cannot be made and the organism cannot replicate. Resistance to β-lactams may be the result of secretion of β-lactamases of which there are three groups - A, B and C. Inhibitors for A and B β-lactamases are available and used as adjunct to β-lactam therapy. There are at this time no inhibitors for Group C β-lactamases (57).

The mode of action of the β-lactam family of antibiotics is a good example that shows how all of these factors are so important for the understanding of resistance mechanisms. β-Lactamases and penicillin-binding proteins (PBP) play a key role in the resistance to β-lactam antibiotics. These proteins take part in the cell wall synthesis and are located no randomly on the outer face of the cytoplasmic membrane. The DD-transpeptidase is one of the activities under the responsibility of the PBPs (58).

Briefly, the compounds belonging to the family of β-lactams interfere with the biosynthesis of the peptidoglycan, the major component of the bacterial cell wall. The structural similarity of the β-lactam to the substrate of the DD-transpeptidase is the basis for the antibiotic being bound to the PBP. The inhibition of this enzymes leads to structural instability and death of bacteria. However before reaching the target, β-lactam antibiotics have to cross the outer membrane by interaction between the antibiotic and the lipid phase or through a specific channel protein (the porin) (59).

Perhaps the most common example of PBP-mediated clinical resistance to β-lactams is methicillin resistance of *Staphylococcus aureus* (MRSA). MRSA is due to the acquisition of a DNA element from an unknown source and its introduction into the organism's chromosome (58). Klitgaard and co-authors observed that the reversal of resistant to oxacillin in MRSA by thioridazine was due to a reduction in the transcription of the *mecA* and *blaZ* genes resulting in a reduced protein level of PBP2a (60)

3. Transport across the cell envelope (its outer and inner membranes)

However, the transport of the majority of compounds across the membrane requires the intervenience of specialized structures – the transport systems. It can mainly be done through three basic types of transport (42).

1) **Simple diffusion**: water, carbon dioxide and oxygen are among the few molecules that can cross the cell membrane by diffusion. The movement across the membrane is done in response to differences in concentration: lower to higher concentration of solute in water (osmosis) and higher to lower concentration in diffusion of the gas (CO_2 and O_2).
2) **Facilitated diffusion**: this process is done via the binding of the molecule to a membrane protein and, by change of the conformation of the protein, the compound is moved across the membrane. An example is the transport of glucose into *E. coli* cell that, once inside the cell, is immediately phosphorylated; if not, it diffuses to the outside.
3) **Group translocation**: It is a process similar to the facilitated diffusion but involves a specific biding of the molecule (ligand) to a membrane protein (receptor), followed by an energy dependent chemical modification of the ligand. Group translocation is unidirectional.
4) **Active transport**: It is used to transport small molecules without any chemical alteration and it is an energy dependent process. The energy can be generated by ATP or ion gradients. An active transport protein can be highly specific for one molecule or recognise and transport chemically unrelated molecules. This type of transport will be discussed in detail in later sections of this thesis and is one of the important contributors to antibiotic resistance.

3.1 Outer membrane proteins and porins

The regulation of membrane permeability of Gram-negative bacteria is a function of membrane proteins. Regulation involves the joint action of porins and efflux pumps. Porins are trimer of identical subunits, each consisting of an anti-parallel β-barrel containing a pore (61), found in Gram-negatives and mycobacteria (Figure 8) that form channels that traverse the outer membrane and end in the periplasm.

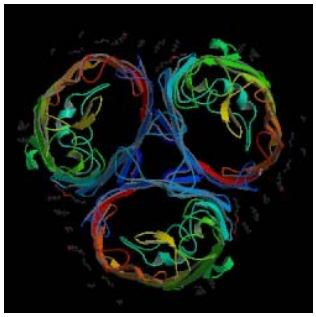


Figure 8 – Structure of OmpF porin trimer.
The looping strands of each porin protein bind with the adjacent loop of the next porin (Shown in the form of a triangle in the center of the three proteins). The constriction channels are the open spaces inside each porin. The internal loop can be seen as "wrapping around" this opening (61). Image produced by the Protein Data Bank.

The tri-barrel structure consists of three identical outer membrane proteins (OMPs). The OMP for each porin is genetically controlled so that the type of OMP that makes up the porin bestows the specificity or non specificity of the porin. As an example, the main porin of *E. coli* is porin F and consists of three OmpF units (61).

Not all OMPs contribute to porin structure. OmpA is an integral component of the cell envelope and provides structural integrity. Other OMPs make up many different porins which are highly specific for given compounds or nutrients that the cell requires for survival. Porins permit the entrance of small solutes, ions and water to the periplasm of

the Gram-negative bacterium. With respect to antibiotic effectiveness, porin F is the porin primarily used by antibiotics to penetrate the outer cell envelope of *E. coli* (62).

OmpF, OmpC, PhoE, OmpD and Omp36 are representative examples of non-selective OMP / porins (63). OmpF and OmpC families show a slight preference for cations, whereas PhoE selects inorganic phosphate and anions (64). LamB or FhuA are selective membrane proteins responsible for the diffusion of sugars and metals. OmpA and TolC form channels to avoid the passage of a wide variety of molecules (63).

Most porins that are involved in antibiotic transport belong to the classical OmpF or OmpC subfamilies. However, there are exceptions, such as OprD of *Pseudomonas aeruginosa* and porins from *Acinetobacter baumannii* and *Neisseria* spp (64).

For example, the well studied OmpF from *E. coli* is considered the main entry for different classes of antibiotics such as β-lactams or fluoroquinolones as well as a large variety of small hydrophilic molecules to be translocated from the cytoplasm to the periplasm of the cell (34;38;59). Indeed, some β-lactam resistant strains of *E. coli* have shown a deficiency in OmpF expression or alterations in its loop structure, caused by mutations. This is very important because the interaction of the antibiotic with the surface of the channel determines its penetration inside the cell (59). In fact, clinical isolates with porin modification were already identified in many Gram-negative strains (64).

The loss, downsizing or mutation of porins are mechanisms through which bacteria can decrease their permeability to antibiotics providing resistance (47). For example, porin-deficient mutants are more resistant to quinolones, tetracyclines, chloramphenicol, nalidixic acid and trimethoprim (38). *P. aeruginosa* has innate low susceptibility to β-lactams due to its low number of porins with distinct physico-chemical properties compared to other strains (64).

It was shown that the expression of OmpC and OmpF, controlled by the concentration of some antibiotics in the environment, regulates the permeability of the outer membrane to glucose and nitrogen under nutrient deficient conditions (65). Most studies had been done in order to define which are the mechanisms involved in that regulation

(33;59;62;65). It was shown that some clinical isolates, from patients undergoing treatment, had changed their membrane permeability due a switch in expression from OmpF to OmpC, a porin whose pore size is smaller, suggesting that a modification in the porin balance had occurred during the treatment (64). OmpC-OmpF balance is strongly regulated by different genetic control systems, such as EnvZ-OmpR and RNA anti-sense regulators (MicF and MicC) (64;66).

Some *in vitro* studies also showed that loss of OmpC is followed by the expression of another subfamily of porins. OmpN type of porin is structurally related to OmpC and OmpF. OmpN pore is a selective filter for charged molecules due to its structural organization. It allows the maintenance of bacterial fitness with the entrance of nutrients but not of the antibiotics. This increases the resistance to the β-lactams (64). OmpX is other important outer membrane protein. It is small and, together with OmpF, is involved in the response to external stress via different regulation cascades (33).

3.2 Efflux pump mechanisms

The cellular efflux systems (Figure 9) are responsible for the extrusion of both the endogenous and exogenous toxic compounds (17;67), playing an important role in the physiology and homeostasis of the cell (19). Some efflux pumps have also been shown to have a role in colonization and persistence of bacteria in the host, as well as in bacterial pathogenicity (68). Efflux pumps are also useful tools for the cell to remove antibiotics conferring resistance to a given drug or class of drugs (36).

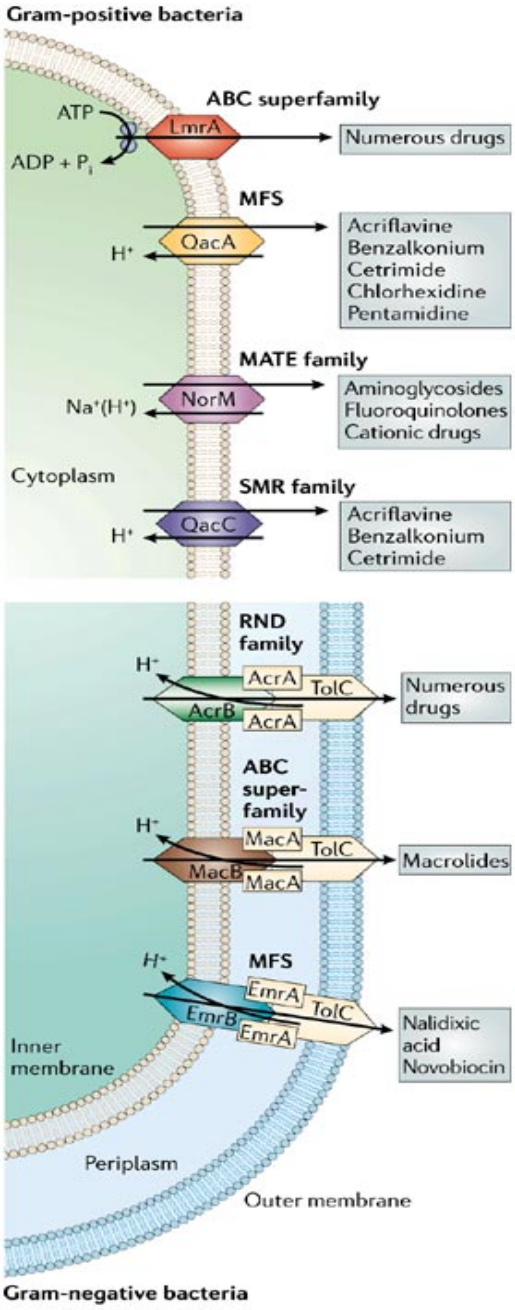


Figure 9 – Schematic representation of the 5 families of membrane transporters in Gram-positive and Gram-negative bacteria.

Source: (68)

According to Marquez *et al* (19) and Bambeke *et al* (67), efflux pumps can be grouped into two major groups: 1) primary active transporters; and, 2) secondary active transporters (antiports, symports and uniports) (67).

3.2.1 Primary active transporters

ATP-binding cassette (ABC) superfamily is the one responsible for the antibiotic efflux among the primary active transporters. It uses the hydrolysis of ATP for the source of protons that energise it. These transmembrane proteins are present in all membrane systems of the cell and play an important role in the transport of toxins, metabolites and drugs (67;69).

ABC transporters are composed of six to, as many as, twelve transmembrane domains (TMDs). Among these domains are two nucleotide binding domains (NBDs) and two domains that recognise the agent to be transported. The remaining domains are integrally involved in membrane structure. NBDs are water soluble proteins associated with the TMDs on one side of the membrane. The TMDs form a transport channel. Their structure and number can vary in the ABC transporters, as well as the number of transmembrane helices (between 8 and 20) (70-72). In contrast, the NBDs are highly conserved with Walker A and B motifs which are characteristics of all the ATP-binding proteins. The signature motifs are specific sites of the ABC transporter where ATP is bound and hydrolysed. The protons released energise the pump for its transport function (70).

The coupling mechanism between the two different domains of the transporter is still unknown. However, some crystallographic studies support the theory that when the ATP is bound and an NBD dimer is formed, TMDs change their conformation that results in their closing to the inside of the cell and opening to the periplasmic side. When the hydrolysis of ATP occurs, the structure returns to its initial state (70).

These typical efflux pumps are usually involved in the resistance to only one drug (Single Drug Resistance – SDR transporters) (19). However ATP transport systems involved in multi-drug resistance have also been reported (67). ABC transporters are also frequent in antibiotic-producing prokaryotes, conferring to them self-resistance to the drug they produce. Streptomyces spp. is an example (19). These transporters when present, are also responsible for the resistance of Gram-positive bacteria to macrolides or bacitracin; examples: Staphylococci (69) and Enterococci (19). The DrrB pump of

Mycobacterium tuberculosis and the MacB pump of *E. coli,* which are responsible for the extrusion of macrolides (69) and the Sav1866 pump of *S. aureus* (70), also belong to this superfamily of ABC transporters.

In mammals, the MDR1 and MRP1 pumps are two important groups of transporters (73) and exist in different kinds of cells of the human organism, including important cells of the immune system such as lymphocytes, monocytes and macrophages (74). The P-glycoprotein (P-gp) belongs to the family of the MDR1 transporters and it is responsible for the extrusion of physiological substrates such as phospholipids and cytokines. Moreover, they are also capable of extruding antibiotics such as fluoroquinolones, macrolides, β-lactams (lipophilic cephalosporins), tetracyclines and trimethoprim (74). When over-expressed in the cancer cell, P-gp confers resistance to cancer chemotherapy (41). The mechanisms responsible for the extrusion of fluoroquinolones, rifamycins and macrolides in cancer cells should also be mentioned as an example for the presence of MRP1 efflux pumps in eukaryotes (74). The special case of P-gp transporters in cancer and their inhibition by compounds isolated from *Carpobrotus edulis* is a subject that will be discussed in detail in the sections dealing with cancer.

3.2.2 Secondary active transporters

This group of multi-drug transporters utilizes the proton (or sodium) motive force – proton gradient across the membrane – as source of energy (67). They are present in bacterial cells and most of them confer intrinsic resistance to antibiotics and, when over-expressed or acquired from external sources (plasmids), result in multi-drug resistance of the bacterium (67) (Figure 10). There are different concepts for their grouping (19;36;67), but it is consensual that these transporters have four main superfamilies (36) according to the followings:

- **MFS** (Major Facilitator Superfamily) transporters are the largest of the families and consist of over 1000 individual, genetically characterised transporters. MFS transporters are responsible for the transport (influx) of sugars, amino acids, and many other nutrients and extrusion (efflux) of intermediate metabolites, cellular toxins and drugs (19;67). The proteins from this superfamily efflux antibiotics such as, erythromycin, nalidixic acid, tetracycline, sulfamides, fluoroquinolones, rifampicin or chloramphenicol, and catalyse uniport, solute/cation (H^+ or Na^+) symport, solute/H^+ antiport or solute/solute antiport (67). It is an ancient (highly conserved), large and diverse family and it is the most common family in the Gram-positive bacteria (72). These drug-efflux pumps can be grouped into smaller groups of which the Bmr of *Bacillus subtilis*, the QacA of *S. aureus* or the MefA of *Streptococcus pyogenes* are examples (72). The NorA pump of *S. aureus*, EmrB of *E. coli*, Tap of *M. tuberculosis* or LfrA of *Mycobacterium smegmatis* (67;69) are other well known members of this family. These proteins are situated in the inner membrane and contain 12- or 14- TMDs (72). These transporters function as single-component pumps (e.g. NorA); however, in some Gram-negative bacteria they function in conjunction with membrane fusion proteins (MFPs) and outer membrane components (e.g. EmrAB-TolC of *E. coli*). The tetracycline efflux pumps are some of the best characterized members of the MFS family. They are found in both Gram-negative and Gram-positive bacteria (72). Efflux of tetracycline by Gram-negative can be due to transposons that carry the efflux pump genes from unknown sources and which have been inserted into plasmids that infect the cell. Some authors suggest that there is an horizontal transfer of these plasmids among different genera of Gram-negative bacteria that share the same environment such as the colon of the tetracycline treated animal (75).

- **RND** (Resistance Nodulation Division) family ensures the efflux of lipophilic and amphiphilic molecules and toxic divalent cations (19;67). These transporters are the main contributors for resistance in Gram-negative bacteria and consist of three different proteins: a transporter protein that is connected to a TolC protein (an outer membrane protein that provides the conduit of the agent from the transporter to the outside of the cell) and two fusion proteins that attach the

transporter firmly to the plasma membrane. The fusion proteins assist the extrusion of the agent from the transporter through the TolC by peristaltic action (19;72;76;77). The best studied RND efflux pump is AcrAB-TolC (78). The AcrAB-TolC is believed to function as follows: The agent present in the outer leaflet of the plasma membrane is recognised by AcrB transporter. The manner by which recognition takes place is currently unknown. Subsequent to recognition and binding of the agent to specific sites of AcrB, protons from the periplasm energise the transporter and the agent is extruded into the TolC conduit. The protons are released to the cytoplasm. The movement of agent through the TolC channel and its reaching the outside of the cell is assisted mechanically by the two fusion proteins. Recent evidence from Nikaido's group indicated that ligands that bind to AcrB have dissociation constants (Kd) that are pH dependent. At low pH the Kd is high, thereby affording after recognition and binding of the agent, rapid dissociation and subsequent binding of a second agent. At high pH the Kd is very small. This is important for the creation of drugs that are to be recognised by the AcrB transporter as a "substrate" which, after binding, will not dissociate readily and hence, inhibiting further activity of the transporter. The complex AcrAB-TolC is the main efflux pump of *E.coli* and also of Salmonella spp. The homologous pump to AcrAB-TolC of *P. aeruginosa* is the MexAB-OprM (76). These pumps are responsible for the efflux of β-lactams, chloramphenicol, erythromycin, fusidic acid, nalidixic acid, rifampicin, tetracycline, aminoglycosides and fluoroquinolones (67) as well as heavy metals, dyes or detergents (72). All the known pumps belonging to the RND family efflux its substrates via a substrate/H^+ antiport mechanisms. The transporter proteins have 12 TMDs with two large periplasmic loops and similar N- and C- terminals (72).

- **SMR** (Small Multi-drug Resistance) family is responsible for the extrusion of lipophilic cationic compounds (19;67). For example the EmrE pump of *E. coli* (that extrudes erythromycin, sulfamides and tetracycline (67)), the Smr of *S. aureus* (72) and the Mmr pump of *M. tuberculosis* (that can remove erythromycin (67)) belong to this family. These proteins have approximately

110 amino acid residues with 4 TMDs and work as tetramers in the membrane. The extrusion of compounds is energized by the proton motive force (72).

- **MATE** (Multi-Antimicrobial and Toxin Extrusion (69)) family members act as a Na^+/drug antiport system (19), like for example the YdhE pump of *E. coli* which can extrude chloramphenicol, fluoroquinolones and trimethoprim (69). These proteins have approximately 450 amino acids and 12 TMD (72).

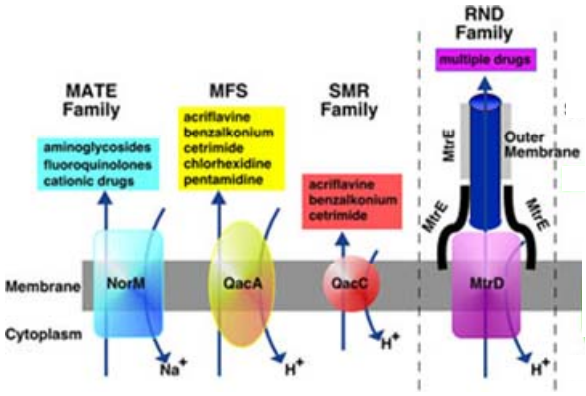


Figure 10 - Schematic representation of each representative families belonging to the secondary membrane transporters.

Adapted from: (79)

4. *Mycobacterium tuberculosis* – an emerging problem of resistance

4.1 The bacillus of Tuberculosis

The bacillus of *M. tuberculosis* was first identified by Robert Koch, in March 24, 1882. At that time the rate of death because of tuberculosis (TB) was 1 per 7 human beings. There are evidences that TB is a very ancient disease, as it was found in the skulls and spines of Egyptian mummies with more than 4000 years. (80).

The identification of the TB bacillus was so important to the evolution of the treatment of the disease that, in 1905, Koch received the Nobel Prize in Medicine for his discovery. After this, lots of research has been done and three more scientists received the noble prize because of their discoveries and their contributions to TB treatment and related aspects of TB (80).

- In 1908, Paul Ehrlich for his discovery of syphilis treatment. This was the start in the search for other chemical substances which could destroy disease-causing microorganisms;

- In 1939, Gerhard Domagk for describing the use of "Prontosil," an organic compound containing sulfur (sulphanilamide), for treatment of bacterial infections;

- In 1952, Selman Waksman for his discovery of streptomycin.

M. tuberculosis is a relatively large non-motile rod-shaped bacterium, with 2 to 4 micrometers in length and 0.2 to 0.5 in width. It is an obligate aerobe and that is the reason why its complexes are mainly found in well aerated upper lobes of the lungs. The mycobacterium is a facultative intracellular parasite, usually of macrophages, and has a slow generation time, 15 to 20h. Among other factors, discussed later, this physiologic characteristic may also contribute to its virulence. It is classified as an acid-fast bacterium and not classified as either Gram-positive or Gram-negative, because it

does not have the chemical characteristics of either, although, as it was discussed in previous chapter, it does contain peptidoglycan in its cell wall. Once stained, this kind of bacteria will retain dyes when heated and treated with acidified organic compounds (e.g. Ziehl-Neelsen stain method). As it can be seen with the light microscope, *M. tuberculosis* often forms cords. It was also Robert Koch who made this observation for the first time. The formation of cords is due to the synthesis of cord factor which is, possibly, of significant importance to its virulence (81).

4.2 The disease

TB is an infectious bacterial disease which, most commonly, affects the lungs. It is transmitted from person to person via micro-droplets expelled when coughing, singing and shouting. Expulsion of *M. tuberculosis* indicates the infective phase of the disease, termed "Active Disease".

In healthy people, infection with *M. tuberculosis* is often asymptomatic since the immune system of the person acts to "wall off" the bacteria. The symptoms of active TB of the lung are coughing, chest pains, weakness, weight loss, fever and night sweats, and haemorrhage. Usually, the latter symptom is the cause for medical attention and subsequent diagnosis (82).

A person gets infected with *M. tuberculosis* from the inhalation of bacilli which is then internalized by the alveolar macrophages (pneumocytes II). At this time the infection is in the lungs, and with severe cavitary disease the bacteria will, via lymphatic and capillaries, reach and infect other organs (disseminated TB). The sites of tissues indicative of disseminated TB reveal granuloma (collection of immune cells / lymphoid matter and epithelial components). Because many *M. tuberculosis* bacillus survive the immune functions that lead to a granuloma, the tissues, if not for the encasement of *M. tuberculosis*, would yield new sites for infection (horizontal tissue transfer) (83).

Infection with *M. tuberculosis* results in the development of active pulmonary TB in only 5 to 10 % of all infections. However, due to strife, poverty, famine and immune-incompetence (HIV/AIDS; pregnancy; therapy for transplantation of tissues and organs) the frequency of active TB is considerably much greater (83). In the absence of the above active TB supporting causes, the 5 to 10 % of infections that progress to active disease status takes place after the 5th decade and is due to the decrease of T cell immune functions that are normally reduced with aging. Reactivation of the infection occurs and this is usually noted to take place in apical part of the right lobe of the lungs. Reactive TB can occur for various reasons such as HIV/AIDS, malnutrition, or a systemic infection that modifies the immune response (82).

4.3 TB and antibiotic resistance

Almost immediately after the start of the use of streptomycin in TB therapy, the TB bacillus acquired resistance to that antibiotic. 10 years later it was already used a combination therapy with streptomycin, isoniazid (INH) and para-aminosalicylic acid (PAS) (80). The addition of INH to rifampin (RIF) formed the most effective therapeutic combination providing that the *M. tuberculosis* was susceptible to these agents. When *M. tuberculosis* is resistant to both INH and RIF it is termed "MDR". Therapy of MDRTB involves the addition of other first line of defence drugs, such as pyrazinamide (PZA), sulfonamide (SM), ethambutol (EMB) and thiacetazone, following the schedule proposed by the World Health Organization (WHO): Regardless of this therapy, mortality as high as 85% takes place within 12 months if the patient is co-infected with HIV, and as high as 70% within two years if infected only with MDR *M. tuberculosis* (84).

Despite the therapies available, TB continues to be a global public health problem, and lots of informative and "fight TB" actions and guidelines have been provided by some organizations, especially the WHO (84).

According to the WHO, more than 400 000 cases of multi-drug resistant TB (MDRTB) emerge every year as a result of under investment to control TB, poor management of patient with anti-TB drugs and especially patient non-compliance (85). In Europe the rate of new MDRTB is 50 new cases and 7 deaths per hour with more than 70000 new cases of MDRTB per year.

MDRTB has progressed to extensively drug resistant TB (XDRTB) as reported for all regions of the world by 2006 WHO report on MDRTB (85). XDRTB is defined an *M. tuberculosis* that is resistant to multiple drugs (INH, RIF and other first line of defence drugs), streptomycin, plus resistant to any one of the fluoroquinolones and at least one of the three injectable second-line drugs (amikacin, capreomycin or kanamycin) (84). The extreme resistance of XDR *M. tuberculosis* is a serious emerging threat to global public health, especially in countries with a high prevalence of HIV (85). Figure 11 shows the distribution of XDRTB up to the first half of 2008, with a huge number of countries with reported XDRTB.

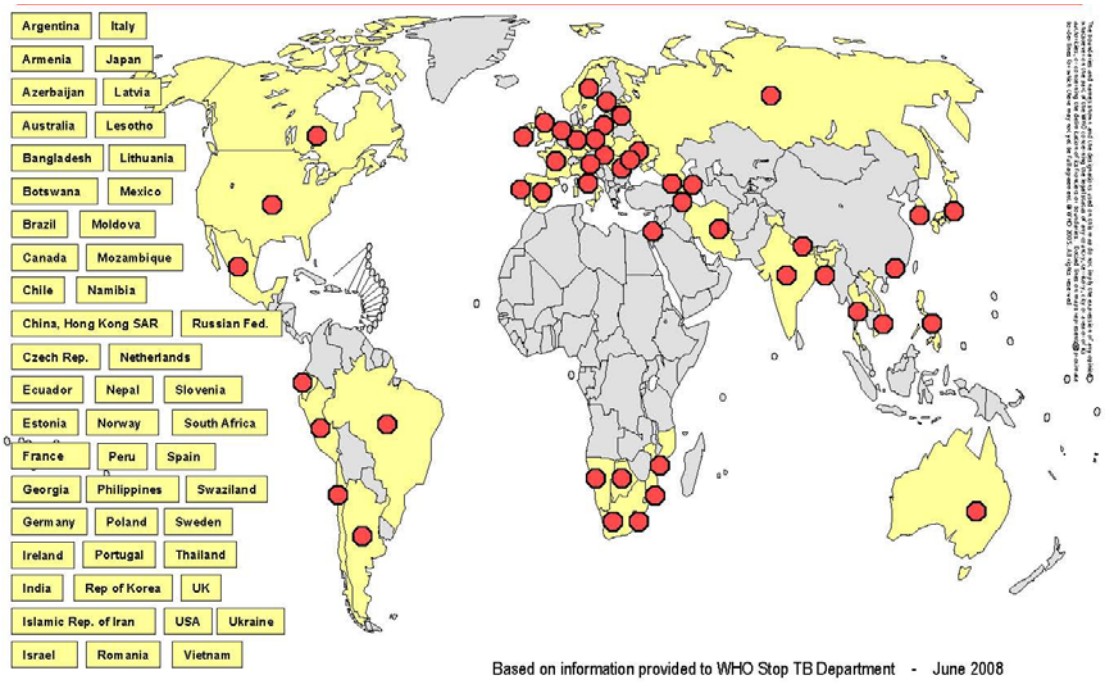


Figure 11 - Distribution of the confirmed cases of XDRTB all over the world, until June 2008.
Source: The stop TB department of WHO (82;84;85).

Studies performed by Perdigão and co-authors revealed that the situation of MDR and XDRTB in Portugal had been underestimated. According to the authors MDRTB had a prevalence of 11% in the region of Lisbon against the 3% reported by the official authorities. Moreover, MDR *M. tuberculosis* strains were characterized as resistant to isoniazid, rifampin, streptomycin and pyrazinamide. From the cases studied in the cited work it was found that in 2003, 53% of the MDRTB detected were already XDRTB isolates, which is a prevalence much higher than others previously reported to other countries (86).

The first MDR strains in Portugal were identified in the 1990s and the majority is genetically related. Its continuous disseminations in the community together with the high frequency of fluoroquinolones and third generation quinolones prescription to treat other respiratory tract infections had also contributed to the high prevalence of XDRTB in Portugal. The excessive use of quinolones is associated with the acquisition of resistance and may have contributed to the selection of resistant *M. tuberculosis* strains (86).

4.4 Cell characteristics, infection and resistance

The high lipid content of the mycobacterial cell wall confers to *M. tuberculosis* an intrinsic resistance, which is characterised by its low permeability to drugs. The most important lipid that is also the major component of the mycobacterial cell wall is mycolic acid, which is much bigger than the common fatty acids. It is covalently linked to arabinogalactan, which is, in turn, covalently linked to peptidoglycan. These characteristics are one of the main reasons for the general resistance of mycobacteria to the majority of the antibiotics (87;88).

There were also identified different *M. tuberculosis* efflux pumps which confer resistance to different classes of compounds such as tetracycline (TetK-L (67), Tap (87)) or macrolides (Mmr or DrrB) (69), fluoroquinolones (Rv1634; Rv2686-2687c-

2688c) (87), aminoglycosides (Tap) (87), rifampin and ofloxacin (Rv1258c) (87), and possibly isoniazid (mmpL7) (87) and ethambutol, erythromycin, streptomycin, chloramphenicol and anthracyclines (DrrAB) (87). The characterization of *M. tuberculosis* efflux pumps revealed that they belong to all the families described in chapter 3.2 (89).

Although some studies have been carried out to identify the importance of over-expression of efflux pumps in clinical isolates (90;91), the correlation between EP and antibiotic resistance is still unclear (92). However, it seems certain that, as described before, the drug concentration inside the cell is dependent on the equilibrium between its intake and efflux (87).

It is known that the acidification of the phagosome is a major mechanism used by macrophages against bacteria, including *M. tuberculosis*. The clinical and cellular outcomes of *M. tuberculosis* and the role of the adaptive immune system are presented in Figure 12 (93).

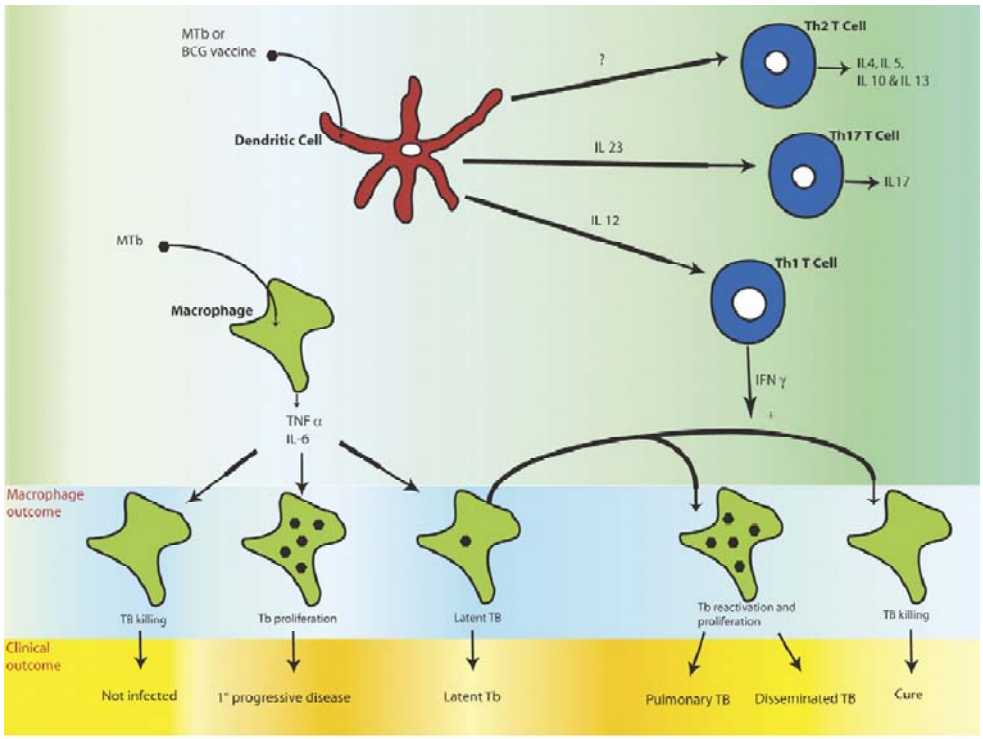


Figure 12 - Clinical and cellular outcomes of *Mycobacterium tuberculosis* and the role of the adaptive immune system.

Source:(93).

An initial infection of the macrophage results in the production of pro-inflammatory cytokines such as interleukins (IL) IL-6, IL-1β, IL-12 and TNF-α (tumor necrosis factor-α). In latent infection, there is a balance between the mycobacterial proliferation and the capacity of the host defence. At this stage the mycobacterium is contained inside the macrophage with no presentation of clinical symptoms. Ten percent of those with latent *M. tuberculosis* infection will eventually develop clinically active disease that manifests as localized pulmonary infection, or disseminated disease, depending on the innate immune response of macrophages and/or dendritic cells. T-cell responses are shaped by interactions with dendritic cells, which depend on the innate immune response to *M. tuberculosis*. Th1 T cells produce interferon-γ (IFN-γ) and promote mycobacterial killing by infected macrophages. Th17 T cells secrete IL-17 and could be important for protective vaccine-induced responses. The role of Th2 T cells in host defence to *M. tuberculosis* infection is less clear. The combination of innate and adaptive responses influences the macrophage response to *M. tuberculosis* infection as well as the clinical outcome (93).

Some authors have also studied the importance of the cell membrane, in particular some proteins, in the survival of the *M. tuberculosis* inside the phagosome and its virulence. *M. tuberculosis* survives within macrophages by preventing fusion of phagosomes with lysosomes, but it also persists within acidic phagolysosomes in activated macrophages (94).

Mycobacteria block phagosome acidification, but interferon-γ (IFN-γ) restores acidification and confers antimycobacterial activity. In the studies performed by Vandal *et al*, *in vitro*, at pH 4.5, *M. tuberculosis* survived in a simple buffer maintaining the intra-bacterial pH and survived when phagocytosed by IFN-γ activated macrophages. Therefore, *M. tuberculosis* resists phagolysosomal concentrations of acid. The use of a strain disrupted in *Rv3671c*, a gene encoding a membrane-associated protein, was sensitive to acid and failed to maintain intra-bacterial pH in acidic *in vitro* conditions, as well as in activated macrophages. Growth of the mutant was also severely attenuated in mice. Thus, *M. tuberculosis* is able to resist acid, owing in large part to Rv3671c, and this resistance is essential for virulence (94).

The use of infected macrophage in the study of the action of new compounds that may be used in therapy is extremely important, mainly in *M. tuberculosis* infection. The demonstration that inhibitors of K^+ and Ca^{2+} transport enhance the killing of intracellular MDRTB and XDRTB by the human macrophage suggests a totally different approach for the design of new antituberculosis agents. In addition to designing agents that have direct activity against these bacteria at intracellular sites, consideration may be given to designing agents that have activity against EPs that would normally transport K^+ and Ca^{2+} into the human macrophage and which, due to the invagination of the plasma membrane during the phagocytosis of the bacterium, now transport these ions from the phagolysosome to the cytoplasm of the phagocytic cell (95).

5. Multi-drug resistance and cancer

Neoplasma are divided in two categories: benign and malignant. The "benign" neoplasm is not benign inasmuch as, due to its rapid growth, it can affect the functions of tissues and organs in the immediate vicinity. The cells of a benign neoplasm do not migrate to distal sites. Malignancy is a neoplasm that due to the loss of inter-digitation between cells has been lost and the cell migrates (metastases) via the lymph or vascular channels to other sites where they may be trapped (lymphoid tissue) or settle and grow, eventually interfering with the functions of the issue or organ that they have colonised. Cancer is a generic term for a large group of diseases that can affect any part of the body. It can be defined as a malignant tumor of potentially unlimited growth that expands locally by invasion and systemically by metastasis (2). Concerning the biological characteristics of cancer cells, it should be emphasized the proliferation of abnormal cells and failure of programmed cell death (apoptosis) which promote cancer progression (82).

According to data from the WHO, 7.9 million of people died from cancer in 2007. This is the second principal cause of mortality in the developed countries, after the cardiovascular diseases (82). However, the cancer rate is increasing also in the low and middle income countries (in particularly South America and Asia) that have already the majority of world deaths (>70%) (82).

There are "external" factors, mainly due to tobacco, alcohol, diet, physical inactivity, pollution, radiation or some infections such as hepatitis B, human papilloma virus or by *Helicobacter pylori* which contribute to increase the number of cancer cases more than the natural ones. The frequency of cancer is also related to genetically determined factors. The increase of cancer however, is primarily due to significant increase of life expectancy (96). 40% of cancer can be prevented only by the changing of lifestyles (82).

The organs/tissues more commonly affected by cancer are prostate, breast and colon, in developed countries, and liver, stomach and cervical, in the in developing countries.

However, with the exception of oat cell carcinoma of the lung, lung cancer and certain death are related primarily to smoking (82). There is a very strong relationship between the development of lung cancer and TB infections. However, as of today, the question of whether one precedes the other is still not known (97;98)

The prevention and early diagnosis are very important in the control of the disease. Treatment of cancer includes different approaches such as surgery, radiation therapy, chemotherapy, or hormonal therapy (99). However, in the majority of the cases, cancer therapy does not result in a complete cure but may increase the life time (5 years or more in 75% of the cancer types) (99). However, the increase of life expectancy is more related to early intervention than to the intervention itself. New approaches for treatment of cancer are required.

Studies of oncogenes and tumor suppressors, known to be involved in the development of human cancers, have improved the treatment of disseminated cancer. These new studies of cancer related genetic targets, has resulted in a molecular approach for therapy of cancer. These improve the response rate in cancer and reduce side effects of anticancer treatment. Therapies have progressed from the use of general cytotoxic agents, such as nitrogen mustard in the 1940s, to the development of natural-product anticancer drugs in the 1960s, such as Vinca alkaloids and anthracyclines, which are more cytotoxic to cancer cells than normal cells, and to the use of specific monoclonal antibodies and immunotoxins targeted to cell surface receptors and specific agents that inactivate kinases in growth-promoting pathways (12).

The failure of therapy in cancer can be due to host factors or specific genetic or epigenetic alterations in the cancer cells. These failures include, for example:

1. low serum levels of drugs because of poor absorption, rapid metabolism or excretion;

2. poor tolerance to side effects of drugs, resulting in a need to reduce doses below optimal levels;

3. inability to deliver drugs to the site of the tumor;

4. and, various alterations in the host-tumor environment that affect response of the tumor (local metabolism of drugs by healthy cells, unusual tumor blood supply that affects the drug deliver, etc) (12).

As already discussed, eukaryotic cells develop resistance in a manner analogous to prokaryotic cells, and this is the major factor that promotes failure in cancer chemotherapy (100). It can affect a wide variety of cancer types and solid tumors, including breast, ovarian, lung, and lower gastrointestinal tract cancers (101).

Every cancer expresses a different array of drug-resistance genes, and exhibits an enormous amount of heterogeneity with respect to drug resistance. In addition, even if tumors are not intrinsically resistant to a specific anticancer treatment, if they face the powerful selection imposed by potent anticancer drugs, and due to that heterogeneity, an overgrowth of drug-resistant variants can occur as well as rapid acquisition of drug resistance (12).

As in bacteria, the phenomenon of resistance in cancer can be due to a variety of factors (100;102):

- Adjustment of the cell to damage produced by drugs by mechanisms that increase repair of DNA systems. Alterations in the cell cycle through changes in the factors responsible for its control; blocking apoptosis or interrupting the signalling cascades;
- Activation of detoxifying systems (usually involving glutathione (GSH) and GSH-S-transferase);
- Reduction of intra-cellular concentration of drug by either increasing its efflux or decreasing its uptake. Drugs that affect membrane lipids such as ceramide can also reduce intracellular concentrations.
- Inactivation of drugs;
- Alteration of drug targets;
- Expression of a major vault protein, termed lung resistance-related protein (LRP), which may regulate nuclear entry of drugs

Although all of these mechanisms are possible, resistance of cancer cells has been mainly described as the result of efflux of the drug used in the treatment (100). The efflux mechanisms of cancer cells will be presented in greater detail in the paragraphs that follow.

The efflux can be the consequence of different mechanisms, also dependent on the cancer type, such as the up regulation of membrane proteins or by down-regulation of, for example, the caspase cascade (103).

In humans, the three major types of multi-drug resistance proteins include members of the ABCB (ABCB1/MDR1/P-glycoprotein), the ABCC (ABCC1/MRP1, ABCC2/MRP2), and the ABCG (ABCG2/MXR/BCRP) subfamily (104) represented by Figure 13.

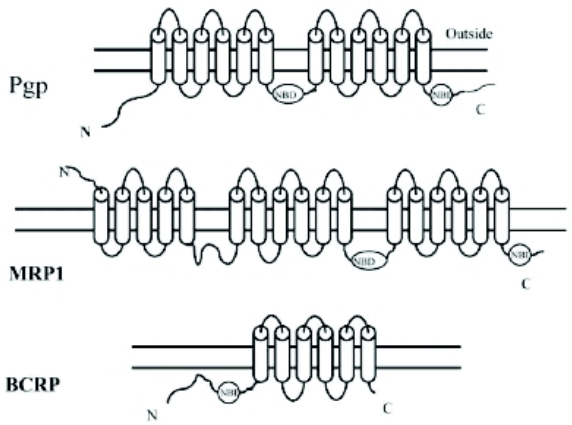


Figure 13 – Membrane topology of the MDR-associated ABC transporters MDR1, MRP1.
Source: (105)

P-gp was purified for the first time in 1979 (106). The genes that code for this protein have a high sequence homology to the bacterial hemolysin transport protein and this relationship was the first step to understand the function of this protein (101).

The P-gp can be expressed in cancers at different rates according to the cancer type. In renal carcinoma, adrenocorticoid, hepatocellular, pancreatic and colorectal carcinoma

the P-gp is usually highly expressed and these cancers are intrinsically resistant to chemotherapy. In other kinds of cancer, such as breast cancer or small-cell lung cancer, high level of this protein is expressed after chemotherapy suggesting an up-regulation of its expression or a selection of already resistant cells (102).

P-gp is an ATP binding protein of the ABC super family of transporters and therefore uses ATP as the energy source. It was one of the first members of the ABC family to be described. It is the product of the *mdr*1 gene in the humans and two different related genes, *mdr*1a and *mdr*1b in the mouse (12;102;107). All the organisms have encoded within their genome pumps from the ABC family which are involved in the transport of nutrients and other biologically important molecules across plasma membranes and intracellular membranes in cells (12).

P-gp is a 170,000 dalton phosphoglycoprotein consisting of two ATP binding cassettes and two transmembrane regions, each of which contains six transmembrane domains. P-gp can detect and bind a large variety of hydrophobic natural product drugs as they enter the plasma membrane. These drugs include many of the commonly used natural product anticancer drugs such as doxorubicin and daunorubicin, vinblastine and vincristine, and taxol (12). When these drugs bind there is an activation of one of the ATP-binding domains and the hydrolysis of ATP causes a major change in the shape of P-gp, which results in the releasing of the drug into the extracellular space. Hydrolysis of a second molecule of ATP is needed to restore the transporter to its original state (12).

Although the molecular mechanism of extrusion a wide range of structurally diverse drugs remains unclear (107), it is presumed that the ATP binding cassette acts as the engine for the transport of various of neutral and positively charged hydrophobic drugs within the plasma membrane (12). This extrusion can be blocked by many non-cytotoxic drugs, including nifedipine, verapamil, quinine, chloroquine, progestogens, tamoxifen, cyclosporine A and its analogues, reserpine and tricyclic anti-depressants (108;109). Most of them cannot be used in therapy because they are toxic for the patients in the doses that are needed for successful combination chemotherapy. For example high doses of verapamil needed to reverse drug resistance produce congestive cardiac failure and heart block (110). Moreover, any drug that interacts with the

substrate-binding region of P-gp can be a competitive inhibitor of the binding of other drugs (12).

MRP1 (multi-drug resistance associated protein 1) was the first member of the MRP family to be identified and confers resistance to a broad spectrum of anticancer drug like anthracyclines, alkaloids, epipodophyllotoxins and methotrexate, as the P-gp, but not to the taxanes (102). MRP1, unlike MDR1, transports negatively charged natural product drugs and drugs that have been modified, for example by glutathione, glycosylation or sulfation. MRP1 is also widely expressed in many human tissues and cancers (12).

The BCRP (Breast cancer resistance protein) is another member of the ABC family and is responsible for the transport of mitoxantrone, topotecan, irinotecan and methotrexate. Unlike MDR1 and the MRP family members, it only has one region with six transmembrane domains and a single ATP-binding cassette but is presumed to function as a dimmer (12;102).

The LRP, although it does not belong to the ABC transporters, it is also, highly expressed in drug resistant cell lines (102)

All of these proteins are found in normal tissue with basal expression and are also involved in the normal physiology of the cell. However, in cancer cells their function increases as well as their importance in the resistance to the chemotherapy (102).

6. New therapy approaches

Many approaches can be followed in order to look for efficient compounds against drug and multi-drug resistant strains. New classes of antibiotics with new targets are options that should be tested. However, much research has been performed to discover new adjuvants to be used with antimicrobial therapy. These compounds, together with the existing antibiotics, could increase the efficacy of the antibiotic against drug and multi-drug resistant strains.

A huge number of small molecules, many of natural origin, are able to reverse multi-drug resistance by inhibiting the functions of EPs and their action has been considered a possible way to reverse MDR. However, while a few compounds have reached clinical trials, none of them has, so far, been cleared for therapeutic use (111).

The adjuvants can act on many biochemical processes involved in influx of the antibiotic into the cell or its extrusion, the assembling of the cell membranes, the membrane potential, among others.

6.1 Efflux modulators

Various examples of efflux pump inhibitors (EPI's) with a wide variety of structures and chemical properties, both synthetic and natural compounds, can be found in the literature (16-20;112-114). Among these are verapamil (74), quinidine (74), reserpine (74), compounds from terpenoids family (112;115;116), phenothiazines and their derivatives (117-119), and certain alkaloids (120).

Some studies are presently in progress in order to recognize the similarities of antibiotic/cytostatic and EPI structures as well as their affinities to efflux pumps (121;122). This kind of work permits to find the occasional structural similarities

between the EPIs (123) and provides important information for the research of new active compounds. It can lead to the finding of 1) EPIs that are not substrate competitive and can directly inhibit efflux pump or 2) EPIs that are substrate competitors. To be a "substrate competitor" means that the EPI is recognized by the different pumps and competes for the active site of the transporter to which the antibiotic is bound. An EPI that competes for the binding site of two or more efflux pumps is a broad spectrum EPI (124).

However, some precautions should be taken with the inhibition of efflux pumps. An effective EPI that inhibits the EPs of a bacterium should not be active in the eukaryotic cells. Because some efflux pumps of prokaryotic and eukaryotic cells are similar, given their genetically conserved regions, structure specificity of EPI compounds for bacterial EPs efflux pumps must therefore be determined. Moreover, the EPIs should not present toxicity to the infected host. *Ex vivo*, *in vivo* and toxicity assays are therefore very important.

6.2 Other membrane interacting compounds

In order to identify compounds that would destabilize the stability of the bacterial membrane and increase its permeability some authors have been following another approach for therapy (125;126). For example *S. aureus* dehydrosqualene synthase is the first enzyme involved in the synthesis of staphyloxanthin (127), the golden carotenoid pigment of that bacterium, which promotes resistance to reactive oxygen species and host neutrophil-based killing. Based on the similarity between this enzyme and the human squalene synthase involved in cholesterol biosynthesis, Liu *et al*. screened inhibitors of squalene synthase on the inhibition of dehydrosqualene synthase and found that the killing of bacteria by the immune system was increased. Furthermore, the virulence of the organism was decreased. This mechanism of inhibition does not affect the viability of host cells because the source of cholesterol provided by external sources,

such as food is sufficient to by-pass the effects of inhibition of human squalene synthase (125).

Squalamine is another example of a compound that interacts mainly with membrane constituents of MDR strains such as LPS composition. Salmi and co-authors studied this compound against a wide variety of wild type and MDR Gram-negative and Gram-positive bacteria. The study with wild type and clinical drug resistant isolates of Gram-negatives showed that despite differences in membrane composition between strains, squalamine was effective against all strains tested and acted as a membranotropic compound (50).

Antimicrobial peptides are constituents of the immune system and are being studied as models for new synthetic antibacterials (128) or membrane active compounds (129). Their role against bacterial and mycobacterial infections was also studied, and showed that: 1) *in vitro*, these compounds increase membrane permeability of mycobacteria, 2) *in vivo*, they can influence the immune response of the host; and 3) they are efficient bacteriostatic factors, once they bind and inactivate a crucial protein involved in the control of bacterial proliferation, the ftsZ (130;131). According to "the antimicrobial peptide database" (132;133), these little peptides are, also, being studied as antiviral/HIV, anticancer and antifungal compounds. Figure 14 shows the possible mechanisms of action of antimicrobial peptides.

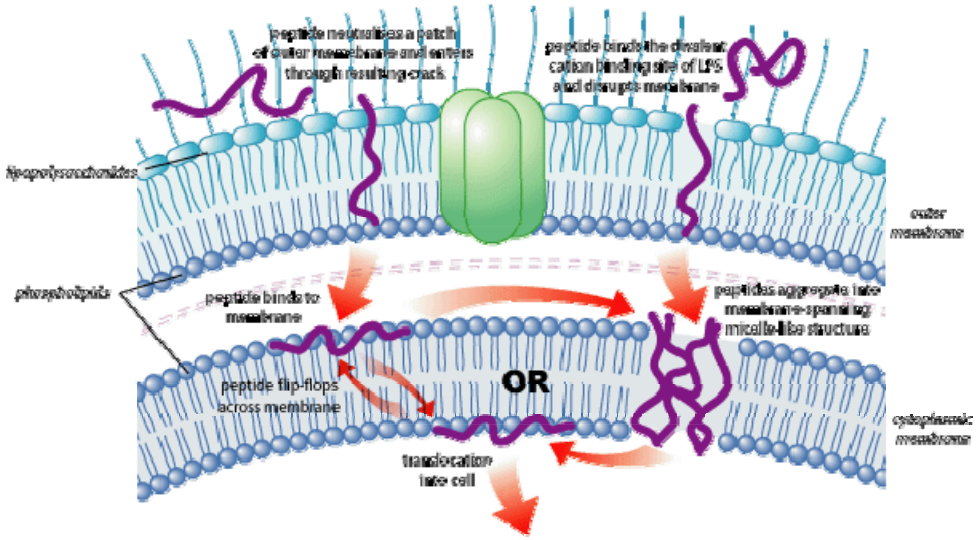


Figure 14 – Proposed mechanism of action for antimicrobial peptides.

In Gram-negative bacteria, it is hypothesized that cationic peptides interact with the highly negatively charged outer membrane. The peptides either bind tightly to the negatively charged membrane lipopolysaccharide (LPS) or neutralize the charge over an area of membrane subsequently distorting the membrane structure. Once this occurs the peptides can translocate across the outer membrane. The bacterial cytoplasmic membrane is also negatively charged. Cationic peptides can insert in a position parallel to the membrane lipids and fold into membrane-bound structures leading to cell lysis (although this is not very likely at physiological concentrations of cationic peptide), channel formation (the peptides reorient themselves in the cytoplasmic membrane in such a way that they form channels leading to a breakdown in membrane integrity), catastrophic breakdown of the membrane integrity or direct killing of the bacterial cell by peptides attacking internal targets after diffusing through both membranes. Source: (27).

Plants are good sources of these kinds of compounds. In the next chapter some examples of the use of plants in therapy will be given.

7. Importance of plants in therapy

Pharmacognosy is the science that studies the use of plants in pharmacy and describes the main characteristics of herbal medicines (phytomedicines) and their clinical use (phytotherapy). In general, pharmacognosy is the science of biogenic or nature-derived pharmaceuticals and poisons (134). Many plant derived pure compounds (natural products) are used in conventional medicine. Many compounds extracted from plants are commonly used. For example: 1) the analgesic morphine was first isolated from *Papaver somniferum*; 2) digoxin isolated from Digitalis spp. is used to treat heart failure; 3) taxol used in anticancer treatment, from *Taxus brevifolia*; 4) quinine used to treat malaria, from Cinchona spp.; 5) caffeine used as stimulant was isolated from *Coffea arabica*; 6) Salicin isolated from Salix spp. was the precursor for the acetylsalicylic acid or aspirin (134). Many other compounds used today in medicine are derived from plants and more of plant source will be discovered and effectively used. Herbal drugs derived from specific parts of plants such as leaves of *Ginkgo biloba* used in cognitive deficiencies, or the flower heads of chamomile (*Chamomilla recutita*) are referred to as phytomedicines and are used for mild gastrointestinal complaints and as an anti-inflammatory agent, respectively (134). Foods that are known to have beneficial effects on health such as garlic, ginger, anthocyanin, flavonoid containing plants or carotenoid containing plants such as tomatoes, carrots and many other vegetables are called nutraceuticals (134).

Natural products and herbal drugs have been used for many centuries and their use recorded in archaeological documents. The traditional use of herbal drugs or natural products in some parts of the world is documented and it is known as traditional medicine. China (135;136), India (134), Japan (136) or some African regions are famous for their “traditional medicines” (137). A very important approach to improve therapy is to explore traditional use of plant material and search new active compounds in those plants.

Another approach is to exploit the knowledge about the environment where the plant grows. As the soil is a media rich in bacteria, fungi and virus it is supposed that plants

produce their own antimicrobials, including antibacterial compounds to protect themselves against these microorganisms (16). Plants are known to produce an enormous variety of small molecule (MW <500 Da) antibiotics, generally classified as 'phytoalexins'. Their structure is diverse having characteristics of terpenoids, glycosteroids, flavonoids and polyphenols (138).

Streptomyces spp is an example of soil bacteria that is taxonomically related to Mycobacterium species. Those plants that are resistant to Streptomyces spp should be studied for compounds that may have activity against Mycobaterium spp, hence, exploring taxonomic "closeness" and specificity is an important area for investigation (16). These relationships can also be useful for exploring chemical similarities between the natural compounds and their derivatives, in a quantitative structure/activity relationship (QSAR). For example 60% of the antimicrobials and anticancer drugs have their origin in natural products (16).

Plants can be investigated for as sources of new antibiotics, new anticancer drugs or adjuvants for therapy. In these investigations attention must be paid to the properties of the plants and also to the characteristics of the bacteria or cancer type, as seen in the previous chapters.

7.1 Plants as sources of chemotherapeutics and modulators of cell mechanisms

Most of the antibiotic classes that are in use nowadays were derived from natural products (Table 1). This prevalence of natural derived compounds is in the main because plants produce biological active compounds needed for their own survival. It should be taken selective advantage from that fact. The potential use of natural products from plants for therapeutic purposes underlies the closely related physical and chemical properties of the substance to their effectiveness against fungi, viruses and bacteria all present in soil and atmosphere where the plant exists (10).

The isolation of compounds from plants has in recent years yield many different kinds of molecules with *in vitro* antibacterial activity against Gram-negative and Gram-positive bacteria (139-143).

Table 1 - Representative classes and examples of antibiotics derived from natural products and their site of action.

Adapted from Butler *et al* **(10)**

Antibiotic class	Example	Mode of action
Cephalosporins	Penicillin	Bacterial cell wall
Bacterial peptide	Bacitracin	Bacterial cell wall
Aminoglycosides	Streptomycin	Protein Synthesis
Cephalosporins	Cephalosporin	Bacterial cell wall
Chloramphenicols	Chloramphenicol	Protein synthesis
Tetracyclines	Chlortetracycline	Protein synthesis
Macrolides	Erythromycin	Protein synthesis
Antimycobacterials	Cycloserine	Bacterial cell wall
Glycopeptides	Vancomycin	Bacterial cell wall
Antimycobacterials	Rifamycin	RNA synthesis

It is important to note that isoniazid is not a derived natural product and it is not included in the table. However, the structure of this very important antibiotic in TB therapy is based on that of nicotinamide, commonly known as vitamin B_2 (10). Moreover, isoniazid is a pro-drug that is converted to its active form by the peroxidase system of the mycobacterium.

Many reviews have been published in the last years about the use of plants or natural sources for new anti-mycobacterial agents (144-147). An important review is that from Coop and Pearce, describing a series of characterized natural compounds that inhibit the growth of mycobacteria, with special focus on the active ones against *M. tuberculosis*.

The isolation from different sources including terrestrial and marine plants, animals and microorganisms lead to the identification of more than 350 compounds distributed among different classes, such as: alkaloids, terpenes, phenolic compounds, steroids, quinones, simple aromatic compounds and peptides, among others (148). Examples of active compounds against mycobacteria are triterpenes such as ursolic or oleanolic acid and its derivatives with minimum inhibitory concentrations (MIC) between 28 and 50 mg/L (148;149).

Some of the active compounds were shown, for example, to inhibit the isocitrate lyase, an enzyme class required for fatty acid catabolism, virulence of *M. tuberculosis*, or the mycolic acid biosynthesis (148;150). Others were shown to inhibit bacterial protein synthesis by binding to the 23S rRNA of the 50S ribosomal unit or intercalating into DNA (148).

However, it is interesting to note that most of the small molecules purified from plants have weaker antibiotic activity (sometimes several orders of magnitudes less) than that of common antibiotics produced by bacteria and fungi. Despite this fact, plants fight infections successfully. Apparently, plants adopt a different strategy – ‘‘synergy’’ – to combat infections. An example is the combined action of berberine and 50-methoxyhydnocarpin, both of which are produced by berberry plants. Berberine, a hydrophobic alkaloid that intercalates into DNA, is ineffective as an antibacterial, because it can be pumped out easily by pumps that confer multi-drug resistance. Hence, the plant produces 50-methoxyhydnocarpin that blocks those pumps and this combination is a potent antibacterial agent (138).

Recently, the search for new compounds from the class of the EPIs, that could improve the TB therapy, has also been described. For example, Lechner *et al* described the activity of an isoflavone, biochanin A (151), epicatechin, myrecitin, quercetin, rutin and other flavonoids (150;151) against mycobacteria efflux pumps (EP). These authors also suggest that, for example, the modulation of the INH activity by flavonoids is not only related with the inhibition of mycobacterial efflux pumps but also with their antioxidant capacity, which will scavenge the H_2O_2 available for the peroxidase KatG so that the INH can be more easily oxidized by that enzyme and be active inside the cell (150).

Thus, as it was presented in previous chapters of this introduction, EP are widespread in all types of cells from prokaryotes to eukaryotes. As TB infection involves these two kinds of cells (mycobacteria and host cell), it is important to keep in mind that adjuvant therapy should focus on a wide variety of different kinds of efflux pumps and be tested against its efficiency *in vitro* and *ex vivo*, before the *in vivo* use (152-155). Additionally, cancer adjuvant therapy should have particular attention to not interfere with the normal metabolism of the healthy cells of the organism.

The plant kingdom has proved to be a valuable source of efflux modulators (16;112). Many natural compounds have been reported as "efflux pumps inhibitors", decreasing the minimum inhibitory concentration (MIC) of antibiotics against bacteria such as *S. aureus* (156;157), Mycobacteria (150), or MDR cancer (114), among others. Reserpine, a natural compound which was isolated from *Rauwilfia vomitoria* in 1954 (120;158), or Toratol™, whose main source is the New Zealand Totara tree (159) are just two examples of numerous commercialized plant derived compounds which are described as EPIs.

In relation to eukaryotic cells, P-gp, together with the MDR1, was shown to be one of the most important membrane proteins involved in cancer resistance to chemotherapy (160). Because of this reason, it has been a target of choice for the improvement of cancer chemotherapy. Nabekura *et al*, for example, studied, *in vitro*, inhibitors of these membrane proteins obtained from dietary phytochemicals that can also act as chemo-sensitizing agents for the MDR and non MDR cancer. Among these compounds were sesamin (from sesame), matairesinol (from soybeans), glycyrrhetinic acid and glabridin (both from licorice) (161).

The class of flavonoids has been described as an active class modulating and reducing the resistance of cancer cell to specific compounds, by inhibition of P-gp or through other mechanisms. However, opposite results were obtained with this class, and some flavonoids were shown to potentiate and increase that resistance. Some structure/activity relationship studies have been done in order to understand their mechanism of action (160).

The group of phenanthrenes have anticancer activity against different kinds of cancer. However, not all compounds of this family have similar activities and some of them were inactive, indicating, once again, that the relationship between the structure of the compound and the place and mechanism of action is an important step in the study of new chemotherapeutics (162).

A large number of those plants, where these compounds were isolated from, has been used for a long time in the traditional medicine of many countries and their chemical and pharmaceutical studies showed the chemical basis of these applications (135-137). African and Chinese traditional medicines have been important sources of knowledge and many plants from these regions have been studied in order to identify their active constituents (163).

The *Carpobrotus edulis*, topic of this thesis, is a particularly interesting example of this. According to literature data, this plant is used in South-African traditional medicine, some of which are of particular interest in the antibacterial research (see section bellow). One particular effect, relevant to this study, is the use against tuberculosis. As it is a very common plant (also in Portugal) and has a fast growth without any special requirements, this plant can be easily used as source of active compounds against infection and cancer. So that, it is interesting to complement the previous studies of this plant and characterize its constituents responsible for the observed activities. This was the essential motive of the choice of *C. edulis* as the subject of study in this thesis.

8. *Carpobrotus edulis*

Few studies can be found in the literature about the genus Carpobrotus and for the species studied in this work. Plants from the genus Carpobrotus (Aizoaceae family) are well known in South Africa and their spread and use in traditional medicine, in that country, has been described by some authors (137;164-170).

The genus Carpobrotus is one of the 143 genera of the Aizoaceae family, which contains about 2300 species (171). It is characterized by succulent leaves and fast growth through extensive areas, mainly sandy or dune soils along coastal areas (170). The most popular species of the genus Carpobrotus is *C. edulis*. Although *C. edulis* has been described mainly in the traditional medicine of South Africa (137;170), this species also occurs in coastal California (172) and in Europe (173). The first specimens of the plant were introduced in Europe between 1681 and 1690 and they are very well known along the Mediterranean coast (171). The Portuguese coast is rich in this plant (Figure 15) with flowers purple and yellow.

Figure 15 - *Carpobrotus edulis.*

Pictures taken in Cabo da Roca, Sintra, Portugal

8.1 Uses in traditional medicine

As the different Carpobrotus species are very similar between each other, they have been used in an undefined manner by different communities (167). In the traditional medicine this species have been used to treat stomach ailments (169), mouth ulcers (166;167;169;170), throat infection (167;169;170), sinusitis (170), pains (169), oral and vaginal thrush (169), burns (166;169), tuberculosis (169;170), dysentery (169;170;174), or as a diuretic (169).

8.2 Activity of Carpobrotus spp.

Some species of Carpobrotus have already been studied by scientific methods to test those properties. Watt *et al* isolated five active compounds from *C. edulis* leaves with activity against *Bacillus subtilis, S. epidermis, S. aureus, Streptococcus pneumonia, Moraxella cattharlis and Pseudomonas aeruginosa*. The authors identified the compounds such as flavonoids rutin, ferrulic acid, hyperoside, cactichin and neohesperidin using standard colouring reagents and comparing UV-light spectra with those from known standard compounds (170).

Ordway *et al* had previously demonstrated that *C. edulis* methanolic extract has immune-modulator activity and efflux pump inhibitor properties against MDR mouse lymphoma cell lines. In the same publication it was also demonstrated that the methanolic extract enhances the killing of phagocytosed *S. aureus* (175). Martins *et al* showed that the same extract inhibits the growth of phagocytosed multi-drug-resistant *M. tuberculosis* and methicillin-resistant *S. aureus*, but does not inhibit the growth of these bacteria *in vitro* (176). However, Springfield *et al* demonstrated that the ethyl acetate fraction of other three Carpobrotus species (*C. muirii (167), C. quadrifidus* (167) and *C. mellei* (168)) have antimicrobial activities against *S. aureus* and *M. smegmatis* in disc diffusion method, in direct bioassay in TLC plates and minimum inhibitory

concentration / minimum bactericidal concentration (MIC/MBC) determination. The extracts were not active against *P. aeruginosa* or *C. albicans* (167).

From these previous studies, it has been shown that the genus Carpobrotus is reach in flavonoids (168;170), hydrolysable tannins, phytosterols and aromatic acids (168), and Springfield *et al*, as well as Watt *et al*, claim that the activity of the Carpobrotus species is mainly due to the presence of flavonoids (168;170).

This Ph.D. research thesis proposes to isolate and identify components from the species *C. edulis* that are active against *M. tuberculosis* and MDR cancer cells.

II. AIMS OF THE STUDY

The first part of the present work aims to contribute for a better understanding of the antibiotic resistance mechanisms of bacteria and the physiological processes that influence and modulate resistance to antibiotics and to other noxious agents in the environment. The main objectives of this work are:

- To study the influence of the environment and growth conditions on the expression of outer membrane proteins of a bacterium;
- To study the effect of different conditions of antibiotic pressure on the acquisition of resistance mechanisms, namely over-expression of efflux pumps;
- To understand the role of efflux pump mediated resistance on the survival of the organism under extreme environmental conditions such as widely differing pH;
- To study the physiological contribution of metabolic energy, protons / proton motive force on the regulation of efflux activity;
- To study the role of ions such as calcium on the efflux pump mechanism of bacteria;
- To study compounds which modulate efflux activity by interfering with key pathways of membrane stability and energy use.

Because efflux is a key point for the efforts against multi-drug resistant infections, the second part of this thesis has for its main objective the search for new compounds that may modulate efflux mediated multi-drug resistance of bacteria and hence, if they are free of toxicity, can be used for therapy of infections caused by these bacteria.

Plants are good sources of active (and most probably non-toxic) compounds against many diseases. Evidence of their use in traditional medicine must be present and the amount of plant material sufficiently must be large, so that complete investigation will yield enough quantities of compounds for evaluation, and that the growth of the plant during the year does not limit its access for investigation.

The plant *Carpobrotus edulis,* chosen to this thesis, satisfies the aspects summarised above:

- It is described in traditional medicine for use against several diseases.
- Some extracts of this plant have been shown to have activity against bacteria, mycobacteria and cancer cell lines.
- Its abundance in the Lisbon area is prominent and its growth is essentially continuous during the entire year.

Therefore, for the second part of the thesis work, the main objectives were to isolate compounds from *Carpobrotus edulis* and evaluate them for activities relevant to their potential use for the therapy of multi-drug resistant bacterial infections and multi-drug resistant cancer. The following protocols were created for the above main aim and its objectives:

1. Extraction and isolation of active compounds of *C. edulis* following a bio-guided purification protocol based upon some activities described in the literature for this species/genus;
2. Characterise compounds isolated for molecular structure and identities.
3. Test the pure compounds for their activity against Gram-negative and Gram-positive bacteria.
4. Test the isolated compounds for activity against mycobacteria.
5. Test the isolated compounds for possible use as therapeutic adjuvants in particular for their activity as efflux modulators in bacteria;
6. Test the activity of the compounds as enhancers of the killing activity of macrophages infected with bacteria;
7. Test the pure compounds for:
    a. Antiproliferative activity against cancer cell lines and multi-drug resistance cell lines;
    b. Capacity to reduce or reverse multi-drug resistance of MDR cancer cell lines to anti-cancer compounds;
    c. Modulate the activity of an over-expressed transporter of MDR cancer cells.

III. MATERIALS AND METHODS

In the following chapter the methods used during the work of characterization of efflux of *E. coli* under different conditions, as well as the isolation of *C. edulis* compounds and evaluation of the activity of the compounds in prokaryotic and eukaryotic cells are presented. The relevant information about the reagents and apparatus used during the experiments are given through the text.

1. General bacteriology procedures

1.1 Bacterial strains

Antimicrobial activity was determined against representatives of Gram-positive, Gram-negative bacteria and mycobacteria strains. The strains used were:

- *Staphylococcus aureus* ATCC 25923 obtained from the American Type Culture Collection (ATCC) ;
- Clinical isolate methicillin resistant *Staphylococcus aureus* (MRSA) strain;
- *Staphylococcus aureus* HPV 107 gently provided by Prof. Hermínia de Lencastre, Molecular Genetics Lab, Instituto de Tecnologia Química e Biológica da Universidade Nova de Lisboa (177-179);
- MRSA COL gently provided by Prof. Hermínia de Lencastre, Molecular Genetics Lab, Instituto de Tecnologia Química e Biológica da Universidade Nova de Lisboa (178-180);
- MRSA COL was adapted to 1600 mg/L of oxacillin, and named as MRSA COL_{OXA}. Its characterization was published in (181);
- *Enterobacter aerogenes* ATCC 13048 gently provided by Professor Jean-Marie Pagès, UMR-MD-1, IFR88, Facultés de Médecine et de Pharmacie, Université de la Méditerranée, Marseille, France. It exhibits an efflux pump that is involved

in macrolides and linezolid efflux and was previously described Chollet et al. (182);

- *Enterococcus faecalis* ATCC 29212 obtained from the American Type Culture Collection (ATCC);
- *Escherichia coli* K-12 AG100 strain (*argE3 thi-1 rpsL xyl mtl delta (gal-uvrB*) supE44) (183) was kindly offered by Hiroshi Nikaido, Department of Molecular and Cell Biology and Chemistry, University of California, Berkely, California, USA.
- *Escherichia coli* AG100 strain was exposed to increasing concentrations of tetracycline (TET) (37) leading to an efflux pump over-expressed strain, *Escherichia coli* $AG100_{TET8}$;
- *E. coli* $AG100_{TET8}$ strain was exposured to 10mg/L of tetracycline during a long period of time leading to the strain *Escherichia coli* $AG100_{TET10}$;
- *Salmonella Enteritidis* 104 and *Salmonella Enteritidis* 5408 were gently provided by Professor Seamus Fanning, Centre for Food Safety, School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Ireland;
- Salmonella strains adapted to 4 e 16 mg/L of CIP respectively, *Salmonella Enteritidis* 104_{CIP} and *Salmonella Enteritidis* 5408_{CIP} were gently provided by Professor Seamus Fanning, Centre for Food Safety, School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Ireland;
- *Mycobacterium tuberculosis* H37Rv (ATCC 27294) strain, which is susceptible to rifampicin, isoniazid, streptomycin and ethambutol.

1.2 Cellular cultures

With exception of any change specified during each specific protocol, *E. coli* strains were grown in Luria Bertani broth (LB) and Luria Bertani agar (LA) media (components for this media were purchased from: ppeptone and Yeast E from Merck, Germany and NaCl from Panreac, Spain); Salmonella, Enterobacter, Enterococcus and

Staphylococci strains were grown in Tryptone Soya broth (TSB) and Tryptone Soya agar (TSA), both purchased from Oxoid, England) and *Mycobacterium tuberculosis* in Middlebrook 7H9 broth media and Middlebrook 7H11 solid media (purchased from Difco, USA).

Stocks of each bacterial strain were maintained in 15% glycerol at -80ºC.

1.3 Determination of Minimal Inhibitory Concentration (MIC)

The MIC determination of the compounds used in the different assays was conducted by the broth micro dilution method in Muller-Hinton broth (MHB) (Oxoid, England), according to Clinical and Laboratory Standards Institute (CLSI) (184) recommendations. A stock solution of the compound to be tested was prepared and an aliquot added to MHB on the 96 well plate. Serial dilutions in the following wells were performed. Then, an over-night bacterial inoculum was diluted to McFarland 0.5 in MHB and added to wells. The MIC, defined as the lower concentration of compound at which the media does not present visible growth of the strain, was determined after 16h and 18h of incubation at 37ºC. This protocol was used to all the strains described above with exception to the *M. tuberculosis* H37Rv which protocol is described below.

The susceptibility of *M. tuberculosis* H37Rv to the pure compounds was tested in the BACTEC 460 - TB system (Becton Dickinson Diagnostic Instrument Systems, Sparks, USA), which detects the production of $^{14}CO_2$ from the ^{14}C substrates of the medium, as a result of the metabolism of the mycobacteria. This detection is finally quantified as a growth index (GI) which varies from 1 to 999. The strain was grown in BACTEC 12B medium supplemented with 0.1 mL of PANTA (antibiotic supplement to avoid contamination by other microorganisms of fast growth) until it reached a maximum GI (GI=999). This inoculum was diluted ½ in phosphate buffer saline (PBS) (purchased from Sigma-Aldrich Química SA, Spain) and fresh 12B medium vials (Quilaban, Portugal), were inoculated with 0.1 mL of that dilution. One bottle was used as absolute

control and the others were inoculated with 0.1 mL of compound at different concentrations. A second control inoculated with 1/100 dilution of the original inoculum was also prepared, to work as a proportional control. The cultures were maintained at 37ºC until the control first reached the maximum GI and the second control reach GI=30 (185). An aliquot of each vial were after plated in 7H11 agar medium and incubated at 37ºC. The colony forming units (CFU) were counted after 3 and 4 weeks of incubation (152;186).

The rate of increase in the GI (ΔGI = GI of one day – GI of the previous day) were determined when the control rich GI=999 and the proportional e control a GI=30. If the ΔGI of the vial with the drug is lower than the one of the control the strain is susceptible to that compound. If it is higher, then the strain is resistant.

For determination of antibacterial activity of a compound by Kirby-Bauer and E-test assays, a McFarland 0.5 inoculum of over-night cultures of the strains was prepared. The strain was, then, swabbed in MHA plates and the E-test or Kirby-Bauer discs applied on the plate. The halls of inhibition were measured after incubation of the plates at 37ºC during 16h and 18h. E-test® was purchased by AB BIODISK, Sweden and Kirby-Bauer discs from Oxoid, UK.

1.4 Semi-automated EB method

1.4.1 Accumulation Assay

Assessment of putative efflux pump activity of *E. coli* AG100 and *E. coli* AG100 $_{TET8}$ strains conducted by the semi-automated ethidium bromide (EB) method was previously described (187). However, it was introduced a modification that affords the evaluation of efflux without the need to centrifuge for the removal of EB. This modification is described in the section that evaluates efflux of EB after addition of glucose or agent that is being evaluated for activity against efflux of EB.

For the assessment of accumulation of EB and conditions that affect it the following was performed. Briefly, strains were cultured in MHB medium until they reached an OD of 0.6 at 600 nm, centrifuged at 13,000 rpm for 3 minutes, the pellets re-suspended in saline, the OD adjusted to 0.6 and 1.0 ml aliquots transferred to micro tubes, the tubes centrifuged and the pellets re-suspended in saline of pH 5, 7 and 8. Aliquots of 0.045 mL transferred to micro tubes of 0.1 mL and 0.045 mL of EB (Sigma-Aldrich Química SA, Spain) in glucose-free saline of pH 5, 7 and 8 added to the respective tubes of same pH. If metabolic energy is required for efflux of EB the absence of glucose assures sufficient accumulation of the agent. The final concentration of EB for all experiments was 1.0 mg/L. Concentrations of EB much greater than 1.0 mg/L exceed the ability of the cell to extrude the agent, the level of intracellular agent rapidly increases and results in its intercalation between the nucleic bases of DNA. EB when bound to DNA is no longer available for extrusion. The tubes were rapidly transferred to the Rotor-Gene 3000^{TM} thermocycler with real-time analysis software (Corbett Research, Australia) programmed for number of 1 minute cycles at a constant temperature of 37ºC. The total average time from the addition of the EB-saline to as many as 12 individual tubes to the transfer of the tubes to the instrument and the instrument restarted did not exceed 60 seconds. Accumulation of EB of each tube was followed on a real-time basis by the assessment of fluorescence emitted. Excitation and emission wavelengths were 535 and 585 nm, respectively. Whereas the medium containing 1.0 mg/L of EB does not appreciably fluoresce, as the concentration of EB builds up in the periplasm of the Gram-negative bacterium, fluorescence is readily detected by the instrument (187). Accumulation of EB at the different pH was followed during a certain period of time.

1.4.2 Efflux Assay

Assessment of efflux of EB at pH 5, 7, and 8 was conducted as follows: accumulation of EB at pH 5, 7 and 8 as described above was first conducted for up to 25 minutes, after which time the instrument was stopped and 0.010 mL of saline at pH 5, 7 and 8 lacking and containing glucose to yield a concentration of 0.4% was added to the

respective tubes. Similar solutions with the tested compound, in presence and absence of glucose were also added to the tubes at this time. The tubes were then transferred to the instrument and the instrument re-started. The total amount of time for this addition to the time the instrument was re-started did not exceed 2.5 minutes. Fluorescence was followed for a minimum period of 10 minutes.

2. Evaluation of OMP from Salmonella

2.1 Growth conditions

Salmonella 104 and 5408 strains were grown over-night, in TSB and TSA. For the extraction, agar cultures were re-suspended in PBS and processed in the same way as broth cultures.

2.2 Protocol of Extraction

Extraction of OMP from Salmonella 104 and 5408 strains was conducted according to the previously published by Amaral *et al* (188). Briefly, cultures were centrifuged at 3600 rpm for 20 min (Hettich Universal centrifuge, USA), and the pellets washed twice with 25 ml of 5 mM phosphate buffer (pH 7.5) containing 140 mM NaCl (Panreac, Spain) and 10 mM $MgCl_2$ (Merck, Germany). The pellets were re-suspended in 10 ml of washing buffer and transferred to pre-weighed centrifuge tubes and centrifuged again. The supernatants were discarded, the tubes weighed and the pellets resuspended in 6 M urea containing 10 mM Tris hydrochloride (pH 7.5) (Sigma, USA) and 5 mM EDTA (Sigma, USA) at a weight:volume of 0.4 g to 1.0 ml of buffer. The tubes were stirred continuously for 1h at room temperature and then centrifuged at 3600 rpm and the

supernatant containing the extracted outer membrane proteins were evaluated for its content by SDS-acrylamide electrophoresis 8.5%.

2.3 Role of antibiotic-promoted stress

2.3.1 Step-wise increasing concentrations of antibiotic

MRSA COL was initially grown in TSB until it reached its maximum optical density (OD) as determined spectrophotometrically at 600 nm. An aliquot of 10 µl was transferred to 10 mL tubes containing 50 mg/L of oxacillin (OXA) (Sigma, USA) in 10 mL of TSB and the culture incubated until it reached full growth at 37ºC (culture 1). An aliquot of 10 µl was transferred from culture 1 to 10 mL TSB tubes containing 100 mg/L of OXA and the culture (culture 2) incubated at 37ºC until it reached the maximum OD (ca. 16 hours). Employing this procedure, MRSA COL was serially grown in TSB containing as much as 3200 mg/L of OXA.

MIC determination for OXA and erythromycin (ERY) were conducted at the beginning of the series and after each step-wise exposure to increasing concentrations of OXA. Susceptibility to kanamycin (KAN), ciprofloxacin (CIP) and amikacin (AMC) were conducted, as previously described. Kirby-Bauer susceptibility was similarly conducted and the zones of inhibition measured in millimetres (mm). The products of each OXA serial culture were sub-cultured in TSB broth containing 40 mg/L of reserpine (RES) and concentrations of ERY that ranged from 0.0 to that of the ERY MIC for each respective serial culture, in similar procedure as the one used for MIC determination, in order to determine if the acquired resistance could be reduced or reverse by the presence of the ERY, an EPI. ERY, KAN, AMC and RES were purchased from Sigma, USA and CIP from Fluka, Switzerland.

2.3.2 Serial passages in the same concentration of antibiotic

E. coli AG100 $_{TET8,}$ when transferred to drug free medium or to medium containing the antibiotic and Phe-Arg-β-naphtylamide (PAβN), assumes tetracycline susceptibility of its parent (37;65). The *E. coli* AG100 $_{TET8}$ strain was exposed to 10 mg/L of tetracycline for 60 serial passages in 500 ml of MHB: strain *E. coli* AG100 $_{TET10.}$

Minimum Inhibitory Concentrations (MIC) of antibiotics and PAβN against the strains employed in this study were conducted by the broth dilution method as per CLSI guidelines (184;189), described in the general procedures section. Antibiotic powders and PAβN were purchased from Sigma–Aldrich Química SA, Spain.

Antibiotic susceptibility to tetracycline during exposure of *E. coli* to tetracycline was determined by broth microdilution and E-test® in accordance to manufacturer's directions and CLSI guidelines (184;189). E-test® was purchased by AB BIODISK, Sweden.

3. pH and energy roles on efflux by Gram - negatives

E. coli AG100 strain, sensitive to tetracycline (MIC of 2 mg/L) was exposed to increasing concentrations of tetracycline. The resulting strain *E. coli* AG100 $_{TET8}$ is resistant to 8 mg/L tetracycline and to other antibiotics of unrelated classes (37;65). This strain subsequently transferred to drug free medium or to medium containing 8 mg/L tetracycline and PAβN assumes initial susceptibility to tetracycline of 2 mg/L (37;65).

The effects of varying concentrations of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), verapamil (VER), chlorpromazine (CPZ), thioridazine (TZ) and PAβN at final concentrations of 5, 80, 15, 15 and 40 mg/L, respectively, on the efflux of EB were carried out as described in the general procedures section for the efflux assays.

Equimolar concentrations (5mM) of Calcium (Ca^{2+}) (in the form of calcium chloride), ethylenediaminetetraacetic acid (EDTA) were also used. For some experiments, the additions at varying concentrations were in matched pH glucose-free medium. This component of the experiment afforded an additional control that would define any role of metabolic energy in conjunction with any effect produced by given concentrations of compound on efflux of EB and the modulation of efflux at a given pH. CCCP, VER, CPZ, TZ, PAβN, EDTA and $CaCl_2$ were purchased from Sigma, USA

4. Search for new active compounds against resistance

4.1 Plant material

Carpobrotus edulis (L.) N. E. Br. (Aizoaceae) leaves were collected at Guincho (Sintra, Portugal). The plant had been authenticated by Prof. António Viveiros (Professor Emeritus of Botany, Plant Biology Department, Sciences Faculty of the University of Lisbon) (176).

4.2 General purification procedure

For the purification vacuum-liquid chromatography in polyamide (ICN Biomedicals GmbH, EcoChrom, Germany) and in silica gel (Silica gel 60 G, 15 *í*m, Merck, Germany) were used. In each case, vacuum was achieved with a water vacuum pump. Gel chromatography was carried out on Sephadex LH20 (Pharmacia Fine Chemicals AB, Sweden) using MeOH as eluent; rotation planar chromatography in silica gel (silica gel 60 GF_{254}, Merck, Germany).

Preparative NP-TLC was performed on Si gel plates (Merck 5715, Merck, Germany) and preparative RP-TLC in RP-$18F_{254S}$ plates (Merck, Germany).

High performance liquid chromatography (HPLC) was carried out on a Waters Millipore instrument on a LiChrospher® 100 RP-18 (10µm) column with MeOH-H_2O (3:7) as mobile phase and detection at 220 nm and 254 nm.

Chromatographic fractions were monitored by normal phase-TLC (NP-TLC) on silica gel plates ($60F_{254}$ plates, Merck, Germany) and reverse phase – TLC (RP-TLC) (RP-$18F_{254S}$ plates, Merck, Germany) and visualized by spraying with concentrated H_2SO_4, followed by heating.

Structure elucidation was carried out by extensive spectroscopic analysis, including 1D and 2D nuclear magnetic resonance (NMR) (^{1}H-^{1}H COSY, HSQC and HMBC) on a Bruker Avance DRX 500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C). The stereochemistry of the compounds was studied by NOESY measurements. 1D NMR spectra were recorded. Two dimensional experiments were performed using the standard Bruker software. The signals of the deuterated solvents were taken as reference.

Electronspray-Impact Mass Spectrometry (ESI-MS) was recorded on a Finnigan MAT 95SQ hybrid tandem mass spectrometer.

The following solvents were used during the purification procedure: methanol, cyclohexane, *n*-hexane, acetone and acetic acid (Molar, chemicals KFT, Hungary); dichloromethane (Fluka, Germany); formic acid (Riedle-de Haën, Germany); iso-propanol (Reanal, Hungary); ethyl acetate and chlorophorm (Merck, Germany) and acetonitrile (Sigma, Germany).

4.3 Extraction and isolation

The fresh plant material of *C. edulis* (5 kg) was homogenized and percolated with MeOH (13 L) at room temperature (aprox. 25ºC). The crude extract was concentrated under vacuum (Rotavapor-RE, Büchi) at 40ºC to 0.5 L and extracted with *n*-hexane four times (0.4 L each). After evaporation fraction (**A)** had a dry residue of 4.5 g. The aqueous methanol phase was extracted with chloroform (5 x 0.5 L) and evaporated to dryness under vacuum yielding a residue of 4.3 g for this fraction **(B)**. The methanol

water phase was also extracted three times with ethyl acetate (3 x 0.4 L). After evaporation, the ethyl acetate fraction **(C)** had a dry residue of 4.4 g. All the fractions were re-dissolved in methanol (Figure 16).

Fraction A and **B** (Figure 16 - A) were joined and adsorbed to 17.6 g of polyamide and chromatographed on a polyamide column (44g) – *column 1.* A stepwise gradient elution was performed from 20 to 80 % of aqueous methanol by increasing methanol by 10 % in each step (10 fractions of 5 mL each).

The fractions obtained with 60, 70 and 80% of MeOH elutions (**D**) were combined, adsorbed onto 2 g of silica gel and chromatographed on silica gel (60 g) – *column 2* using a gradient system of 0.1 L of *n*-hexane:$CHCl_3$:MeOH (70:30:0, 60:40:0, 50:50:0, and 50:50:1), and $CHCl_3$:MeOH:H_2O (95:5:0, 90:10:0, 80:20:0 and 70:26:4). 10 mL fractions were collected.

The fractions obtained with *n*-hexane:$CHCl_3$:MeOH (50:50:1, v/v/v) and $CHCl_3$:MeOH (19:1, v/v) – (**E**) were further purified on Sephadex LH 20 (50 g) – *column 3*, using MeOH as elution solvent. Fractions of 2 mL were collected. Aliquots 15 to 23 (**F**) were combined and then fractionated by using a rotation planar chromatography (RPC) on silica gel eluted with *n*-hexane:dichloromethane (50:50, v/v) and increasing concentrations of methanol (¼, ½, 1, 2, 3, 5, 10 and 50). It was used 0.1 L of each solvent and collected 5 mL of each fraction. Fractions 6 to 13 eluted with *n*-hexane:CH_2Cl_2:MeOH (50:50:¼, v/v/v) (13.1 mg) – (**G**) and fractions 57 to 80 *n*-hexane:CH_2Cl_2:MeOH (50:50:5, 50:50:10 and 50:50:50, (v/v/v)) (14.3 mg) – (**H**) yield compounds **1** (13.1 mg) and **2** (14.3 mg), respectively. Fraction 46 to 53 eluted with *n*-hexane:CH_2Cl_2:MeOH (50:50:3, v/v/v) (22.3 mg) – (**I**) was purified by preparative TLC with a solvent system of *n*-hexane:CH_2Cl_2:MeOH (40:60:2, v/v/v) to obtain **3** (12.4 mg).

From *column 1* the fractions obtained with 50% MeOH (**J**) were chromatographed on a silica column (30 g – *column 4*) by using dry-loading technique (0.8 g of silica for adsorption) and the same gradient system as that of *column 2*. The fraction obtained with $CHCl_3$:MeOH (95:5, v/v) (18.8 mg) – (**L**), was further purified by preparative TLC with a mobile phase of c-hexane:EtOAc:EtOH (60:40:4,v/v/v) to obtain compound **4** (10.6 mg). The elution with *n*-hexane:$CHCl_3$ (1:1, v/v) originated a fraction (**M**) (19.4 mg), that yield compound **3** (15,8 mg) by crystallization, in MeOH. Also from *column*

4, the fractions eluted with *n*-hexane:$CHCl_3$:MeOH(1:1:1, v/v/v) (3.5 mg) – (**N**) was submitted to a preparative TLC with the solvent system *n*-hexane:$CHCl_3$:MeOH (50:50:3, v/v/v) and yielded compound **3** (2.9 mg).

Fraction **C** (Figure 16 - B) was adsorbed onto 8.6 g of silica gel and chromatographed in a silica gel (40 g) column – *column 5* with a gradient system of CH_2Cl_2:MeOH (95:5, 9:1, 85:15, 8:2, 7:3, v/v), CH_2Cl_2:MeOH:H_2O (70:30:1, v/v/v) and MeOH:H_2O (8:2). 50 mL of each solvent were used and from each it was collected 10 fractions of 5 mL. The fraction eluted with CH_2Cl_2:MeOH (85:5 and 8:2, v/v) (133 mg) - (**O**) was fractionated in a silica gel (40 g) column – *column 6* with a gradient of $CHCl_3$:MeOH (95:5 to 7:3, v/v) and from this, the one eluted with $CHCl_3$:MeOH (8:2, v/v) – (**P**) was purified by RP-HPLC with MeOH-H_2O (3:7) as solvent system and compounds **5** (7,7 mg) and **6** (5,6 mg) were obtained.

From *column 5*, the fraction eluted with CH_2Cl_2:MeOH:H_2O (70:30:1, v/v/v) – (**Q**) was chromatographed in a Sephadex LH20 - *column 7*, and 5 mL aliquots were collected. It was collected aliquots of 5mL each. Aliquots 12 and 13 were combined (**R**) and subjected to preparative TLC in EtOAc: HCOOH:H_2O (95:10:5, v/v/v). The most polar fraction (**S**) was again separated by preparative RP-TLC in MeOH:H_2O (1:1, v/v) and afforded compounds **5** (6.3 mg) and **6** (3.2 mg).

Aliquot 21, from *column 7* (**U**) was separated by preparative RP-TLC eluted with ACN:H_2O: CH_3COOH (30:70:0.5, v/v/v) yielded also compound **7** (3.6 mg).

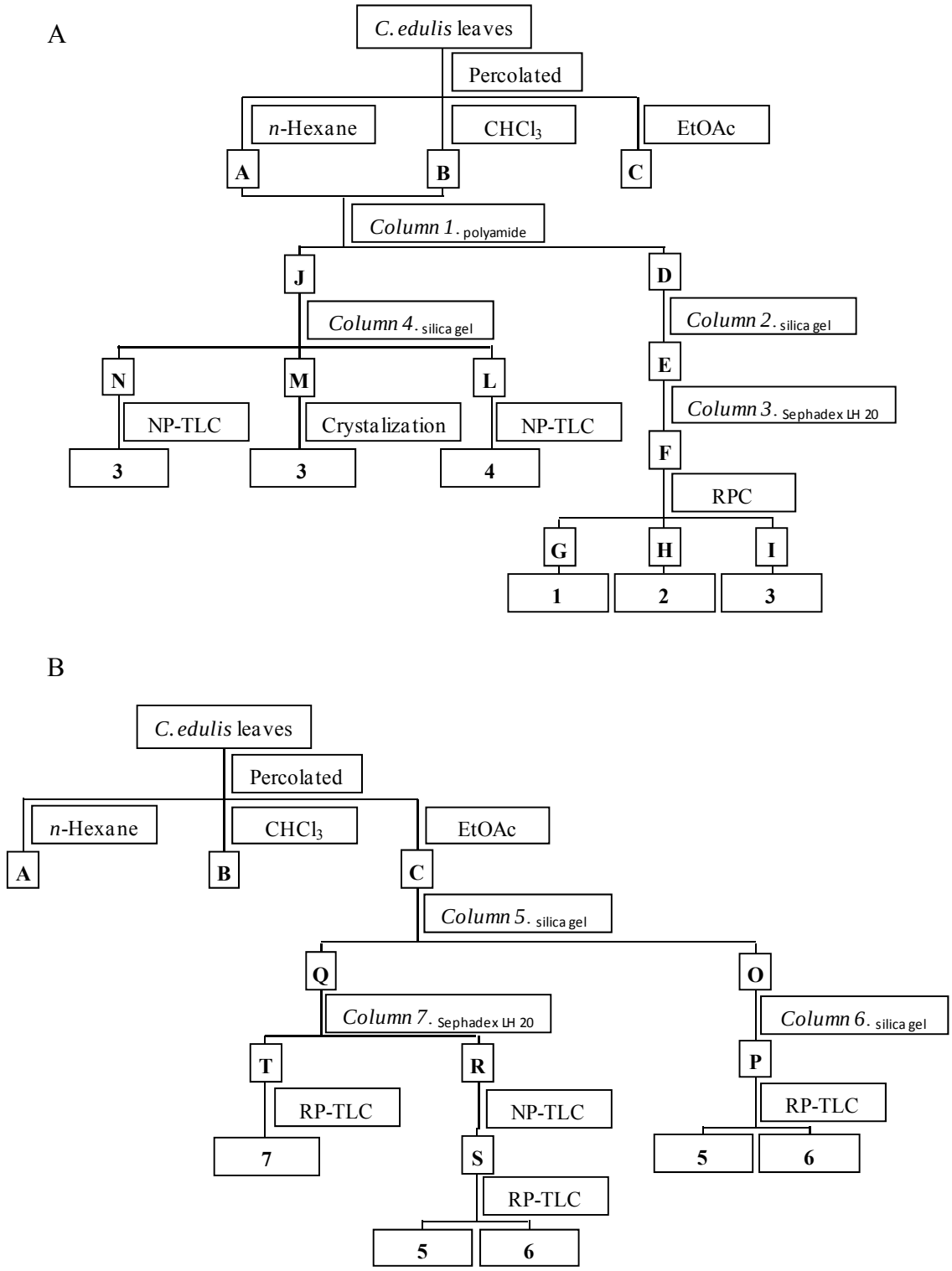


Figure 16 –Protocol of purification of the compounds from the plant *C. edulis*.

Purification of the (A) hexane and (B) ethyl acetate fractions of the methanolic extraction.

4.4 Activity measurements against bacteria

4.4.1 Minimum Inhibitory Concentration of *C. edulis* compounds

MIC determination was performed as previously described in this thesis. Each *C. edulis* compound was tested to a maximum concentration of 200 mg/L. MIC of *C. edulis* compounds were tested against all the strains mentioned at the beginning of the Materials and Methods section of this thesis, as presented by Table 12 of the Results section.

4.4.2 Modulation of resistance

A) Synergistic effect on the MIC

Effect of the *C. edulis* compounds on the reduction of the MIC of a given antibiotic to which the strain was made resistant. The assay was performed as per an antibiotic MIC determination in the presence and absence of each *C. edulis* compound at final concentrations of ½ and ¼ of its MIC.

B) Semi-automated EB method

The accumulation and efflux modulation activity of each compound was assessed by the semi-automated EB method using the Rotor-Gene 3000^{TM} thermocycler with real-time analysis software (Corbett Research, Australia) (187), as described in the general procedures section.

Briefly, *E. coli* AG100, *E. coli* AG100 $_{TET8}$, *S. enteritidis* 5408$_{CIP}$ and *S. aureus* COL were tested for the activity of the compounds for modulation of EB accumulation by these strains. Each compound was tested at 50 mg/L in presence and absence of glucose

and at pH 7. *C. edulis* compounds were also tested for their influence of efflux of EB by both *E. coli* strains.

4.4.3 Toxicity assays

Pure compounds were also evaluated for toxicity using the Trypan Blue exclusion method. The compounds that cause less than 10% of the lymphocyte population to stain blue after 3 days of culture were considered non-toxic (190). Lymphocytes were obtained from human whole blood from healthy donors by ficoll-saline gradient centrifugation at 1400 rpm during 20min (Rotanta 46R Hettich Zentrifuge, Germany) in histopaque 1077-1. The monocytes were separated, washed three times with Hank's Balanced Salts solution (HBSS) and count for the number of viable cells by the trypan blue method in a burker chamber, on the microscope. The concentration of cells were adjusted to $1x10^7$ cells/mL in Roswell Park Memorial Institute medium (RPMI) – 1640 supplemented with plasma from the same donor obtained in the centrifugation and 0.09 mL were added to each well of a 96 wells plate. 0.01 mL of the pure compounds at final concentration of 1, 0.1 and 0.01 were added to each well. Dimethyl sulfoxide (DMSO) was used to the dilution of the compounds and was presented in the assay at a maximum concentration of 10%. The concentrations of 10, 1 and 0.1% of DMSO were tested. The cells were maintained at 37ºC, under 5% CO_2 (Heraeus instruments incubator, USA) and its number was counted in the following 3 days, as described above. Histopaque, HBSS, RPMI and DMSO were purchased from Sigma-Aldrich, USA.

4.4.4 *Ex-vivo* assays

The *ex vivo* activity of the compounds will be assessed by the method previously published (152;176). Briefly, human macrophages were derived from monocytes of

human whole blood that were obtained, as described for the toxicity assay, from human whole blood from healthy donors by ficoll-saline gradient centrifugation at 1400 rpm during 20 min in histopaque 1077-1. The monocytes were separated, washed three times with Hank's Balanced Salts solution (HBSS) and count for the number of viable cells by the trypan blue method. After counting, the number of cells was adjusted to $1x10^6$ cells/mL in supplemented RPMI. 1 mL of the cellular suspension was distributed in each well of a 24 well microplate (Sarstedt, Germany). After incubation for 3 days at 37ºC, under 5% CO_2 (Heraeus instruments incubator, USA), the macrophage cells had adhered to the bottom of the wells. The media with the non-adherent cells, as the lymphocytes, was removed and the macrophages washed 3 times with RPMI. The cells were maintained under 5% CO_2 at 37ºC during 3 more days. The wash step was repeated and followed by the addition of *S. aureus* ATCC, MRSA or *M. tuberculosis* H37Rv cells at $1x10^7$cell/mL. The plates were then incubated for 30 min and cells were washed, as before, to avoid the presence of any unphagocytosed bacteria. Fresh supplemented RPMI medium, containing varying concentrations from 0.0 and 1 mg/L of each compound, was added. One set of triplicate wells will receive sodium dodecyl sulfate (SDS) (Sigma-Aldrich, USA) to lyse the macrophages and release the bacteria. The contents were subjected to colony forming units (CFU) to determine the number of bacterial cells at zero time. The plates were returned to the incubator. For the S. aureus strains the replicate wells were treated with SDS, and the contents of the wells processed for CFU, after 3 and 6h. For *M. tuberculosis* H37Rv assays this procedure was done after 24, 48 and 72h (1, 2 and 3 days).

The content of the wells for *M. tuberculosis* assay were also used to inoculate a BACTEC™ 960 tube (BD BBL™ MGIT™ Mycobacteria Growth Indicator tubes, Becton Dickinson and Company, USA) and its growth was monitorized by the MGIT960TB system with the Epicenter V5.53A software equipped with TB eXIST (eXtended Individual Susceptibility Testing) (Becton Dickinson, USA). This system allows the real time monitoring of growth and the time for detection of a positive culture is directly proportional to the amount of cells in the inoculum. Faster growth detection more cells in the inoculum. It provides a qualitative and comparative measure of the amount of mycobacterial cells present in the inoculum. BACTEC 960 MIGIT tubes were inoculated with 0.8 mL of supplement (BACTEC™ MGIT™ 960 SIRE

supplement, Becton Dickinson and Company, USA) and 0.5 mL of the well suspended content of each well, and the growth of the strain in each tube followed along the time.

4.5 Activity measurements on eukaryotic cell lines

4.5.1 Cell lines

Parental (PAR) and multi-drug resistant (MDR) cell lines used in the biological assays were the L5178 mouse T-cell lymphoma cells and the L5178 mouse T-cell lymphoma cells transfected with pHa MDR1/A retrovirus, as previously described (191). MDR cell lines were selected by culturing the infected cells with 60 μg/L of colchicine which maintain the expression of the MDR phenotype (192). Both cell lines were cultured in McCoy's 5A medium supplemented with 10% heat inactivated horse serum, L-glutamine and antibiotics (penicillin and streptomycin), at 37ºC and 5% CO_2 atmosphere. Colchicine, L-glutamine, penicillin and streptomycin were purchased from Difco, USA.

4.5.2 Antiproliferative assay

The antiproliferative activity of the compounds was tested in triplicate by the MTT assay (193). The MTT assay is a colorimetric assay that can be used to determine cytotoxicity of potential medicinal agents and other toxic materials, since those agents would result in cell toxicity and therefore metabolic dysfunction. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in living cells. A solubilisation solution (sodium dodecyl sulphate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance of this coloured solution can be quantified by

measuring at a certain wavelength. This reduction takes place only when reductase enzymes are active, which is taken as an indicator of the number of viable cells with metabolic activity.

The assay was performed in 96-well plates in serum free McCoy's 5A medium. The compounds were added to the wells of the third column of the plate and serial diluted in the following wells. Then it was added the MDR1 cells or the PAR cells to a final concentration of 6000 cells per well. After 72h of incubation under CO_2 and at 37ºC (Heraeus instruments incubator), it was added the MTT (Sigma, USA) to a final concentration of 10% per well, and after 4h of incubation, in the same conditions, it was added SDS 10% to a final concentration of 5% in the well. The optical density at 540 nm and 630 nm was read, after 24h hours of incubation in the same conditions, using a Dynatec MRX vertical beam ELISA reader (Dynatec, USA).

4.5.3 Inhibition of P-gp on *mdr*1 gene transfected mouse lymphoma cells

The activity of the fractions was evaluated by following the accumulation of rhodamine 123 on *mdr1* gene transfected mouse lymphoma cells (103). L5178 MDR cell line and L5178 Y parental cell line were cultured in McCoy's 5A medium (Sigma, USA) with and without colchicine, respectively. Briefly, the cells were re-suspended to a final concentration of 1 million cells in 0.5 mL of serum free McCoy's 5A medium. Each sample was treated with the purified fractions and incubated for 10 min. Rhodamine 123 (Sigma, USA) was added to each tube, at a final concentration of 5.2 μM. The samples were incubated for 20 min at 37ºC in water bath, centrifuged at 2000 rpm for 2 min and the pellet re-suspended in 0.5 mL of PBS. The PBS washing procedure was repeated twice. The fluorescence of the samples was measured by flow cytometry (Becton Dickinson FACScan). Verapamil (EGIS, Hungarian Pharmaceutical Company, Hungary) at 10 mg/L was used as the positive control of activity. Each compound was previously solved in DMSO and the activity of the solvent was measured as reference.

4.5.4 Checkerboard assay for interaction of compounds with anti-cancer agents.

The most active compound isolated from *C. edulis* for modulation of P-gp was determined for its synergistic effect on the activity of an anti-cancer agent against an MDR cell line. The assay was performed by the checkerboard microplate method for the study of drug interaction between the pure compound and doxorubicin on MDR cancer cells. This method is similar to the antiproliferative assay but doxorubicin is serially diluted in the wells as previously described (horizontal direction) and the compound diluted in the vertical direction. Cell suspension was added to each well to a final concentration of 5 x 10^4 cells per well. Plates were incubated for 72 h at 37°C under CO_2 and cell growth rate determined through MTT staining as previously described in this section.

IV. RESULTS

The results obtained will be presented in this section. The first part of the Results section focuses on the influence of the growth conditions on the characteristics of the cell envelope. Next section focuses on: influence of the growth media conditions (agar *versus* broth); resistance profile of *E. coli* under specific conditions of pH, temperature, calcium availability; the relationship between efflux pumps and outer membrane proteins in the resistance to antibiotics; the relationship between the efflux pumps that are over-expressed and the challenge of MDR bacteria to the same concentration of antibiotic for prolonged periods of time. From these results important information was collected to guide the following experiments in search of new compounds that may modulate efflux mediated multi-drug resistance of bacteria and cancer cells

In the second section of Results, the main subject of this dissertation, it will be presented the purification and the identification of the compounds isolated from *Carpobrotus edulis*. The purification procedure was done at the Pharmacognosy Institute of the Faculty of Pharmacy of the University of Szeged under supervision of Professor Judit Hohmann.

The third section of Results presents studies of the antibacterial activity of the previously isolated compounds on bacteria and the synergism between the pure compounds and anti-bacterials against prokaryotes. The evaluation of the compounds as modulators of the efflux activity of the bacterial cells will be presented.

Because TB is an intracellular infection (194), and previous studies showed that the methanol extract of *C. edulis* enhanced the killing activity of macrophages infected with *M. tuberculosis* or *S. aureus* strains (175;176), the compounds were tested for their capacity to enhance the killing of intracellular bacteria by non-killing macrophages. *S. aureus* ATCC and MRSA were used as a model because our previous studies demonstrated that the effects of compounds on intracellular *S. aureus* predicted the effect of the compounds on a *M. tuberculosis* infection of the macrophages. These assays were done at the Institute of Medical Microbiology of University of Szeged. Similar assays with *M. tuberculosis* H37Rv are still in progress at the moment of the writing of this dissertation.

Finally, and because it was already described by other authors that there are similarities between some prokaryotic efflux pumps and the eukaryotic efflux pump P-pg (195), the compounds were also tested for their inhibitory activity against the human P-gp that was coded by the human gene *mdr1* subsequent to the transfection of mouse lymphoma cells with this gene. These assays were done at the Institute of Medical Microbiology of University of Szeged under supervision of Professor Jozseph Molnár.

1. Growth conditions and the bacterial cell envelope

1.1 Growth environment and OMP expression

The investigation of the activity of any compound on a cell or organism, including bacterial cell, should take into account the behaviour and characteristics of that cell as well as the environment in which the cell exists.

The cell envelope plays a crucial role in this aspect: lipid content, its porins and efflux systems are the major components of the cell envelope and these are known to be modified under different environmental conditions (19;23;24;33;34). The first two limit the entrance of substances into the cell. Decrease in the availability of nutrients increases the number of porins and the expression of the OMPs that are assembled into these units (23;196). Moreover, the activation and modulation of OMP genes by nutritional conditions result in an increase of large pore porins which favour the penetration of a larger gamut of nutrients (23;196). Exposure of the bacterium to toxins, antibiotics or other noxious agents results in the down-regulation of porins (62;197) either by reduced expression (48;198;199) or increased proteases that degrade the OMPs prior to their being assembled into the porin unit (200).

Exposure of Salmonella strains to CPZ, a resistance modulator that has been studied in this thesis, has been shown to alter the composition of the outer cell membrane (188). Other studies have shown that media of high hydrostatic pressure affect the composition of the cell envelope (201). Extractions of outer membrane proteins from the cell envelopes of *S. enteritidis* that were cultured in solid and liquid media of the same type were conducted and the distribution of the extracted outer membrane proteins compared.

The outer membrane proteins were extracted (page 70 of the Material and Methods chapter) from two strains cultured in TSB and TSA and the extracted proteins subjected to 8.5% SDS-polyacrylamide gel electrophoresis (PAGE). The distribution of the extracted proteins from the two *Salmonella enteritidis* strains 104 and 5408 is presented

by Figure 17. From Figure 17, it may be noted that for both strains when grown in broth the major proteins specie is a 56 kDa that is almost absent when these strains are grown in agar. However, the strains do differ with respect to the amount of this protein: the *S. enteritidis* 104 strain clearly contains a greater amount. These extractions and subsequent electrophoretic analyses were repeated three times. As will be seen later, the *S. enteritidis* 5408 strain has reduced permeability to antibiotics and other noxious agents due to its high lipopolysaccharide content of the outer cell membrane (202;203). The extracts were studied by Prof Dr Jean-Marie Pages of UMR-MD1, Transporteurs Membranaires, Chimiorésistance et Drug-Design Facultés de Médecine et de Pharmacie, France, for the determination of whether they contained components of the main efflux pump of Salmonella AcrAB and the main porins of this organism. The extracts did not contain AcrA, AcrB, and TolC and the porin content was similar regardless of the type of culture conditions employed (203). However, the identity of the main 56 kDa protein has been made by Foulaki (204) and will be discussed later.

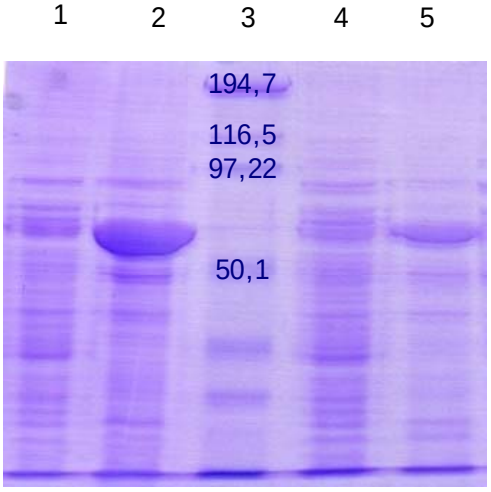


Figure 17 - Gel SDS-PAGE 8,5% of *S. enteritidis* 104 and *S. enteritidis* 5048.

1. *S. enteritidis* 104 – growth in agar; **2.** *S. enteritidis* 104 – growth in broth; **3.** Marker – Pre-stained SDS-PAGE standards Broad Range (kDa), Bio-Rad; **4.** *S. enteritidis* 5048 – growth in agar; **5.** *S. enteritidis* 5048 – growth in broth.

1.2 Role of antibiotic-promoted stress

1.2.1 Step-wise increasing concentrations of antibiotic

It was previously shown that exposure of *E. coli* to stepwise increases in tetracycline concentrations induces resistance from 2.0 mg/L to 12 mg/L of TET (37). Accompanying this induced resistance are significant increases in resistance to many other antibiotics and non-antibiotic agents. Similarly exposure of *M. tuberculosis* sensitive to isoniazid (INH) to increasing concentrations of this antibiotic increases the MIC from 0.2 mg/L to more than 40 mg/L (205). However, increased resistance to INH was not accompanied by resistance to any other drug employed for the therapy of pulmonary tuberculosis.

If it is assumed that events at the level of the bacterial cell envelope that result in increased efflux pump activity are independent of a chromosomal mutation that confers high-level resistance of the bacterium to a given antibiotic, then prolonged exposure of that bacterium to increasing concentrations of the antibiotic to which it is resistant may induce the appearance of an MDR type efflux pump. Similar experiments were done with a Methicillin-resistant *Staphylococcus aureus* (MRSA).

MRSA COL strain whose resistance to 400 mg/L oxacillin (OXA) is due to the acquired *mecA* element (60) was exposed to stepwise increasing concentrations of OXA ranging from 50 mg/L to 3200 mg/L and at each level of increased resistance to OXA, a parallel increase of resistance to ERY was evident as demonstrated by Table 2. The increased resistance to ERY could be completely eliminated by the addition of 40 mg/L of RES to the culture (Table 2).

Table 2 - Minimum Inhibitory concentration of erythromycin for MRSA COL strain during the adaptation passages in increasing concentrations of oxacillin.

Inducing resistance of methicillin-resistant *Staphylococcus aureus* COL strain to erythromycin (ERY) by prolonged exposure to increasing concentrations of oxacillin (OXA), and reversal of ERY resistance by the efflux pump inhibitor reserpine (RES)

OXA (mg/L)	MIC (mg/L)	
	ERY	ERY + 40 mg/L RES
0	<0.4	<0.4
50	<0.4	<0.4
100	<0.4	<0.4
200	1.0	<0.4
400	1.0	<0.4
800	4.0	<0.4
1600	>16.0	<0.4
3200	30	<0.4

Kirby–Bauer susceptibility assays showed that the strain that had been adapted to 1600 mg/L OXA produced resistance to KAN, AMC, ofloxacin (OFX), norfloxacin (NOR) and RIF (Table 3).

Table 3 - Changes in sensitivity to other antibiotics as determined by the Kirby–Bauer susceptibility assay after adapting *Staphylococcus aureus* COL strain to 1600 mg/L oxacillin

	Zone of inhibition (mm)					
	KAN (30µg)	AMC (30µg)	OFX (5µg)	NOR (10µg)	RIF (5µg)	ERY (15µg)
Before exposure to OXA	27	13	29	28	36	32
After exposure to OXA	0	0	0	0	29	0

1.2.2 Serial passages in the same concentration of antibiotic

In the previous experiment it was seen that a strain initially resistant to an antibiotic when exposed to increasing concentrations of that antibiotic promotes the acquisition of resistance to other antibiotics. The next question asked was "What happens in terms of resistance profile if a strain was maintained at the same concentration of antibiotic to which it was adapted?" The chosen strain for the answering of this question was the *E. coli* $AG100_{TET8}$. This strain presents an MDR phenotype after the adaptation procedure that was mediated by the over-expression of the AcrAB efflux system as well as by an increase in the expression of genes that code for AcrB, AcrF and other transporters as well as regulators soxS, rob, marA, marB and ompX (65). This strain was chosen because initially it was not resistant to the antibiotic, which is not the case of the MRSA COL which due to the acquisition of the *mecA* element has extremely high resistance to a beta-lactam (60). With respect to the *E. coli* $AG100_{TET8}$ strain, adaptation to tetracycline was solely due to over-expression of genes that control the permeability of the organism to noxious agents and not due to any mutation (65). Resistance of this strain to tetracycline as well as to the antibiotics that contributed to its MDR phenotype could be reversed by transfer to drug free medium or by the addition of an agent that competes with an AcrAB substrate such as PAβN (37;206) or an inhibitor of an efflux pump such as thioridazine or chlorpromazine (37). Serial passage of the *E. coli* $AG100_{TET8}$ in medium containing the same concentration of antibiotic, 10 mg/L of tetracycline, resulted in increase of the MIC of tetracycline from 10 to over 200 mg/L (181). This strain now named *E. coli* $AG100_{TET10}$ was evaluated for its response to PAβN and noted that after the 10^{th} serial passage in medium containing 10 mg/L of tetracycline, the resistance to tetracycline could not be altered.

Table 4 - The effect of serial exposure of the *E. coli* $AG100_{TET8}$ strain to 10 mg/L of tetracycline on the MIC of this antibiotic.

	$AG100_{TET8}$			$AG100_{TET10}$			
Passage number	1	10	20	30	40	50	60
E-Test MIC	10	64	64/96	96	96/128	128	256

The activity of the global regulator genes and genes that code for the transporters of eight efflux pumps of the *E. coli* $AG100_{TET8}$ and *E. coli* $AG100_{TET10}$ relative to those of the *E. coli* AG100 un-exposed to tetracycline was accessed by real time Reverse Transcription polymerase chain reaction (rtRT-PCR) (37;65). The results showed that, whereas the activity of the regulators *marA* and *ompX,* stress genes *rob* and *soxS* and the transporter genes *acrB* and *acrF* of the *E. coli* $AG100_{TET8}$ strain were significantly elevated, those of the *E. coli* $AG100_{TET10}$ were practically identical to the ones of the wild type *E. coli* AG100 strain suggesting that the resistance of the *E. coli* $AG100_{TET10}$ strain was not mediated by the efflux pumps of that strain.

Evaluation of the *E. coli* $AG100_{TET8}$ and *E. coli* $AG100_{TET10}$ strains for resistance to other antibiotics (ex. ampicillin, ciprofloxacin, norfloxacin, nalidixic acid, streptomycin, etc.) demonstrated that both strains were resistant to these antibiotics and therefore maintained a MDR phenotype. These results were confirmed by evaluation of phenotypic resistance of the *E. coli* $AG100_{TET8}$ and *E. coli* $AG100_{TET10}$ strains by micro-array relative to that of the wild-type *E. coli* AG100 (gently performed by Fanning and co-workers, Ireland). Briefly, increased resistance to antibiotics that target the cell envelope, gyrase and the ribosome is shown for the *E. coli* $AG100_{TET8}$ strain; resistance to these antibiotics is even greater for the *E. coli* $AG100_{TET10}$ strain (Table 5).

Table 5 - Phenotypic Array evaluation of *E. coli* AG100$_{TET8}$ and *E. coli* AG100$_{TET10}$ strains.

Test	Increase in TDRU*[1]		Class of antibiotic and target
	TET8	TET10	
Lomefloxacin	19450	**41154**	fluoroquinolone, DNA unwinding (gyrase and topoisomerase)
Enoxacin	**40488**	**44797**	
Ofloxacin	18895	21316	
Norfloxacin	20519	21240	
Ciprofloxacin	21984	21352	
Nalidixic acid	**36125**	**41336**	quinolone, DNA unwinding (gyrase and topoisomerase)
Oxolinic acid	19749	21048	
Cinoxacin	**38867**	**40375**	
Pipemidic Acid	19522	21029	
Kanamycin	18700	20991	aminoglycoside, protein synthesis (30S ribosomal subunit)
Sisomicin	19532	21523	
Tobramycin	21158	22107	
Chlortetracycline	18629	21997	tetracycline, protein synthesis (30S ribosomal subunit)
Demeclocyline	**41074**	**45607**	
Penimepicycline	18013	20270	
Rolitetracycline	**38366**	**40913**	
Oxytetracycline	20910	20558	
Geneticin (G418)	20325	21749	aminoglycoside, protein synthesis
Doxycycline	19558	20840	tetracycline, protein synthesis
Cefazolin	19308	22623	1st generation cephalosporin, cell wall
Cephalothin	19623	23278	
Cefuroxime	20110	22397	2nd generation cephalosporin, cell wall
Cefotaxime	**58973**	**61403**	3rd generation cephalosporin, cell wall
Cefoperazone	**60936**	**60582**	
Amoxicillin	19914	23548	β-lactam, cell wall
Cloxacillin	**40098**	**44947**	
Nafcillin	19322	21372	
Oxacillin	**37677**	**41972**	
Carbenicillin	**41948**	**45618**	
Aztreonam	20157	20760	
Phenethicillin	**40768**	**40166**	

[1] TDRU, tetrazolium dye reduction units, the increase in the area under the kinetic plot in comparison to the wild-type parent strain AG100 is given; an increase of ≥20,000 TDRU is considered significant. The highlighted TDRUs are highly significant and suggest high resistance to the corresponding agents.

2. pH and energy roles on efflux by Gram-negatives

E. coli AG100 and its progeny *E. coli* $AG100_{TET8}$ were previously characterized for alteration in the expression of regulator and transporter coding genes during the adaptation process. These genes collectively control the permeability of the organism to noxious agents such as antibiotics, biocides and detergents (65). In addition, although genes that code for porins remain at the level of the unexposed control, the amount of porins retrieved from cells during the adaptation process decreased (37;65). The decrease was hypothesised to be due to the increase of proteases that degraded the OMPs prior to their assembling into the porin unit (37;65). Based upon the previous findings and relating the membrane alterations when a bacterium is exposed to a noxious agent and the genetic response towards this exposure, attention was now focused on the role of pH and energy in these responses, using a real-time assay to quantify and compare the efflux activity in the adapted and parental strains.

The evaluation of influx and efflux of an AcrAB efflux pump substrate such as ethidium bromide (EB) was conducted by the method described by Viveiros *et al*. (187). EB is a common substrate of efflux pumps (207-209) and does not affect the cell viability or cellular function at the concentrations used in the assay. The signal of the EB inside the cell can be detected by real time fluorescence spectroscopy. It allows the assessment of the transport of the EB molecule across the cell membrane which results in fluorescence as a consequence of its increased concentration in the periplasm of the cell (187) (accumulation assays). The method also affords the evaluation of EB extrusion (efflux assays). It must be noted that the concentration of EB employed in this method does not produce fluorescence by its presence in the medium.

The method provides assessment of influx and efflux on a real-time basis. Moreover, it can be readily used for the simulation of physiological conditions that are known to affect permeability, accumulation and efflux. The application of the EB method to Gram-negative strains that differed with respect to the degree of efflux pump expressed afforded the characterization of these strains at the level of their capacity to extrude the fluorochrome substrate EB.

The conventional assay employed by others involves the fluorometric analyses of EB retained at pH 7, at room temperature and buffer without an energy source (210). This method does not approach physiological conditions that are known to affect the efflux apparatus of a Gram-negative bacterium. To correlate *in vivo* responses to antibiotic that involve extrusion the assay should be conducted at 37ºC, with metabolic energy and an ionic strength of the medium that corresponds to that of the medium in which the organism is cultured. In the work to be described, the accumulation and efflux assays were first conducted at 37ºC, pH 7.4 in the presence and absence of metabolic energy.

2.1 Role of glucose

Glucose was used in the assays as an energy source (Figure 18). Because the importance of metabolic energy has not been explored by the use of the conventional EB assays, its role will be focused on the Discussion Section. Nevertheless, because the cells will face starving conditions in an EB type of assay will also place the organism under stress, the presence of glucose will restore the osmotic conditions and provide the cell a carbon (energy) source needed for the maintenance of a proton motive force (PMF) energy source of protons that drive the RND efflux pump of the Gram-negative bacterium (211;212). The PMF, proton gradient and concentration of protons on the surface of the cell and in the periplasm will be subjects discussed in the Discussion Section.

The influence of glucose on the accumulation of EB by *E. coli* AG100 and the adapted to tetracycline strain *E. coli* $AG100_{TET8}$ at pH 7 can be seen by Figure 18. The strains were incubated in the presence of EB, with and without glucose, during 30 min and the fluorescence was recorded during that period. In the presence of glucose both strains present less fluorescence. However, the curves have similar behaviour in both conditions. The accumulation of EB by the parental strain *E. coli* AG100 is comparatively higher and constant than for the adapted strain, *E. coli* $AG100_{TET8,}$ whose efflux systems are over-expressed.

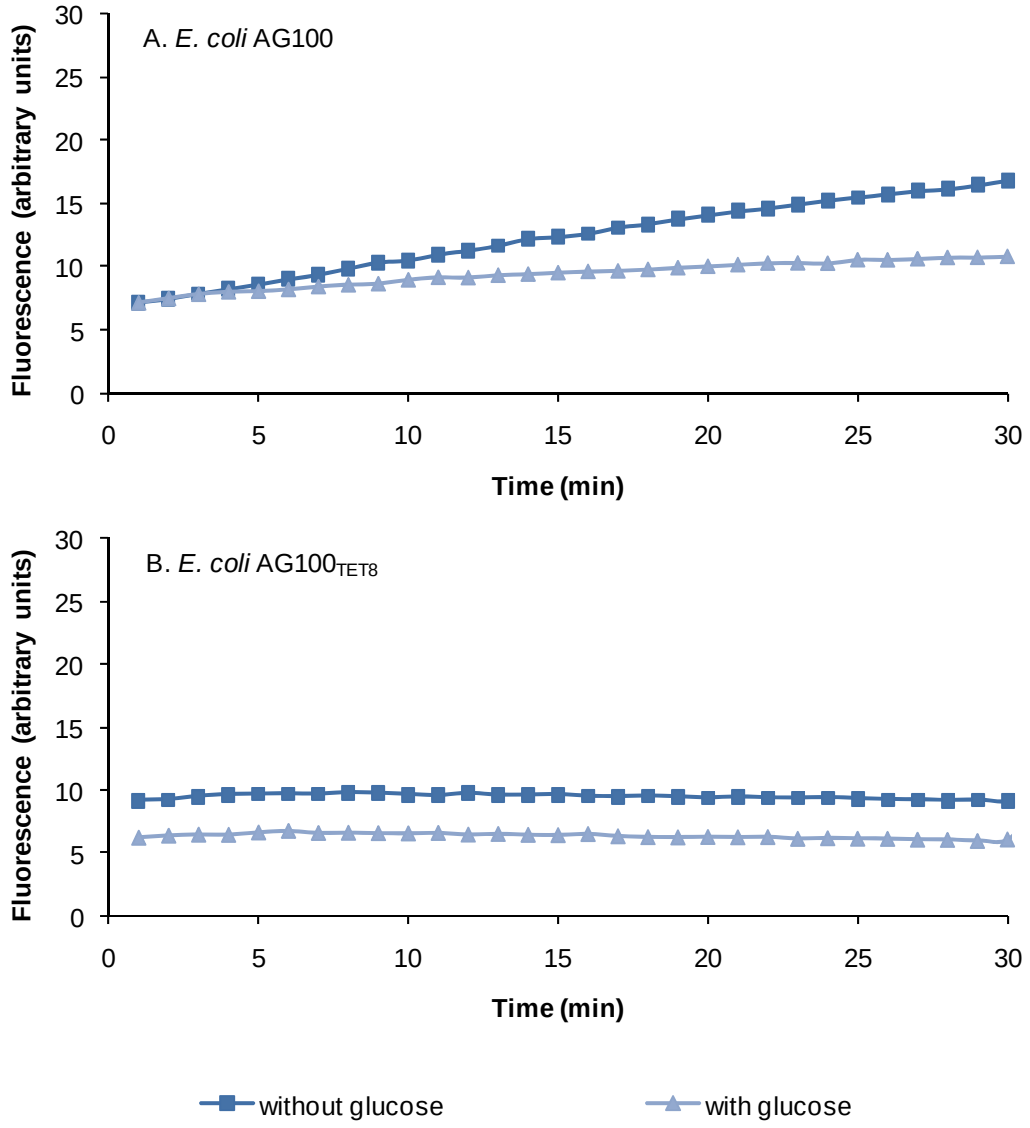


Figure 18 - The effect of glucose on the accumulation of EB by *E. coli* AG100 (A) and *E. coli* $AG100_{TET8}$ (B).

A variation of the experiment of Figure 18 was done to evaluate the influence of glucose on the efflux of EB as shown by Figure 19. Firstly, accumulation of EB was permitted in the absence of glucose. Secondly, the instrument is paused and additions of glucose-free saline and glucose containing saline were made, and the instrument re-started. Whereas the addition of glucose-free saline blank does not affect the rate of increased fluorescence, the addition of glucose containing saline immediately reduces

fluorescence to a basal level. This reduction of fluorescence is due to the presentation of metabolic energy needed by the efflux pump system of the cell, without which efflux of EB cannot take place. A description of this efflux assay may be found at Section 1.4.2 of Materials and Methods.

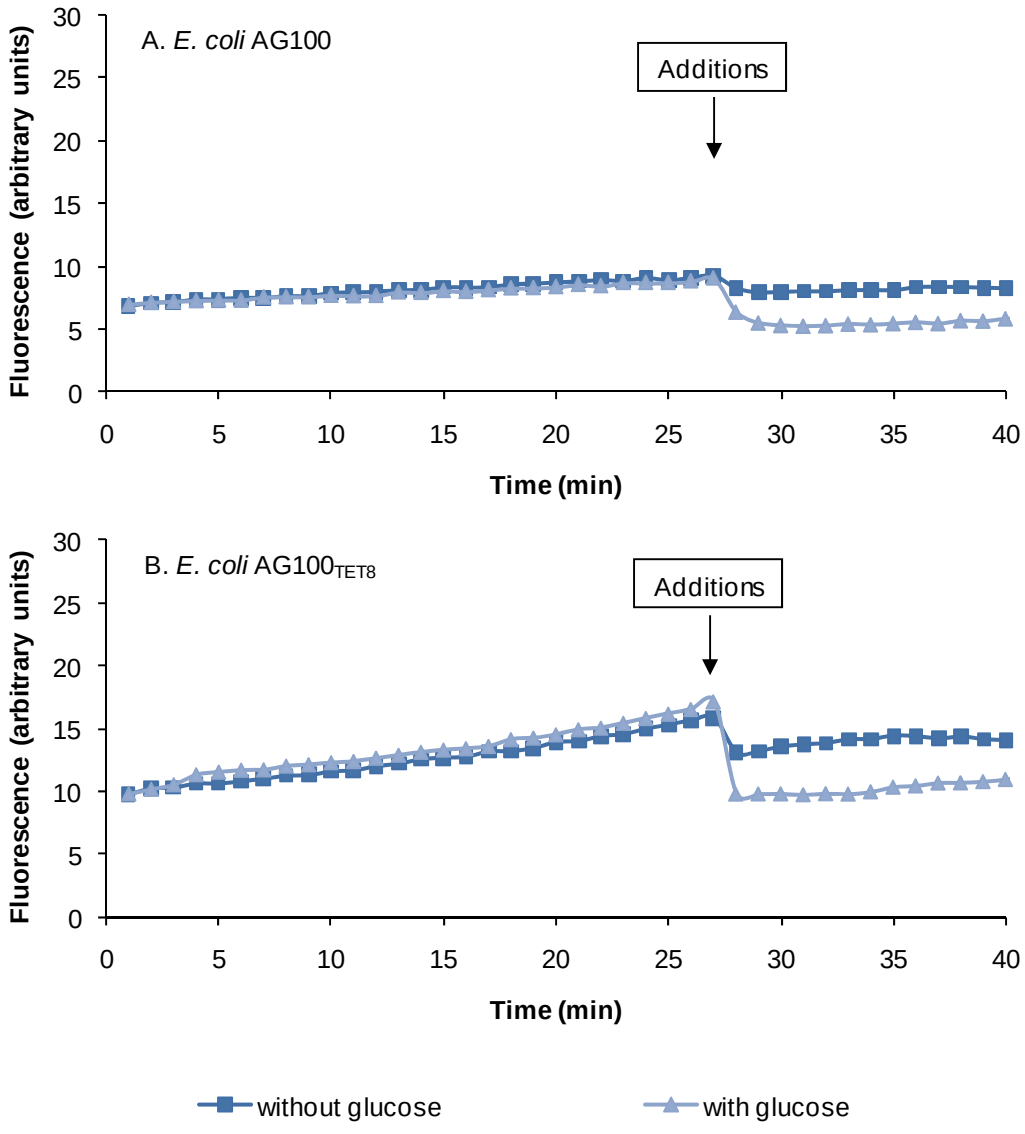


Figure 19 - The effect of glucose on the efflux of EB by *E. coli* AG100 (A) and *E. coli* AG100$_{TET8}$ (B). Accumulation of EB in glucose-free saline for 25 minutes. Instrument is stopped and glucose-free and glucose-saline is added and the instrument restarted.

Because glucose is vital for the function of the efflux pump system of *E. coli,* all of the EB assays to be described were done in the presence and absence of glucose.

2.2 Role of pH

Efflux of antibiotics by bacteria is performed by a variety of transporters that use different energy sources to pump the noxious compounds out of the cell. This variety of efflux pumps ensures the survival of the bacterium in widely diverse media. For example bacteria that colonize the human digestive tract need to survive the exposure to noxious agents such as toxic bile salts (ex: duodenum and colon) (213).

The ingestion of food containing a Gram-negative or positive bacterium that subsequently results in the colonisation of the colon means that the bacterium needs to successfully pass portions of the gastro-intestinal tract that present noxious agents in a milieu that differs widely in pH. Obviously, because the organism does colonise the colon, passage through the gastro-intestinal tract does not pose a problem. Moreover, it is known that wide ranges of pH do not alter the PMF of bacteria (54). Thereby assuming that efflux dependent upon the PMF will not be affected by pH, the question of whether pH modifies the efflux pump activity when the organism is under stress imposed by a noxious agent has not been asked before. This question has been investigated and answered in this thesis. The modulation of accumulation and efflux of EB by *E. coli* AG100 and *E. coli* $AG100_{TET8}$ by pH and glucose is described below.

Firstly, as evident by Figure 20, whereas no significant accumulation of EB during the first 25 minutes takes place in absence of glucose, at pH 8 the degree of accumulation is inversely dependent upon metabolic energy. At pH 5 the addition of glucose or its absence does not affect efflux.

Efflux of EB at pH 8 needs metabolic energy as it is illustrated with the addition of glucose to the cells after 25 minutes of accumulation of EB in glucose free-saline and pH 8. The addition of glucose-saline pH 8 causes fluorescence to drop to that initially

present during the accumulation period of the assay. The addition of glucose-free saline at pH 8 does not produce a drop of fluorescence and the rate of EB accumulation noted in the absence of glucose during the accumulation phase is maintained. Because of the dependence on metabolic energy for extrusion of EB, these results suggest that the intrinsic efflux pump system of the *E. coli* AG100 strain that is operating at pH 8 is one of an ABC superfamily type (214). At pH 5, the glucose does not have effect on the efflux suggesting that at this pH of 5 the PMF is the driving force for efflux.

Similar results were obtained with the *E. coli* $AG100_{TET8}$ strain that over-expresses the AcrAB efflux pump as compared to its parent *E. coli* AG100 (37;65). As evident by Figure 20B, glucose is needed for efflux of EB at pH 8 but plays no role at pH 5.

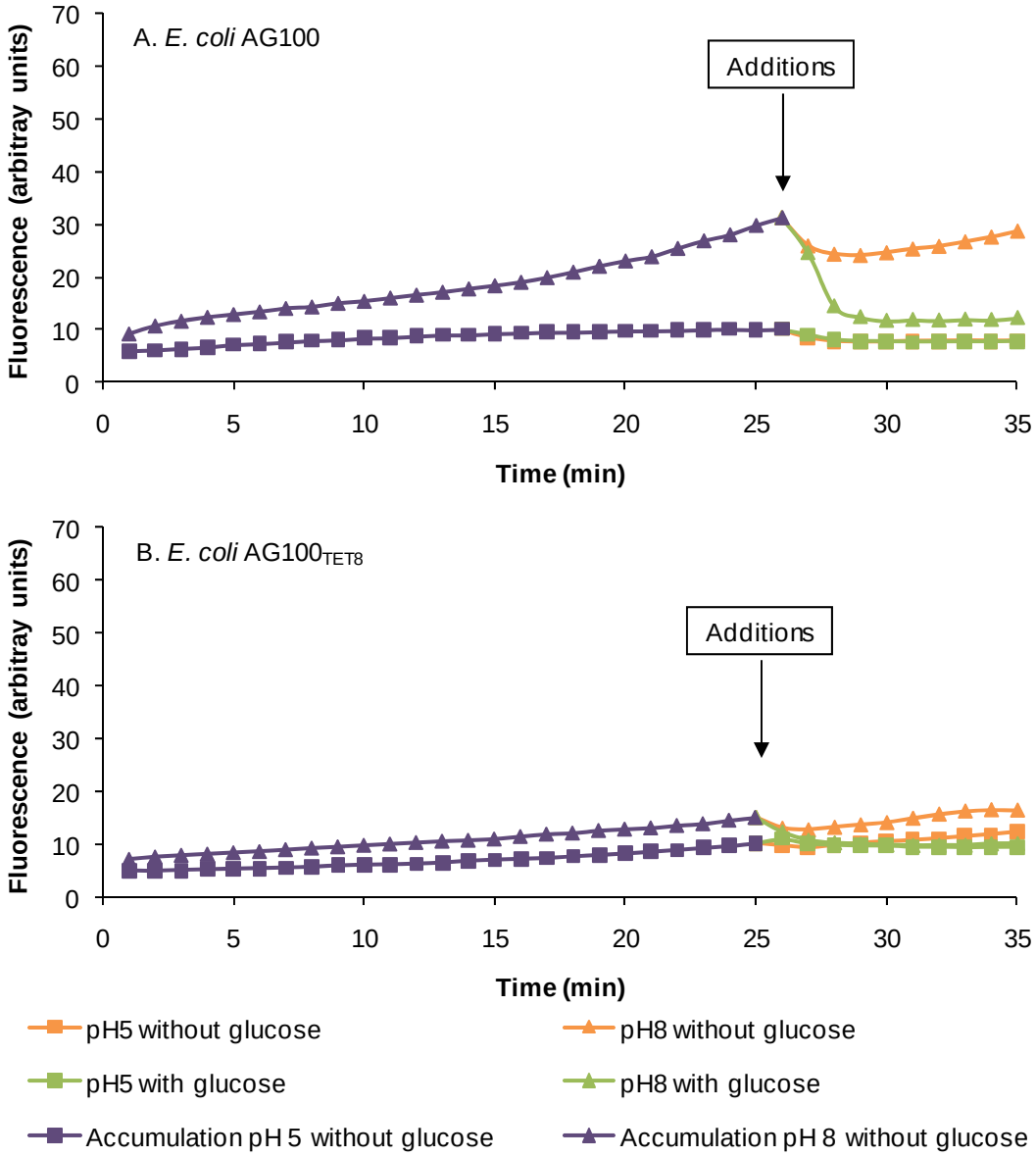


Figure 20 - The effect of pH and the need for metabolic energy for efflux of EB by *E. coli* AG100 (A) and *E. coli* AG100$_{TET8}$ (B).

Accumulation of EB in glucose-free saline at pH 5 and 8 for 25 minutes. Instrument is stopped and glucose-free and glucose-saline pH 5 and 8, respectively, is added and the instrument restarted.

The accumulation of EB by cells grown in MHB of pH 5, 7 and 8 was conducted in order to determine if growth conditions would affect the accumulation and efflux of EB at varying pH of the assay. As evident by the example presented by Figure 21, cells grown in MHB of pH 8 when assayed for accumulation at pH 5, 7 and 8 in presence and

absence of glucose yielded the same data as if the cells had been grown at pH 5 or 7. These results were similarly obtained with cells grown at pH 5 or 7 and evaluated for accumulation at pH 5, 7 and 8 (data not shown). It can be conclude from these experiments that the accumulation and efflux of EB is primarily regulated by the pH of the assay and the presence/absence of glucose and not by the pH of the medium in which they had been cultured.

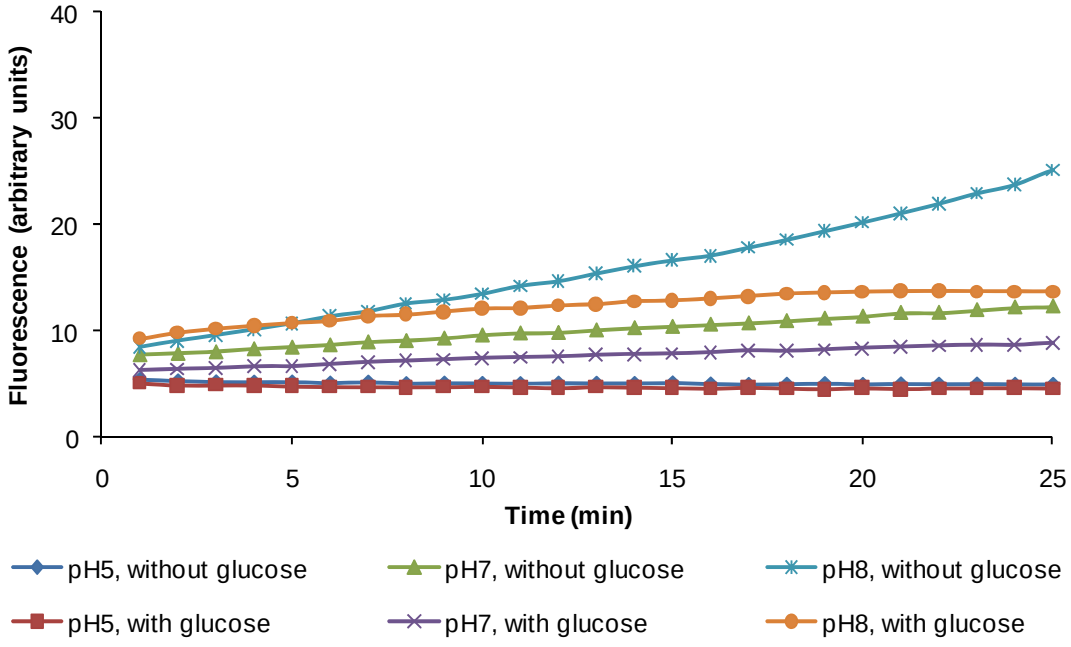


Figure 21 – Accumulation of EB in glucose and glucose free media pH 5, 7 and 8 by *E. coli* AG100.
E. coli AG100 cells were grown at pH 8 and, then, submitted to different pH during the assay.

2.3 Role of efflux modulators

Some efflux modulators, commonly called EPIs (efflux pump inhibitors), were also tested in order to evaluate the efflux of EB by the parental and their MDR progeny. The modulators used were the proton un-coupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), Phe-Arg-beta-naphtylamide (PAβN), verapamil (VER) and phenothiazines (thioridazine (TZ) and chlorpromazine (CPZ)).

2.3.1 CCCP

The demonstration of an RND type efflux pump of a Gram-negative is usually conducted with the un-coupler of the proton motive force CCCP at pH 7 and in the absence of metabolic energy. Given the demonstration that at pH 8 metabolic energy optimizes efflux, the activity of varying concentrations of CCCP at pH 5 and 8 on the efflux of EB after the fluorochrome has accumulated in the absence of glucose has been studied and the results obtained described by Figure 22 for the *E. coli* AG100 and *E. coli* $AG100_{TET8}$ that over-expresses its AcrAB efflux pump (37;65).

At pH 5 and 8 the addition of CCCP prevents efflux and increases the rate and extent of accumulation of EB by *E. coli* AG100 and *E. coli* $AG100_{TET8}$ in a concentration dependent manner. However, whereas at pH 8 a very low concentration of CCCP produces a very steep increase of EB retained (efflux is inhibited) at pH 5 a far greater concentration of this agent is needed for a similar retention of EB (Figure 22A and Figure 22B).

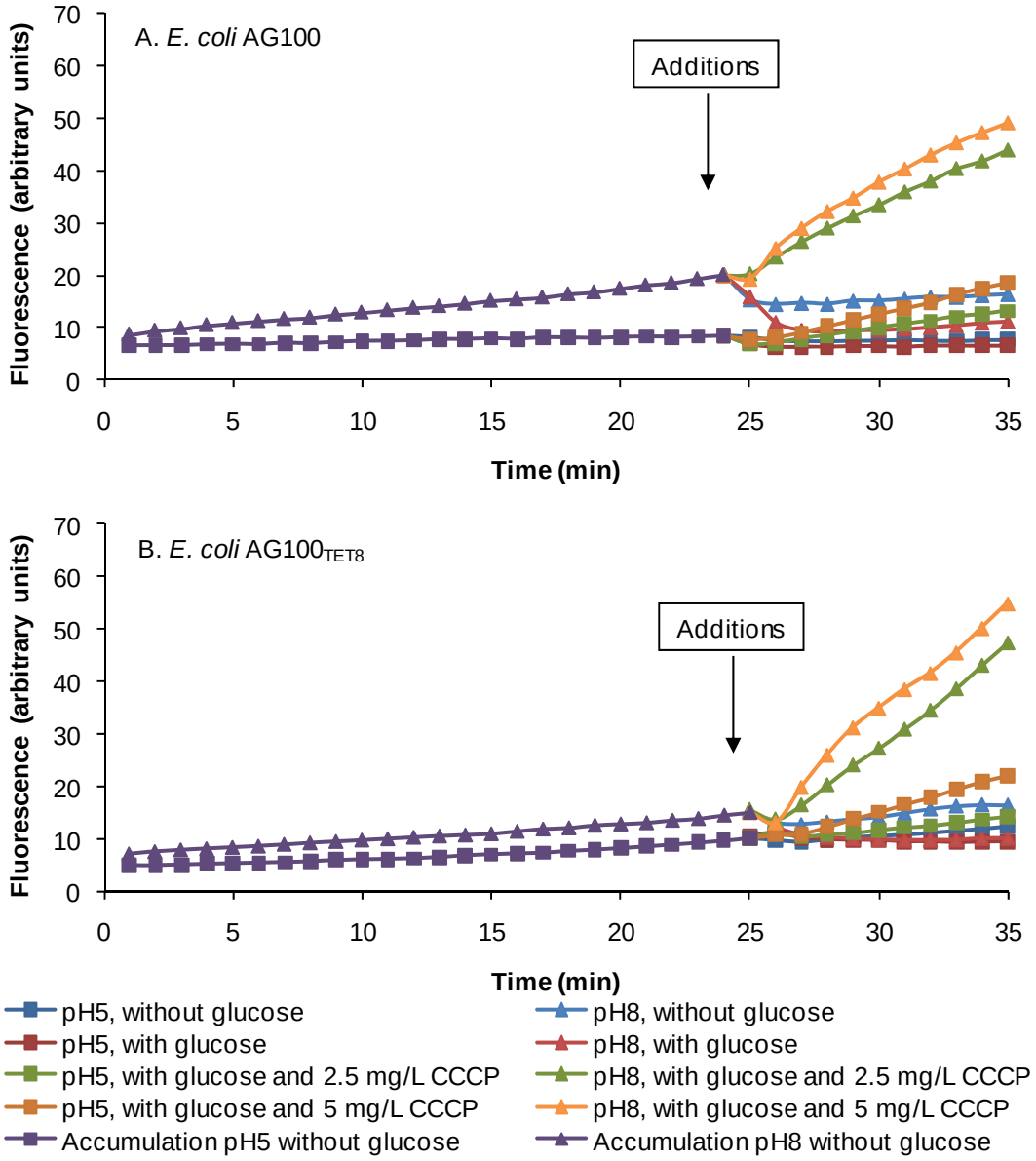


Figure 22 – Effect of CCCP concentrations on efflux of EB by *E. coli* AG100 (A) and *E. coli* AG100$_{TET8}$ (B) at pH 5 and 8.

The same protocol of accumulation was followed as for Figure 20. After 25 minutes, saline with glucose and CCCP was added to the tubes. The control without CCCP and without glucose is also presented.

Slopes of EB accumulation in the first 25 minutes are presented in Table 6 and Table 7 presents the slopes of increased accumulation (amount of increased fluorescence per 10 minutes after the addition of varying concentrations of CCCP) for both parental and its MDR progeny. Comparing the difference in slopes between the period of accumulation

and the period after additions, it is observed that, at pH 5 and for both strains, they are maintained with no considerable difference (as observed also in the graph of Figure 22) when in absence of CCCP and without glucose. The same occurred when glucose is added to *E. coli* AG100. However for *E. coli* AG100$_{TET8}$ the addition of glucose promotes efflux at both pH. At pH 8 this is also observed for the *E. coli* AG100.

When CCCP is added, even in presence of glucose, the slopes increase for both strains at both pH 5 and 8 providing evidence of increased accumulation due to reduction of efflux activity. However this increase is greater at pH 8 than at pH 5. At pH 5 the effect of CCCP is similar for the *E. coli* AG100 and *E. coli* AG100$_{TET8}$. At pH 8 the MDR strain is more affected by the addition of CCCP than the *E. coli* AG100 strain as evident by the difference in the slope of accumulation.

Table 6 – The slopes (rates) of EB accumulation by *E. coli* AG100 and *E. coli* AG100$_{TET8}$.

Conditions of accumulation			Slope (fluorescence/min)	
pH	glucose	CCCP (mg/L)	*E. coli* AG100	*E. coli* AG100$_{TET8}$
5	without	0	0.09	0.21
8	without	0	0.46	0.31

Table 7 – Slopes of EB accumulation / Efflux after the addition of CCCP (Figure 22).

Conditions of efflux			Slope (fluorescence/min)	
pH	glucose	CCCP (mg/L)	*E. coli* AG100	*E. coli* AG100$_{TET8}$
5	without	0	0.02	0.34
	with	0	0.02	-0.09
	with	2.5	0.67	0.48
	with	5	1.18	1.41
8	without	0	0.23	0.53
	with	0	0.23	-0.06
	with	2.5	2.18	3.83
	with	5	2.52	4.13

2.3.2 PAβN

PAβN has been used to reveal the presence of efflux pump activity in Gram-negative bacteria. In this case PAβN is considered as an inhibitor of RND type of efflux pumps. Considering the previous results that the efflux of EB at pH 5 is independent of metabolic energy and dependent upon the PMF, this compound should, then, inhibit the efflux of EB at pH 5. However, previous studies suggested that PAβN is not an inhibitor of an efflux pump but rather a competitor of other efflux pump substrates for extrusion (65;215). Preferential extrusion of PAβN would result in an increasing of concentration of the antibiotic which eventually reaches an active concentration against the organism.

Previous studies showed that PAβN affects the accumulation of EB in a concentration dependent manner at pH 7 but do not have an effect in the efflux of EB by the strain *E. coli* AG100 (187).

In this study was observed the effect of PAβN in the accumulation of EB by the parental and MDR *E. coli* strains at pH 5 and pH 8. Addition of PAβN has no effect on the efflux of EB neither at pH 5 nor at pH 8, as presents on Figure 23 for *E. coli* AG100. It is visible that the efflux is only affected by the presence of glucose.

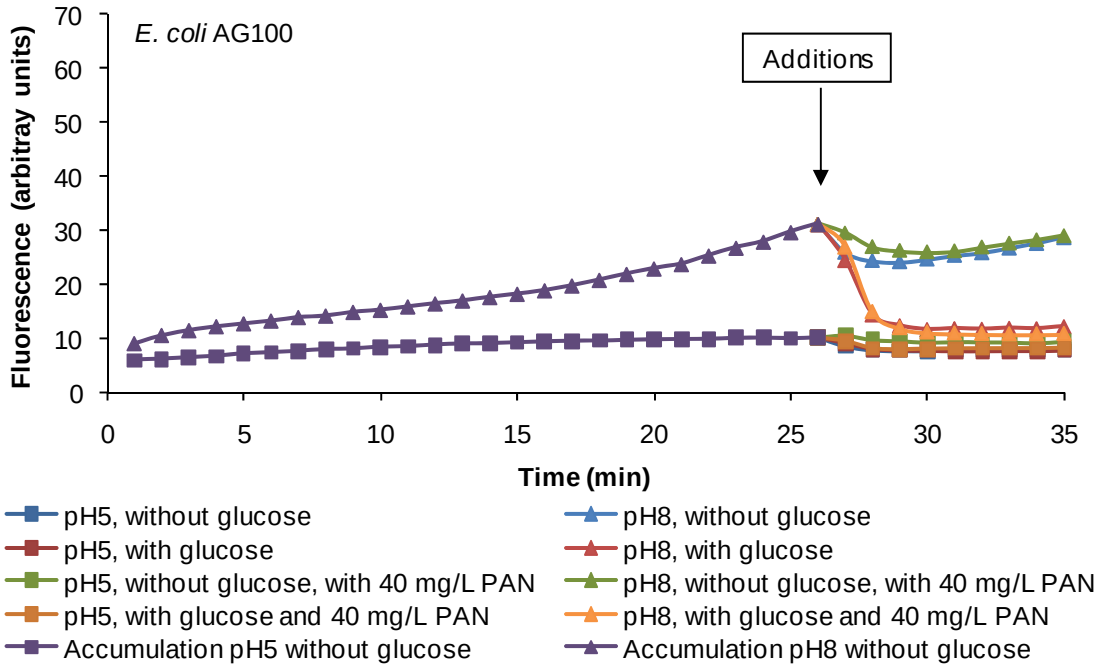


Figure 23 - Effects of PAβN on efflux of EB by *E. coli* AG100 at pH 5 and 8.
The same protocol of accumulation was followed as for Figure 20. After 25 minutes media with glucose and PAβN was added to the tubes.

Based on the accumulation results and because PAβN has no effect on efflux of EB, it was considered the hypotheses of competition between PAβN and EB. If PAβN competes with EB, as the concentration of PAβN is increased more EB would be expected to accumulate, as it was observed. In that situation a derivation of a Km for PAβN relative to EB at pH 5 should be possible. pH 5 was chosen because at this pH metabolic energy is not needed. Moreover, the dissociation constant of EB from the AcrB transporter is lowest at pH 5 (216), a condition that is necessary for continuous efflux of EB.

As described by the Figure 24, as the concentration of PAβN is increased from 1 to 40 mg/L, the amount of EB accumulation by *E. coli* $AG100_{TET8}$ is proportionately increased.

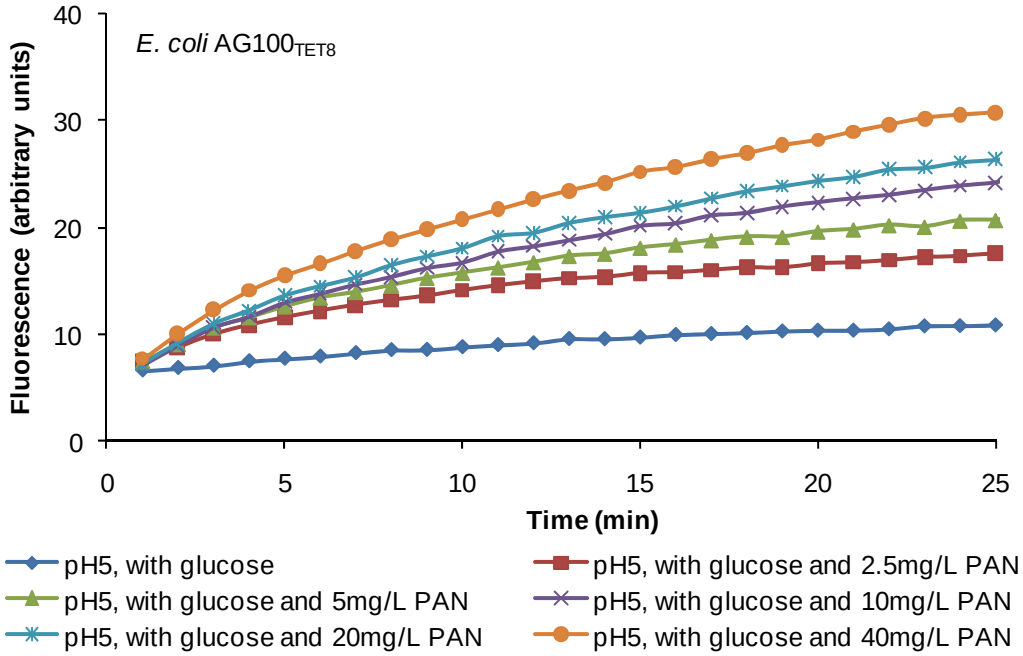


Figure 24 - Effects of different concentration of PAβN on efflux of EB by *E. coli* AG100$_{TET8}$ at pH 5 and 8.

Employing Michaelis-Menten formulae, the Km for PAβN representing competition between PAβN and EB was calculated and obtained the value of 4.21 mg/L (Figure 25).

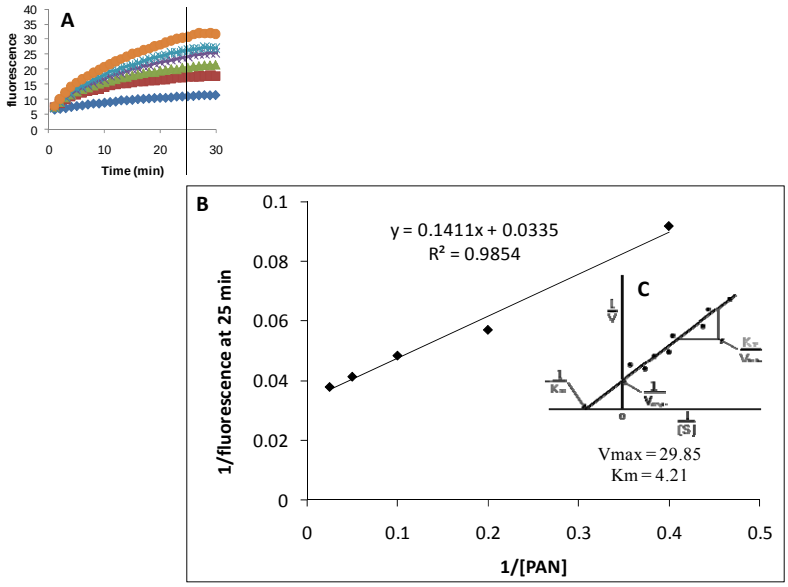


Figure 25 - Competition between EB and PAβN: calculation of Km for PAβN relative to EB.
Increasing concentrations of PAβN from 1 to 40 mg/L caused increase of fluorescence (A). This data was then used for the derivation of the PAβN Km initially plotted by (B) and data employed in the Lineweaver-Burk plot (C).

2.3.3 Verapamil

Verapamil inhibits ABC transporters of *S. aureus* (217) and mycobacteria (218). However, there is little information regarding the effects of verapamil on efflux activity of a Gram-negative such as *E. coli*. Considering the possibility that the study of agents for inhibitory activity against efflux pumps is always conducted at neutral or near neutral pH, and because at pH 8 efflux of EB by *E. coli* is dependent upon metabolic energy, suggesting the involvement of an ABC type transporter, the effects of concentrations of verapamil on efflux of EB were evaluated.

As evident from Figure 26, and consistent with previously presented data, at pH 8 efflux of EB is dependent upon the presence of metabolic energy. The addition of verapamil in the absence of glucose, promotes a concentration dependent inhibition of efflux which causes proportional increases in the accumulation of EB. The inhibitory effect on efflux by verapamil is significantly decreased when metabolic energy is present.

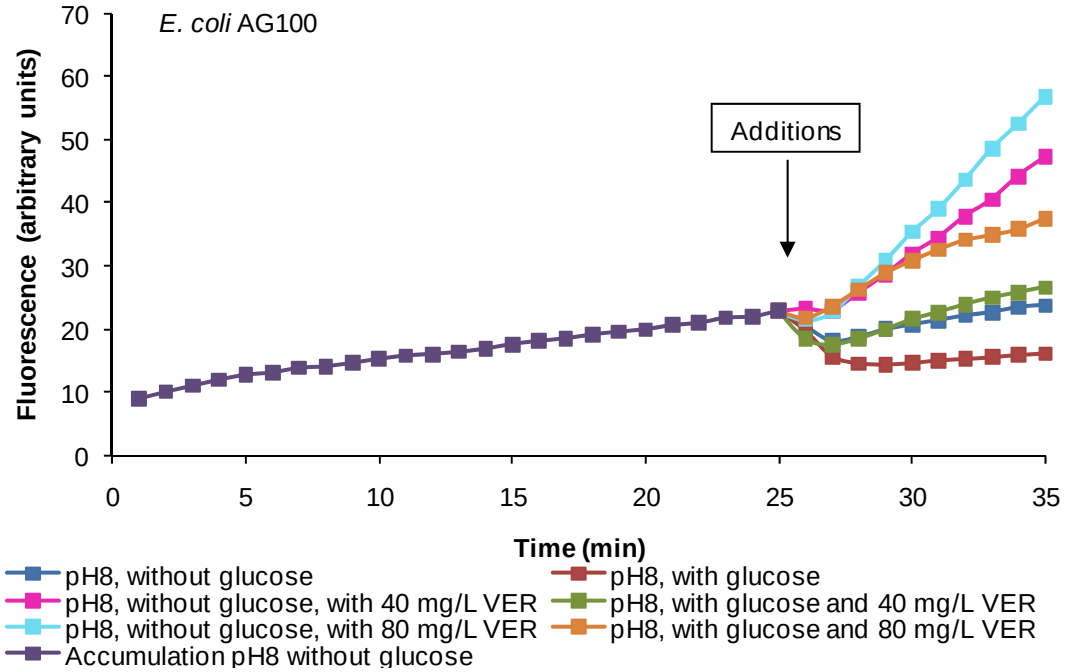


Figure 26 - The effects of concentrations of verapamil on the efflux of EB by *E. coli* AG100 at pH 8.

2.3.4 Phenothiazines

Phenothiazines are calcium channels inhibitors and have been evaluated for EPI activity by other authors (20;152;154). Because calcium plays a crucial role in cell signalling and transport activity of the cell, the phenothiazines thioridazine and chlorpromazine were used in this study to observe their effect on accumulation and efflux of EB. As evident from Figure 27 and Figure 28, TZ and CPZ increase the accumulation of EB in a concentration dependent manner; CPZ promotes a much greater effect than TZ on the accumulation of EB. The accumulation of EB by *E. coli* AG100 is more influenced by the presence of these phenothiazines than the adapted strain. However, because higher concentrations of CPZ are needed to increase EB accumulation by the *E. coli* $AG100_{TET8}$ strain, one may conclude that this result is due to the over-expressed AcrAB transporter of this MDR strain. Similar results were obtained with the addition of CPZ and TZ in the presence and absence of glucose (Figure 29 and Figure 30).

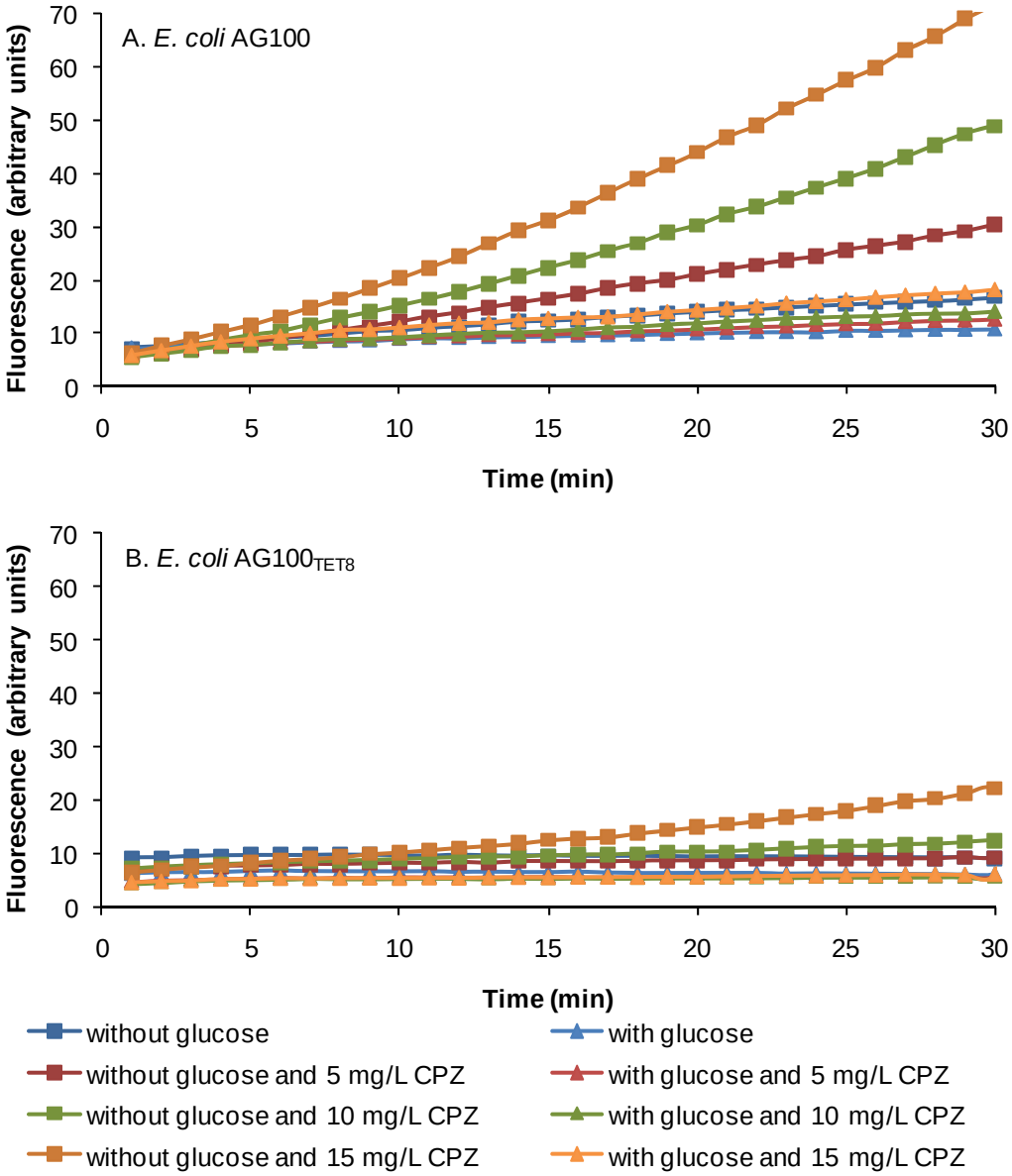


Figure 27 - Accumulation of EB by *E. coli* AG100 (A) and *E. coli* AG100$_{TET8}$ (B) at pH 7 and in the presence and absence of glucose and different concentrations of CPZ.

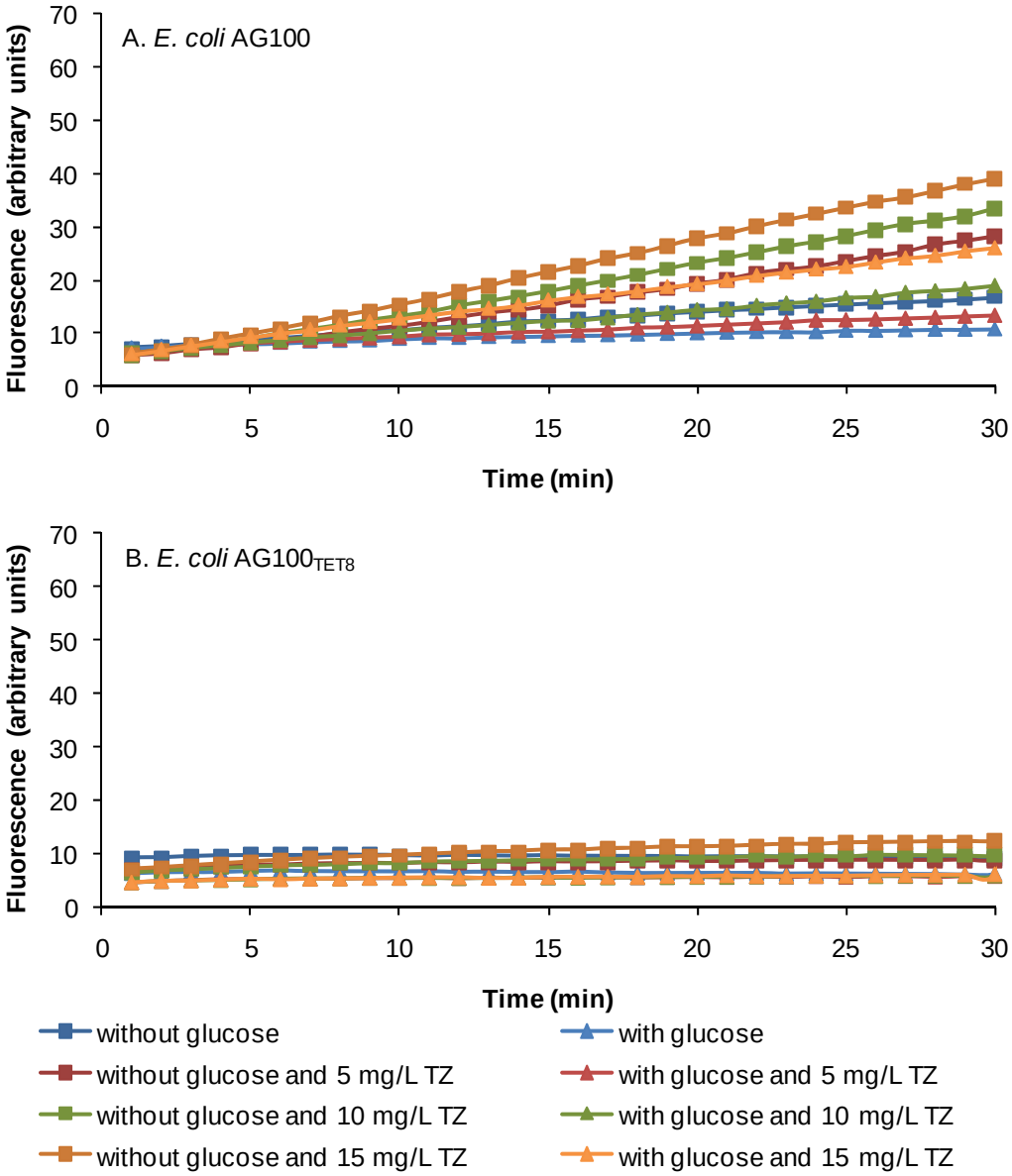


Figure 28 - Accumulation of EB by *E. coli* AG100 (A) and *E. coli* AG100$_{TET8}$ (B) at pH 7 and in the presence and absence of glucose and different concentrations of TZ.

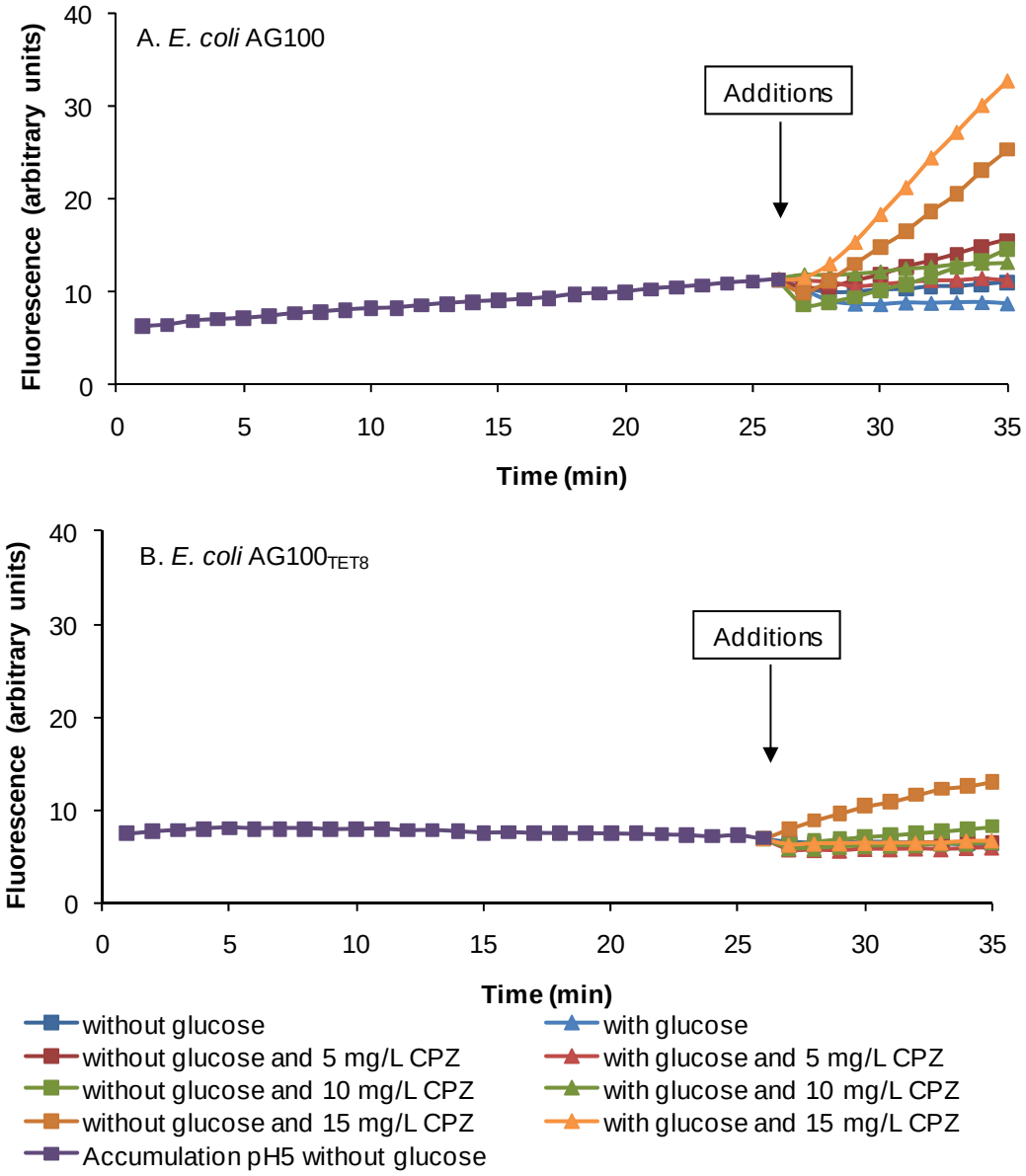


Figure 29 - Efflux of EB by *E. coli* AG100 (A) and *E. coli* AG100$_{TET8}$ (B) at pH 7 and in the presence and absence of glucose and different concentrations of CPZ.

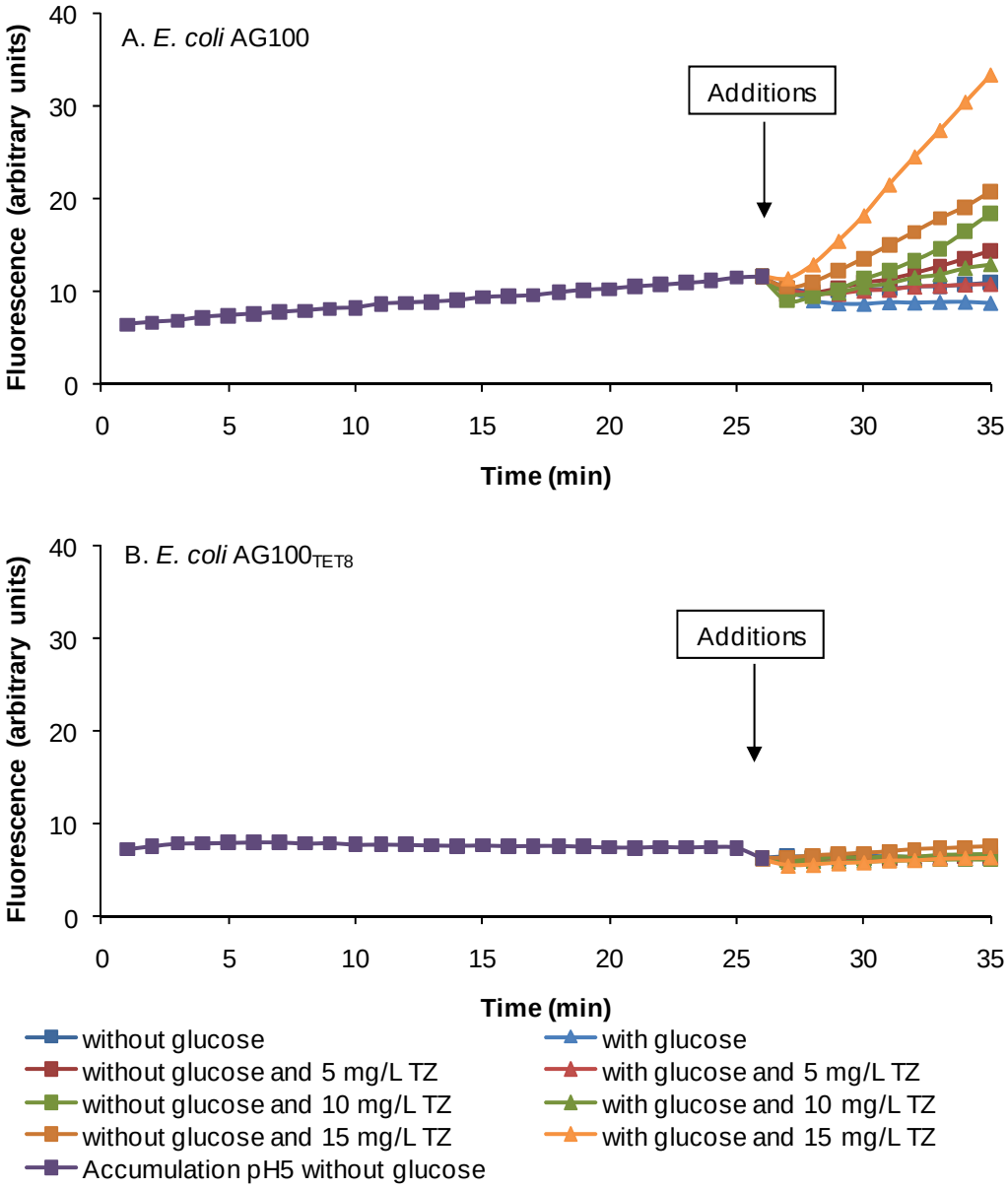


Figure 30 - Efflux of EB by *E. coli* AG100 (A) and *E. coli* AG100$_{TET8}$ (B) at pH 7 and in the presence and absence of glucose and different concentrations of TZ.

Because it was already observed that pH plays an important role in the efflux characteristics of these strains, the effects of TZ on efflux was also studied at pH 5 and pH 8 (Figure 31 and Table 8). As shown by this figure 15 mg/L of TZ influences the efflux of EB at both pH. However, this effect is significantly greater with *E. coli* AG100 than that with *E. coli* AG100$_{TET8}$ (Table 8). The effect of TZ on the efflux is also

dependent on the presence of glucose as was observed with the accumulation assays. Similar results were obtained with CPZ.

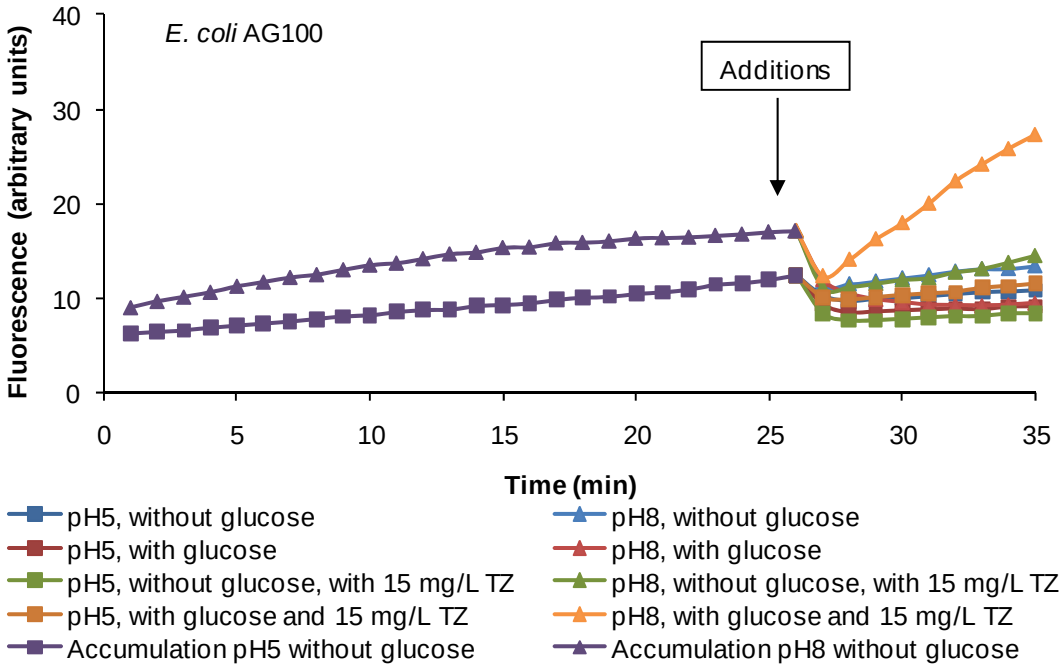


Figure 31 - Efflux of EB by *E. coli* AG100 at pH 5 and 8, in the presence and absence of glucose and different concentrations of TZ.

Table 8 - Slopes of EB accumulation / Efflux after the addition of TZ by *E. coli* AG100 (Figure 31) and *E. coli* AG100$_{TET8}$.

Conditions of efflux			Slope (fluorescence/min)	
pH	glucose	TZ (mg/L)	*E. coli* AG100	*E. coli* AG100$_{TET8}$
5	without	0	0.118	0.144
	with	0	0.026	0.192
	without	15	0.057	0.209
	with	15	0.212	0.259
8	without	0	0.264	0.342
	with	0	0.258	0.162
	without	15	0.426	0.956
	with	15	1.927	0.352

2.4 Role of Calcium

Phenothiazines such as CPZ and TZ inhibit the binding of Ca^{2+} to enzymes involved in furnishing energy from the hydrolysis of ATP (154). Ca^{2+} plays a crucial role in the biochemical pathways of the cell and is of extreme importance for cell signalling, for the membrane transport channels and for activity of some type of ATPases, as well (219;220). Because, as shown by the previous section, the phenothiazines CPZ and TZ have major effects on the accumulation and efflux of EB, and these effects are modified by metabolic energy, the role of Ca^{2+} in the modulation of EB accumulation and efflux was evaluated. Because CPZ has a greater effect on accumulation of EB by the *E. coli* AG100 strain, CPZ will be used in the study of the role of Ca^{2+} on the efflux of EB by this strain. Moreover, because EDTA is a chelating agent and has the ability to "sequester" metal ions such as Ca^{2+} and Fe^{3+}, this agent was also used in the experiments described below to study the role of Ca^{2+}.

The results obtained are presented by Figure 32 and Figure 33. At pH 8 the amount of EB accumulated inside the cell is glucose dependent (metabolic energy) and greater than at pH 5. At pH 8, CPZ enhances the retention of EB, especially with the omission of glucose. The CPZ promoted retention of EB at pH 8 can be nullified by the addition of calcium to the medium. The role of calcium in the accumulation and efflux of EB is further illustrated with the addition of EDTA, which by binding the calcium that is present, promotes the increase of EB retained. The addition of calcium to an EDTA containing medium nullifies the accumulation of EB promoted by EDTA. The simultaneous presence of CPZ and EDTA synergistically increases accumulation of EB. At pH 5 the effects of CPZ, EDTA and calcium are minimal on the accumulation and efflux of EB.

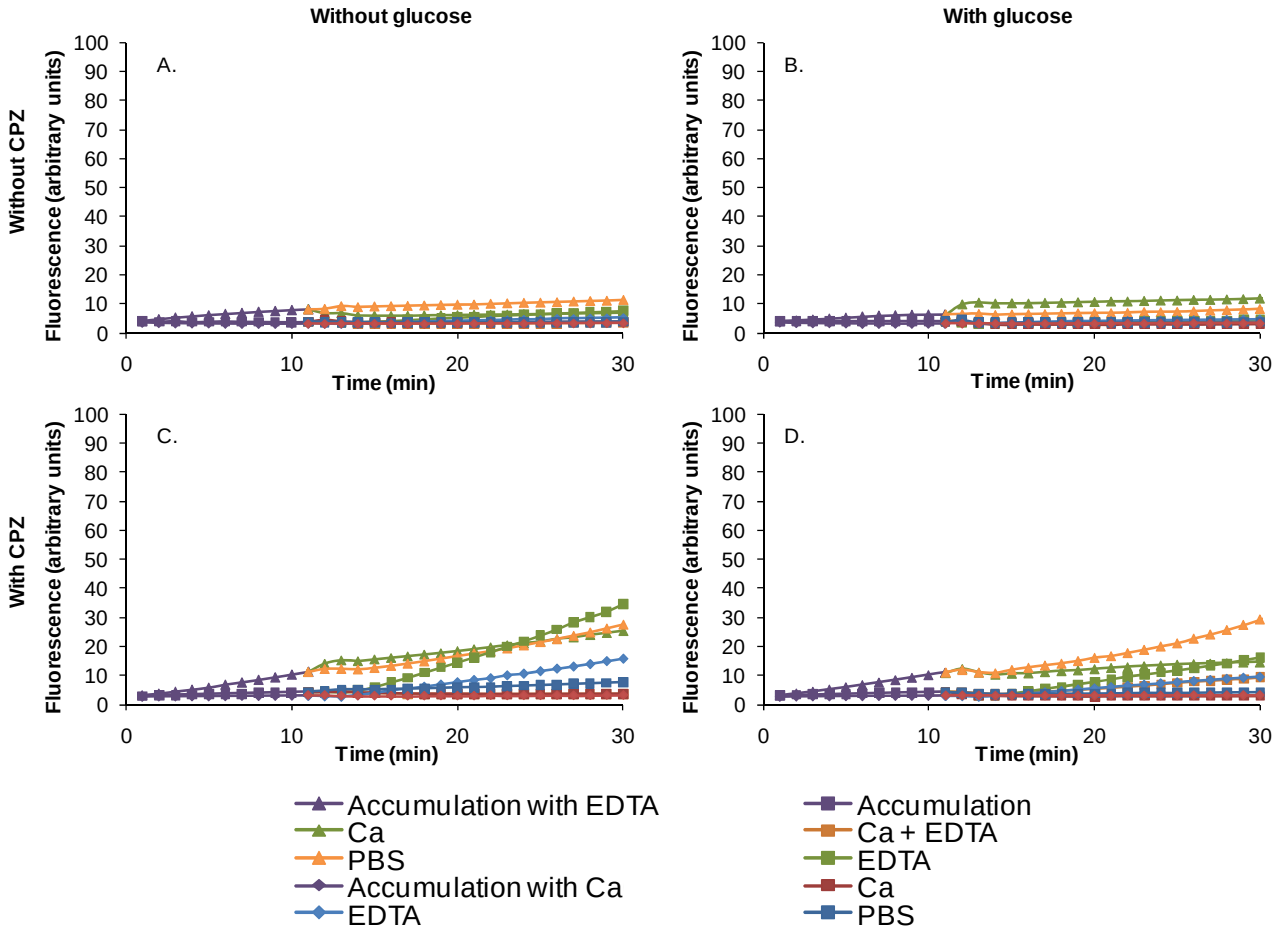


Figure 32 – Calcium role on the efflux of EB by *E. coli* AG100 at pH 5.
Accumulation was done in saline pH 5 (■), saline pH 5 with 5mM EDTA (▲), saline pH 5 with 5mM $CaCl_2$ (♦) and (A) without 25 mg/L CPZ and without glucose, (B) without 25 mg/L CPZ and with 0.4% glucose, (C) with 25 mg/L CPZ and without glucose, (D) with 25 mg/L CPZ and with 0.4%glucose. Addition of saline free pH 5, saline pH 5 with 5mM $CaCl_2$ and saline pH 5 with 5mM EDTA were made after 10 minutes of accumulation of EB, in medium as defined above, and identified by the legend of the graphs A, B, C and D.

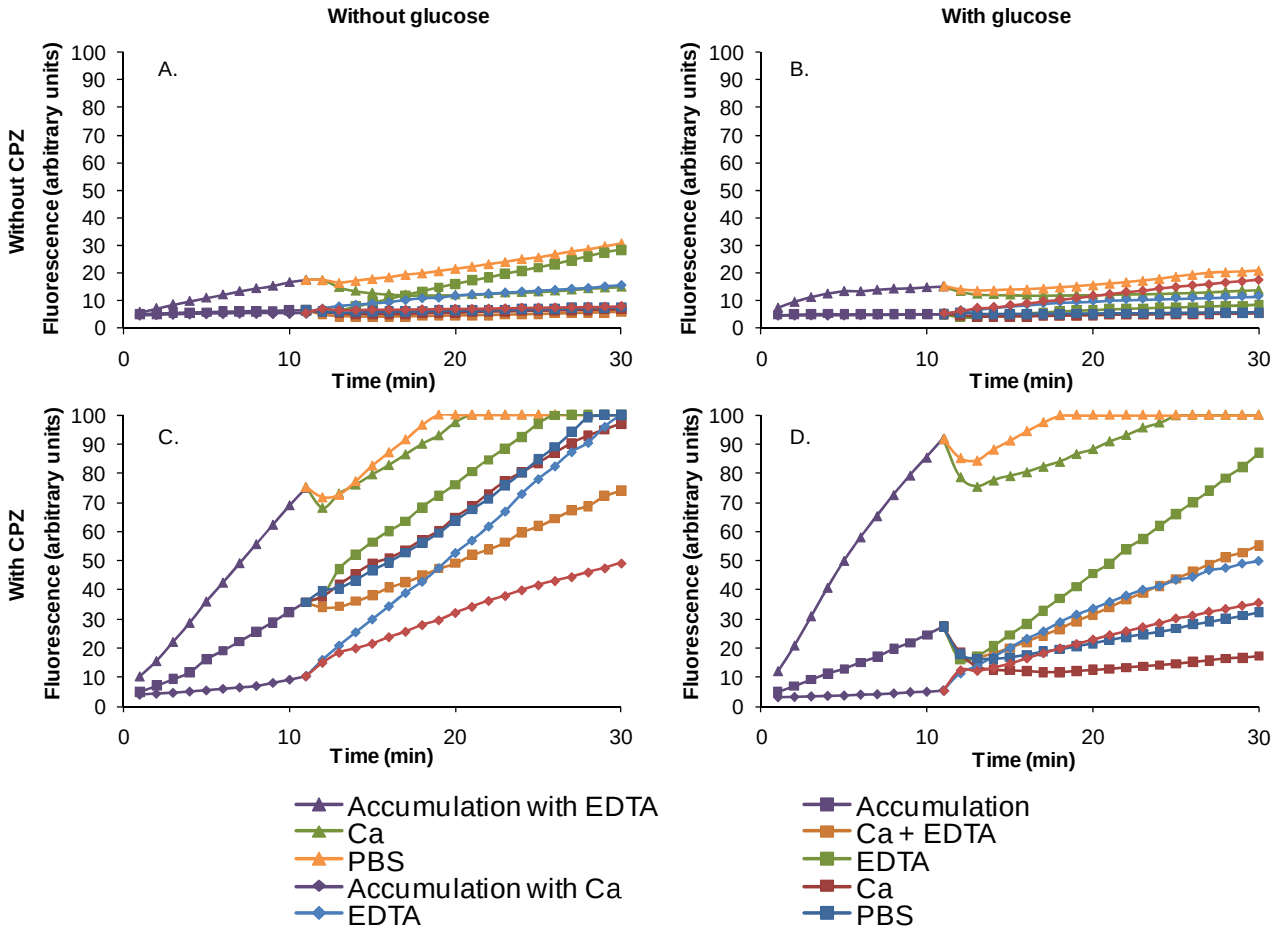


Figure 33 – Calcium role on the efflux of EB by *E. coli* AG100 at pH 8.
Accumulation was done in saline pH 8 (■), saline pH 8 with 5mM EDTA (▲), saline pH 8 with 5mM $CaCl_2$ (♦) and (A) without 25 mg/L CPZ and without glucose, (B) without 25 mg/L CPZ and with 0.4% glucose, (C) with 25 mg/L CPZ and without glucose, (D) with 25 mg/L CPZ and with 0.4%glucose. Addition of saline free pH 8, saline pH 8 with 5mM $CaCl_2$ and saline pH 8 with 5mM EDTA were made after 10 minutes of accumulation of EB, in medium as defined above, and identified by the legend of the graphs A, B, C and D.

The slopes corresponding to Figure 32 and Figure 33 are presented by Table 9 and Table 10, respectively, in order to provide a more easily observed comparison between the effects resulting from conditions in the medium and additions made.

Table 9 - Slopes of EB accumulation / efflux corresponding to the different conditions and influence of CPZ, Calcium and EDTA at pH 5.

Conditions of accumulation					Slope (fluorescence/min)	Conditions of efflux		Slope (fluorescence/min)
pH	glucose	CPZ (mg/L)	EDTA (mM)	$CaCl_2$ (mM)		EDTA (mM)	$CaCl_2$ (mM)	
5	without	0	0	0	0.012	0	0	-0.004
						0	5	-0.032
						5	0	**0.235**
						5	5	-0.018
	without	0	5	0	**0.381**	0	0	0.087
						0	5	**-0.073**
	without	0	0	5	0.029	0	0	-0.012
						5	0	**0.166**
	without	25	0	0	**0.134**	0	0	0.094
						0	5	**-0.111**
						5	0	**1.468**
						5	5	**-0.118**
	without	25	5	0	**0.791**	0	0	0.622
						0	5	0.472
	without	25	0	5	0.033	0	0	0.001
						5	0	**0.693**
	with	0	0	0	0.002	0	0	0.024
						0	5	-0.026
						5	0	0.070
						5	5	0.057
	with	0	5	0	**0.242**	0	0	**0.051**
						0	5	**0.036**
	with	0	0	5	0.036	0	0	-0.004
						5	0	**0.166**
	with	25	0	0	0.108	0	0	**-0.001**
						0	5	-0.059
						5	0	**0.675**
						5	5	0.232
	with	25	5	0	**0.792**	0	0	0.717
						0	5	**0.187**
	with	25	0	5	0.033	0	0	-0.004
						5	0	**0.418**

NOTE. **Highlight in blue identifies significant effect produced on accumulation.**

Highlight in red identifies compound that significantly reversed the inhibitory effect produced on accumulation by an agent in the medium.

Highlight in green identifies compound that significantly enhanced the inhibitory effect produced on accumulation by an agent in the medium.

Table 10 - Slopes of EB accumulation / efflux corresponding to the different conditions and influence of CPZ, Calcium and EDTA at pH 8.

Conditions of accumulation					Slope (fluorescence/min)	Conditions of efflux		Slope (fluorescence/min)
pH	glucose	CPZ (mg/L)	EDTA (mM)	$CaCl_2$ (mM)		EDTA (mM)	$CaCl_2$ (mM)	
8	without	0	0	0	0.155	0	0	0.119
						0	5	0.107
						5	0	**1.290**
						5	5	0.120
	without	0	5	0	**1.172**	0	0	0.892
						0	5	**0.542**
	without	0	0	5	0.058	0	0	0.088
						5	0	**0.424**
	without	25	0	0	**3.149**	0	0	3.158
						0	5	2.980
						5	0	4.121
						5	5	2.183
	without	25	5	0	**6.608**	0	0	**4.639**
						0	5	**3.382**
	without	25	0	5	**0.608**	0	0	**1.906**
						5	0	**4.403**
	with	0	0	0	0.021	0	0	0.037
						0	5	0.076
						5	0	**0.241**
						5	5	0.080
	with	0	5	0	**0.674**	0	0	0.615
						0	5	**-0.027**
	with	0	0	5	0.077	0	0	**0.448**
						5	0	**0.233**
	with	25	0	0	**2.181**	0	0	**0.756**
						0	5	**-0.248**
						5	0	**4.039**
						5	5	2.085
	with	25	5	0	**8.012**	0	0	**2.708**
						0	5	**1.764**
	with	25	0	5	0.223	0	0	**1.587**
						5	0	**2.956**

NOTE. **Highlight in blue identifies significant effect produced on accumulation.**

Highlight in red identifies compound that significantly reversed the inhibitory effect produced on accumulation by an agent in the medium.

Highlight in green identifies compound that significantly enhanced the inhibitory effect produced on accumulation by an agent in the medium.

In the first part of this thesis it was gathered information about the antibiotic resistance mechanisms of bacteria and the physiological processes that influence and modulate resistance to antibiotics and to other noxious agents, namely it was studied the influence of the environment and growth conditions on the expression of outer membrane proteins of a bacterium, the effect of different conditions of antibiotic pressure on the over-expression of efflux pumps, the role of efflux pump mediated resistance at different pH, the physiological contribution of metabolic energy, protons / proton motive force and ions such as calcium on the regulation of efflux activity and the activity of compounds which modulate efflux by interfering with key pathways of membrane stability and energy use. With this information and experimental procedures it was possible to design an effective strategy to search and detect new compounds isolated from plants that may modulate efflux mediated multi-drug resistance of bacteria. The focus of attention will be the plant *Carpobrotus edulis*.

3. Search for new active compounds against resistance

3.1 Purification and identification of the compounds

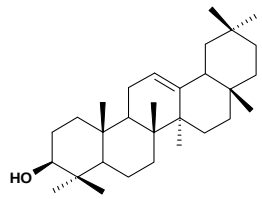
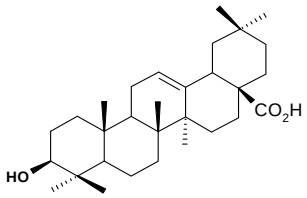
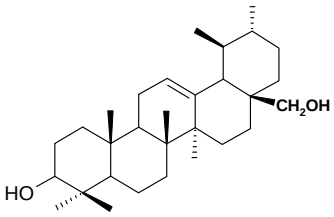
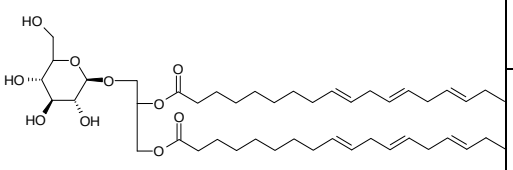
The different techniques used for the isolation of compounds from *C. edulis* are described in the Methods section. From that schematic procedure seven compounds were purified.

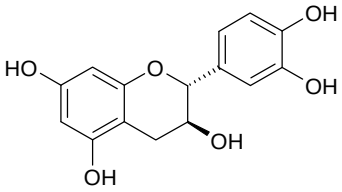
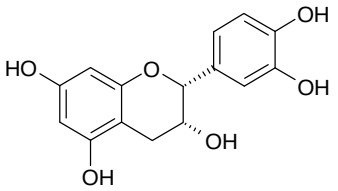
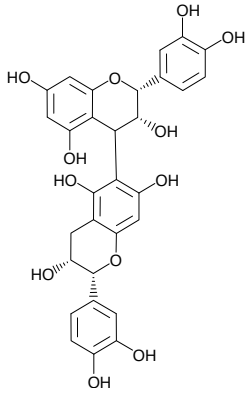
After purification, the structures of compounds were determined by NMR spectroscopy. Their identification was done on the basis of one- and two-dimensional NMR studies, including ^{1}H-NMR, JMOD, NOESY, HSQC and HMBC characterisation and the data obtained compared with that present in the literature for the same compounds (221-229).

Table 11 summarises the structure of the compounds, their common and International Union of Pure and Applied Chemistry (IUPAC) recommended names and also their molecular weight and chemical formula. The compounds purified, already described in the literature, have been isolated from other plants. However, this is the first time that they have been isolated and described from Aizoaceae family. Table 11 provides the references for the previous characterization and identification of these compounds.

From the hexane/acetone fractions of the methanolic extraction four compounds were purified and characterised: β-amyrin (**1**), oleanolic acid (**2**), Uvaol (**3**) and Monogalactosyldiacylglycerol (acyls = linolenoyl group) (**4**). From the ethyl acetate fraction of the same extract three flavonoids were similarly isolated and characterised: catechin (**5**), epicatechin (**6**), and procyanidin B5 (**7**).

Table 11 - Structures of the isolated compounds from the methanolic extract of *C. edulis*.

Structure	Characteristics		Ref.
1 	**IUPAC name**	(3S,6aR,6bS,8aS,11R,12aS,14aR,14bR)-11-(hydroxymethyl)-4,4,6a,6b,8a,11,14b-heptamethyl-1,2,3,4a,5,6,7,8,9,10,12,12a,14,14a-tetradecahydropicen-3-ol	(226)
	Common name	**β-amyrin**	
	Chemical formula	$C_{30}H_{50}O$	
	MW (g/mol)	426.72	
2 	**IUPAC name**	(4aS,6aR,6aS,6bR,8aR,10S,12aR,14bS)-10-hydroxy-2,2,6a,6b,9,9,12a-heptamethyl-1,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydropicene-4a-carboxylic acid	(223)
	Common name	**Oleanolic acid**	
	Chemical formula	$C_{30}H_{48}O_3$	
	MW (g/mol)	456.70	
3 	**IUPAC name**	(3S,4aR,6aR,6bS,8aS,11R,12S,12aS,14aR,14bR)-8a-(hydroxymethyl)-4,4,6a,6b,11,12,14b-heptamethyl-2,3,4a,5,6,7,8,9,10,11,12,12a,14,14a-tetradecahydro-1H-picen-3-ol	(227; 229)
	Common name	**Uvaol**	
	Chemical formula	$C_{30}H_{50}O_2$	
	MW (g/mol)	442.73	
4 	**IUPAC name**	[2-[(Z)-hexadec-7-enoyl]oxy-3-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxypropyl](9Z,12Z)-octadeca-9,12-dienoate	(228)
	Common name	**MGDG** (Monogalactosyldiacylglycerol) (acyls = linolenoyl group)	
	Chemical formula	$C_{45}H_{74}O_{10}$	
	MW (g/mol)	774.53	

Structure	Characteristics		Ref.
5 	**IUPAC name**	(2S,3R)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol	(222; 224)
	Common name	**Catechin**	
	Chemical formula	$C_{15}H_{14}O_6$	
	MW (g/mol)	290.27	
6 	**IUPAC name**	(2R,3R)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol	(222; 224)
	Common name	**Epicatechin**	
	Chemical formula	$C_{15}H_{14}O_6$	
	MW (g/mol)	290.27	
7 	**IUPAC name**	(2R,3R,4S)-2-(3,4-dihydroxyphenyl)-4-[(2R,3R)-2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-3,4-dihydro-2H-chromen-6-yl]-3,4-dihydro-2H-chromene-3,5,7-triol	(225)
	Common name	**Procyanidin B5**	
	Chemical formula	$C_{30}H_{26}O_{12}$	
	MW (g/mol)	578.52	

3.2 *In vitro* activity of the isolated compounds on bacteria

The minimum inhibitory concentration (MIC) of each compound against pathogenic bacteria was determined in order to define the antibacterial activity of the isolated compounds[2]. A maximum concentration of compound of 200 mg/L was used. As shown by Table 12, the majority of the bacteria tested were resistant to more than 200 mg/L of each compound. Greater concentrations of the compounds were not tested because

[2] Compound 7 (procianidin B5) was not included in the study due to timing reasons of its identification because of which it was not possible to test the compound before the end of this thesis.

higher concentrations of these compounds would not be expected to have clinical significance as shown by other studies (16).

The compound oleanolic acid was very active against *E. faecalis* with an MIC of 6.25. The same compound showed also moderate activity against the *S. aureus* strains that differed with respect to their antibiotic susceptibility pattern: MRSA COL, MRSA COL_{oxa} and HPV 107. The latter two strains were also the most sensitive strains to all the compounds tested. Moreover, comparison of all the compounds tested, oleanolic acid had the greatest activity against the reference *M. tuberculosis* H37Rv strain.

Because the methanolic extract of *Carpobrotus edulis* was previously shown not to have *in vitro* antibacterial activity against *S. aureus* nor *M. tuberculosis* strains but enhanced the killing of these bacteria post-phagocytosis (175;176), regardless of the presence or absence of *in vitro* activity, all the compounds were evaluated for ability to reduce or reverse resistance of pathogenic bacteria to antibiotics to which they were resistant as well as evaluated for activity on the efflux pump system of these bacteria by the semi-automated EB method. These results will be described in the next section.

Table 12 – Minimum inhibitory concentration of *C. edulis* purified compounds on Gram-negative, Gram-positive and mycobacteria strains.

Strain	β-amyrin	Oleanolic acid	Uvaol	MGDG	Catechin	Epicatechin
***E. coli* AG100**	>200	>200	>200	>200	>200	>200
***E. coli* AG100$_{TET8}$**	>200	>200	>200	>200	>200	>200
***S. enteritidis* 104**	>200	>200	>200	>200	>200	>200
***S. enteritidis* 104$_{CIP}$**	>200	>200	>200	>200	>200	>200
***S. enteritidis* 5408**	>200	>200	>200	>200	>200	>200
***S. enteritidis* 5408$_{CIP}$**	>200	>200	>200	>200	>200	>200
***E. faecalis* ATCC 29212**	>200	**6.25**	200	>200	>200	>200
***S. aureus* ATCC 25923**	>200	>200	>200	>200	>200	>200
MRSA clinical strain	>200	>200	>200	>200	>200	>200
MRSA COL	>200	**50**	200	200	>200	200
MRSA COL$_{oxa}$	200	**25**	**100**	**50**	**100**	**100**
***S. aureus* HPV**	>200	**25**	200	**50**	>200	**100**
***M. tuberculosis* H37Rv**	>200	**100**	>200	>200	200	>200

NOTE: significant MIC values are highlighted (**bold**)

3.3 Modulation of resistance in bacteria

One of the approaches to find new therapies against multi-drug resistance is to search for compounds that increase the susceptibility of the organism to the antibiotics to which it is resistant. The use of such compounds as adjuvants results in a form of synergism which renders the inactive antibiotic active (138).

The assays that evaluate the modulation of antibiotic resistance by a non-antibiotic are performed as follows: firstly, the MIC of each antibiotic to which the bacterium is resistant and the compound that is to be assayed for modulation of resistance is determined; secondly, the assay is repeated for each antibiotic at concentrations from its MIC to one that is deemed as "clinical susceptibility" in the absence and presence of a concentration of the non-antibiotic that has no effect on the growth of the bacterium. The concentrations chosen for each compound were equal to or less than one halve of their MIC. The results of this modulation assay are presented by Table 13 to Table 17. A minimum fourfold reduction of the MIC of an antibiotic by a given compound was considered significant and that value is highlighted in the tables.

3.3.1 The modulation of antibiotic resistance of Gram-negative strains

The ability of oleanolic acid, uvaol, and epicatechin to decrease resistance of *E. coli* $AG100_{TET8}$ strain to tetracycline is described by Table 13. These compounds decreased the MIC of tetracycline from 25 to 6.25 mg/L. However, their activities were not equal inasmuch as the reduction of the MIC by some of these compounds required higher concentrations (example: the effective concentration of epicatechin was 100 mg/L as opposed to that of oleanolic acid which was 50 mg/L). With respect to oleanolic acid, the concentration of 50 mg/L apparently had reached a saturation of the target (efflux pump) such that higher concentrations of this compound would not increase its effectiveness.

Table 13 – Effect of compounds isolated from *C. edulis* on the MIC of tetracycline on *E. coli* $AG100_{TET8}$.

MIC (mg/L)													
Strain	TET	TET + Compound (mg/L)											
		β-amyrin		Oleanolic acid		Uvaol		MGDG		Catechin		Epicatechin	
		50	100	50	100	50	100	50	100	50	100	50	100
***E. coli* $AG100_{TET8}$**	25	12.5	25	**6.25**	**6.25**	12.5	**6.25**	12.5	12.5	25	**6.25**	12.5	**6.25**

Evaluation of the isolated compounds for reduction of the MIC of CIP against Salmonella strains resistant to ciprofloxacin demonstrated that significant reductions could be achieved only by uvaol, MGDG and epicatechin and only for the strain *S. enteritidis* 5408_{CIP} (Table 14 and Table 15). It is important to note that *S. enteritidis* 104_{CIP} strain that has been induced to high level resistance to CIP is not affected by any of the compounds. Because resistance to CIP of this strain is the result of increased AcrB transporter, mutations in gyrase 1A and two-mutations in the stress gene *soxS* (202), the inability of the compounds to reduce resistance of the *S. enteritidis* 104_{CIP} to CIP suggests that the mutated targets beyond the efflux pump itself, such as gyrase, are not sensitive to the isolated compounds.

Table 14 – Effect of compounds isolated from *C. edulis* on the MIC of ciprofloxacin on *S. enteritidis* 5408_{CIP}.

MIC (mg/L)													
Strain	CIP	CIP + Compound (mg/L)											
		β-amyrin		Oleanolic acid		Uvaol		MGDG		Catechin		Epicatechin	
		50	100	50	100	50	100	50	100	50	100	50	100
***S. enteritidis* 5408_{CIP}**	>50	25	50	25	25	**6.25**	**6.25**	**6.25**	**12.5**	25	25	**12.5**	**12.5**

Table 15 – Effect of compounds isolated from *C. edulis* on the MIC of ciprofloxacin on *S. enteritidis* 104_{CIP}.

		MIC (mg/L)											
		CIP + Compound (mg/L)											
Strain	CIP	β-amyrin		Oleanolic acid		Uvaol		MGDG		Catechin		Epicatechin	
		50	100	50	100	50	100	50	100	50	100	50	100
***S. enteritidis* 104_{CIP}**	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50

3.3.2 The modulation of antibiotic resistance of Gram-positive strains

A methicillin resistant *Staphylococcus aureus* (MRSA) clinical strain was used to test the capacity of the *C. edulis* compounds to reduce the MIC of oxacillin, an antibiotic to which that strain is resistant. Resistance of MRSA to β-lactams is due to the acquisition of *mecA*, a genetic element that carries the resistant gene to this class of antibiotics (60;230;231). The origin of this genetic element remains unknown. Because the *mecA* element is known to be lost during the process of exposure to an antibiotic of a different class (232), the response of an MRSA strain such as the MRSA COL strain that is already resistant to 400 mg/L of oxacillin to increasing concentrations of a beta-lactam was studied in section 1.2.1 of the Results. In the present section, MRSA COL strain that had been induced to high level resistance to oxacillin (MIC > 1600 mg/L) was challenged with each of the compounds for the purpose of determining whether one or more compounds were able to reduce resistance of this strain to oxacillin. The results are presented in Table 16 and demonstrate that uvaol reduces the MIC of oxacillin. The ability of these compounds to have a similar effect on an MRSA clinical strain was studied and as noted by Table 17, none of the compounds reduce the MIC of oxacillin. These experiments show that the ability of any of the compounds isolated from *Carpobrotus edulis* to reduce the resistance of an MRSA strain to a given antibiotic depends upon the presence of an over-expressed efflux pump system, a system that has been induced in the MRSA COL when the organism was exposed to increasing

concentrations of a β-lactam (181) and is not over-expressed in the clinical MRSA strain studied.

Table 16 – Effect of compounds isolated from *C. edulis* on the MIC of oxacillin on MRSA COL_{OXA}.

		MIC (mg/L)										
		OXA + Compound (mg/L)[3]										
Strain	OXA	β-amyrin		Oleanolic acid	Uvaol		MGDG		Catechin		Epicatechin	
		50	100	10	25	50	12.5	25	25	50	25	50
MRSA COL_{oxa}	1600	1600	1600	1600	**<100**	**<100**	1600	1600	1600	1600	1600	1600

Table 17 – Effect of compounds isolated from *C. edulis* on the MIC of oxacillin on MRSA clinical strain.

		MIC (mg/L)											
		OXA + Compound (mg/L)											
Strain	OXA	β-amyrin		Oleanolic acid		Uvaol		MGDG		Catechin		Epicatechin	
		50	100	50	100	50	100	50	100	50	100	50	100
MRSA	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500

3.4 Activity on the accumulation of EB

3.4.1 Gram-negative bacteria

The semi-automated EB method affords the real time detection of the EB accumulation inside the cell by following the evolution of fluorescence of EB during a period of time. It can be used to evaluate a compound for the modulation of accumulation / efflux of

[3] Minimum concentration of OXA used was 100 mg/L and no growth was observed for uvaol. The results of the assay were read after 48h when the strain control also grow.

EB. For this phase of the method, the modulating activity of the compound increases accumulation and decreases efflux of EB, presumably by having an effect, direct on the EP or otherwise on the activity of an efflux pumps system. Because of the multiplicity of efflux pumps in Gram-negative bacteria, one cannot at this time specify any given efflux pump as being the one that is affected. However, because the main efflux pump of Gram-negative bacteria such as *E. coli* and Salmonella is the AcrAB pump, and because for these bacteria representatives that have an over-expressed AcrAB pump are available at the laboratory, the results described may reflect the activity of any compound studied to primarily be one that affects the main efflux pump AcrAB (37;65). Employing this rationale, the semi-automated EB method has been used to screen for the activity of the purified compounds against the presumptive AcrAB efflux pump of *E. coli* strains that have been genetically characterised for the degree of expression of genes that regulate and code for the AcrB transporter (37;65;187).

The strains used in this assay were the *E. coli* AG100 and the *E. coli* $AG100_{TET8}$ as the main strains characterized in the first part of this dissertation, and are examples of susceptible and MDR Gram-negative strains, respectively.

The effect of each compound on the accumulation and efflux of EB by *E. coli* strains was restricted to medium of pH 7.4. This pH was selected inasmuch as if the compounds are to serve an eventual clinical role for activity against *E. coli* that has colonised the colon, the pH under which they are to have activity in a physiological system is essentially one that is close to neutrality.

The compounds catechin and epicatechin were the most effective compounds for increasing the accumulation of EB on the strain *E. coli* AG100 (Figure 34 and Figure 35), and hence, assumed to inhibit the intrinsic efflux pump system of *E. coli* AG100. However, the effects of epicatechin and catechin on the accumulation of EB are inhibited by the presence of glucose. These two compounds do not affect the accumulation of EB by the MDR *E. coli* $AG100_{TET8}$ strain at the concentrations used. The compound oleanolic acid had a modest effect on the accumulation of EB (Figure 36) and this effect was also inhibited by glucose.

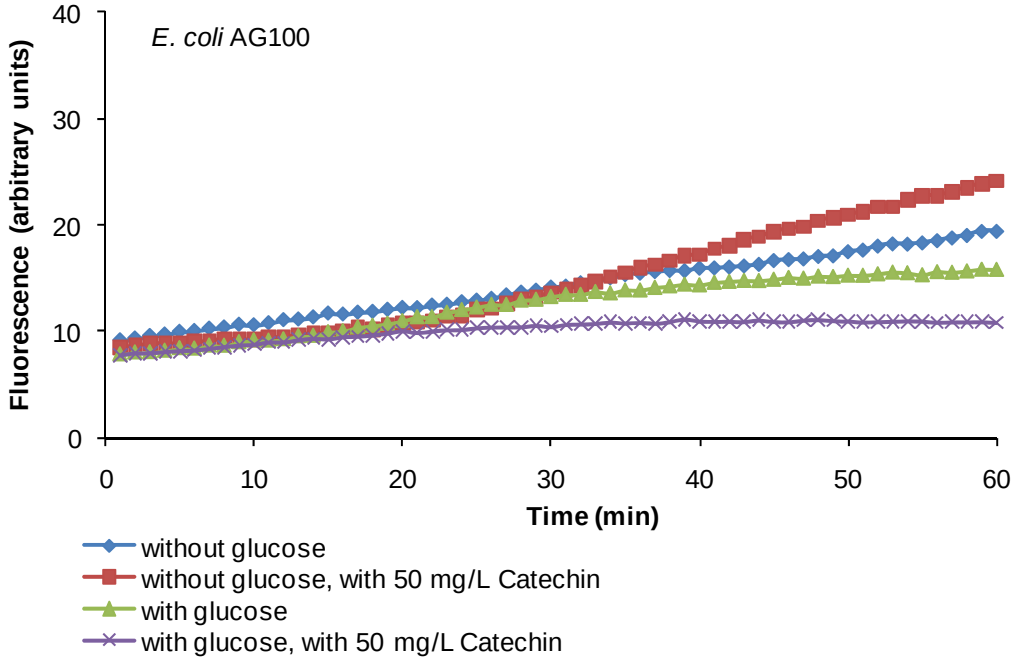


Figure 34 – Effect of catechin on the accumulation of EB by *E. coli* AG100 in presence and absence of glucose.

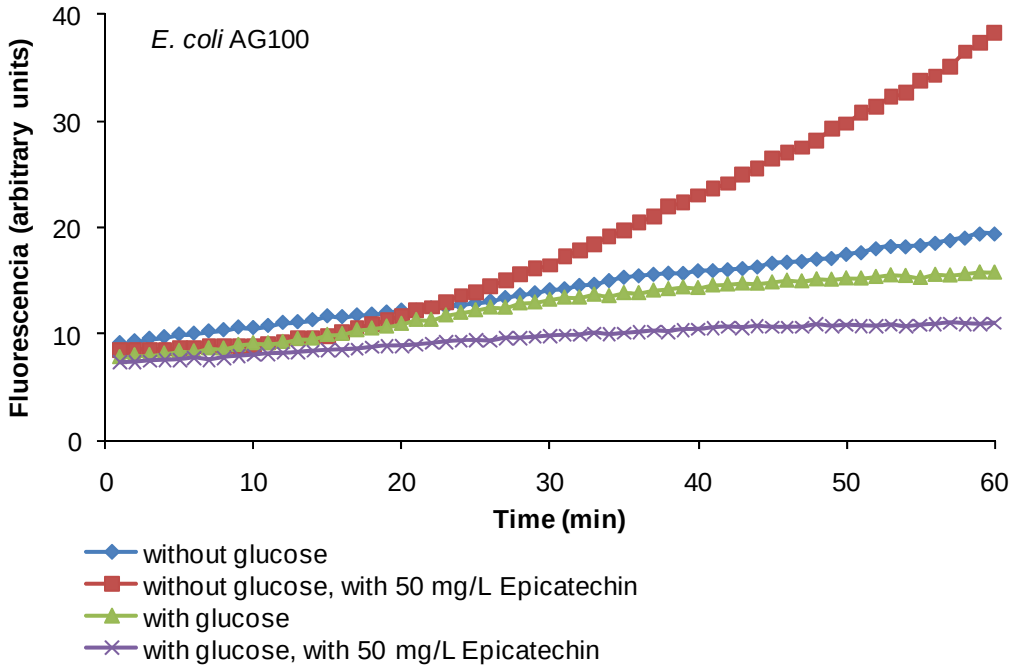


Figure 35 – Effect of epicatechin on the accumulation of EB by *E. coli* AG100 in presence and absence of glucose.

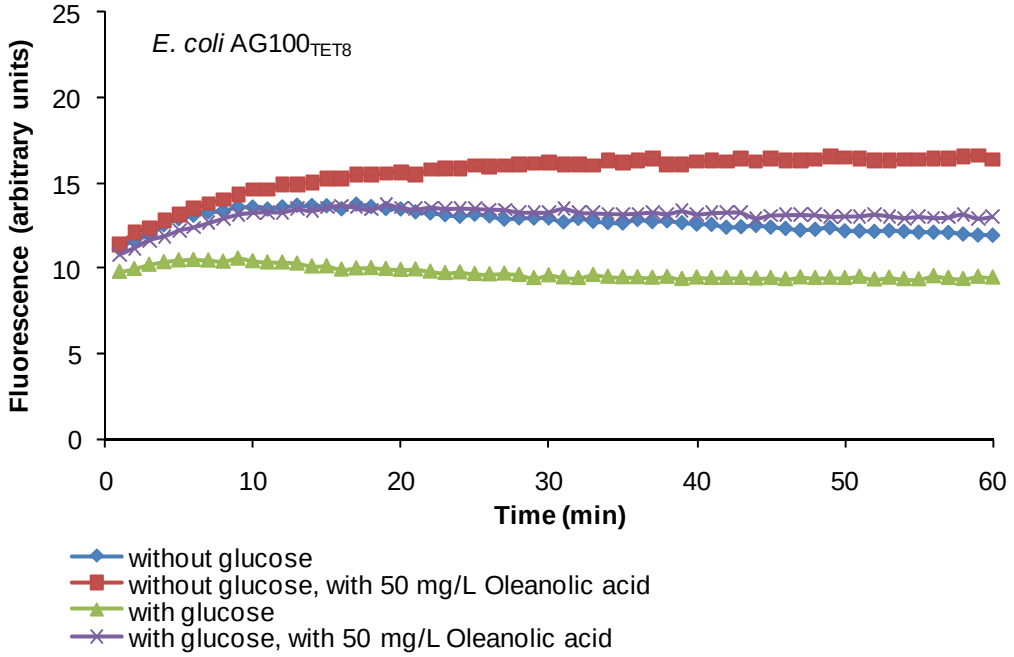


Figure 36 – Effect of oleanolic acid on the accumulation of EB by *E. coli* AG100$_{TET8}$ in presence and absence of glucose.

In the efflux assay conducted at pH 7, the addiction of oleanolic acid or epicatechin promotes the accumulation of EB by inhibiting its efflux (Figure 37 and Figure 38). These compounds and catechin produced similar effects against the adapted strain *E. coli* AG100$_{TET8}$ (Figure 39, Figure 40 and Figure 41).

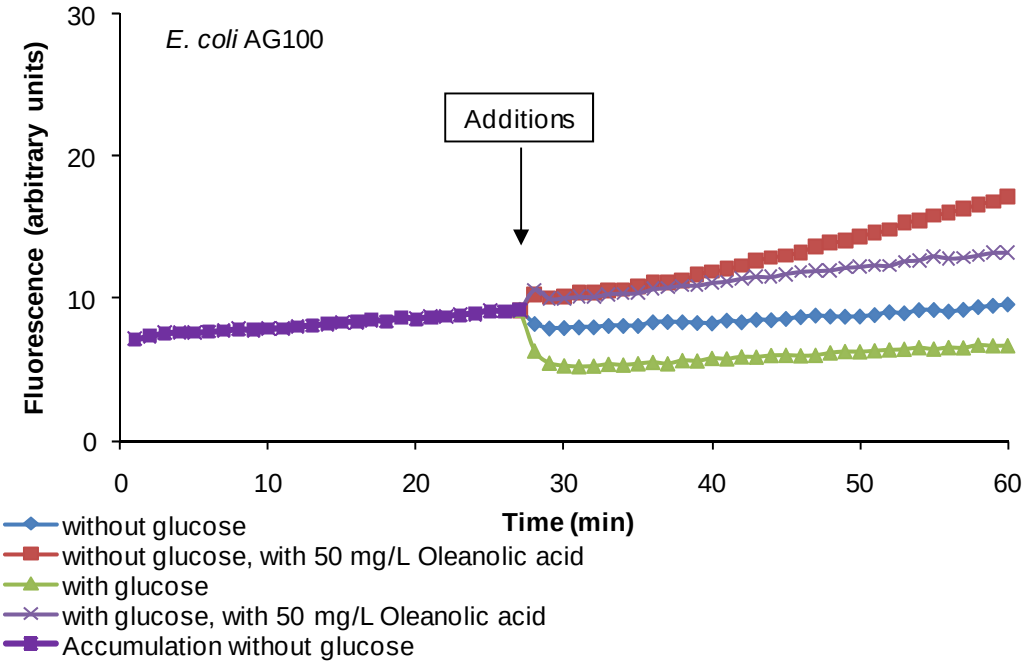


Figure 37 - Effect of oleanolic acid on the efflux of EB by *E. coli* AG100 in presence and absence of glucose.

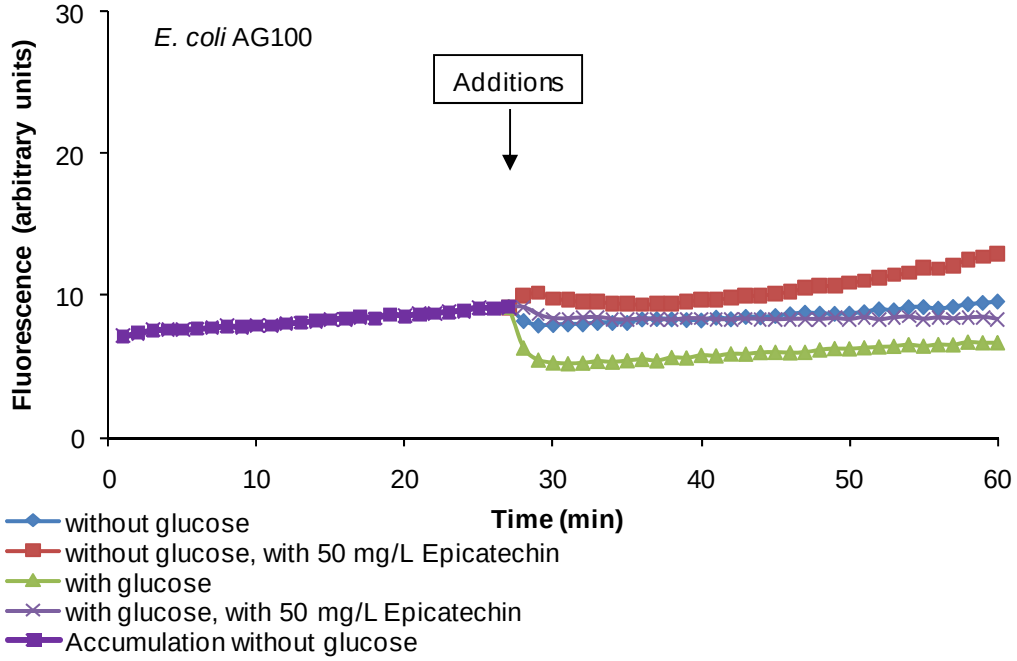


Figure 38 - Effect of epicatechin on the efflux of EB by *E. coli* AG100 in presence and absence of glucose.

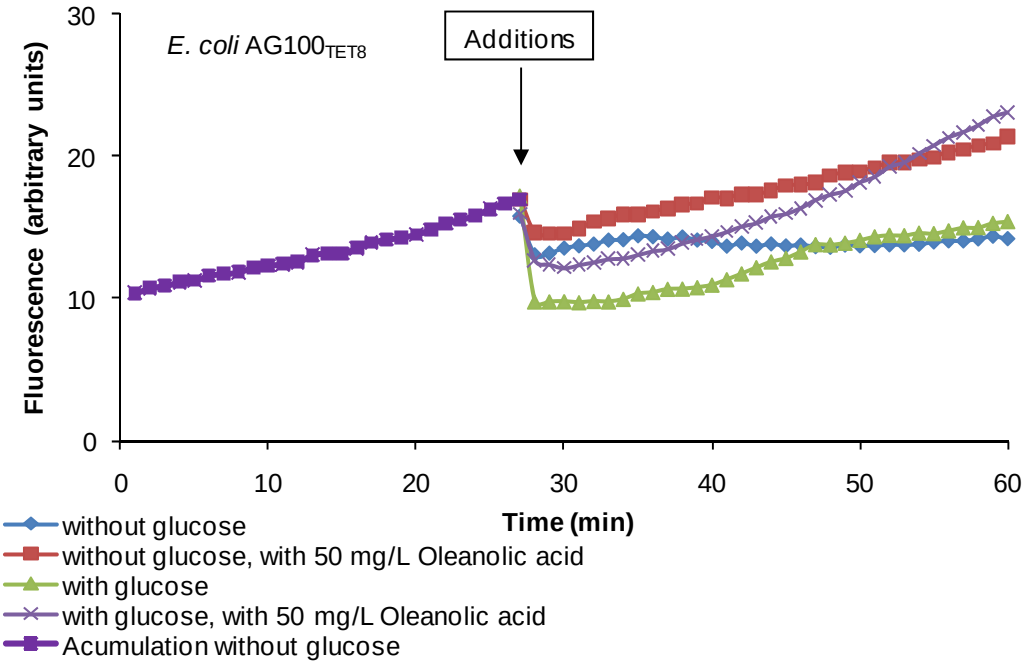


Figure 39 - Effect of oleanolic acid on the efflux of EB by *E. coli* AG100$_{TET8}$ in presence and absence of glucose.

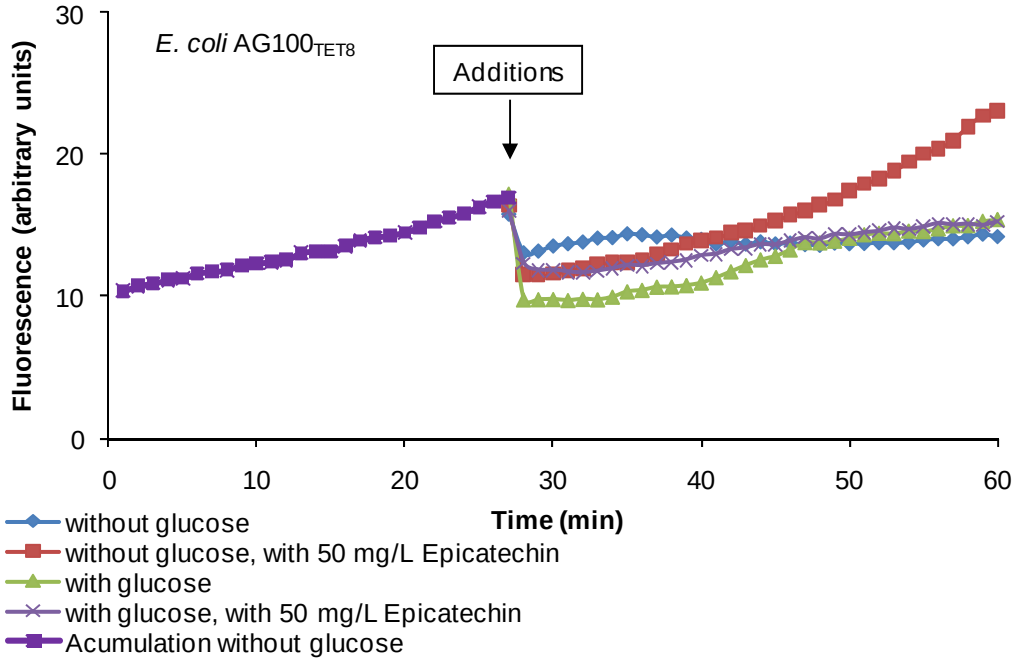


Figure 40 - Effect of epicatechin on the efflux of EB by *E. coli* AG100$_{TET8}$ in presence and absence of glucose.

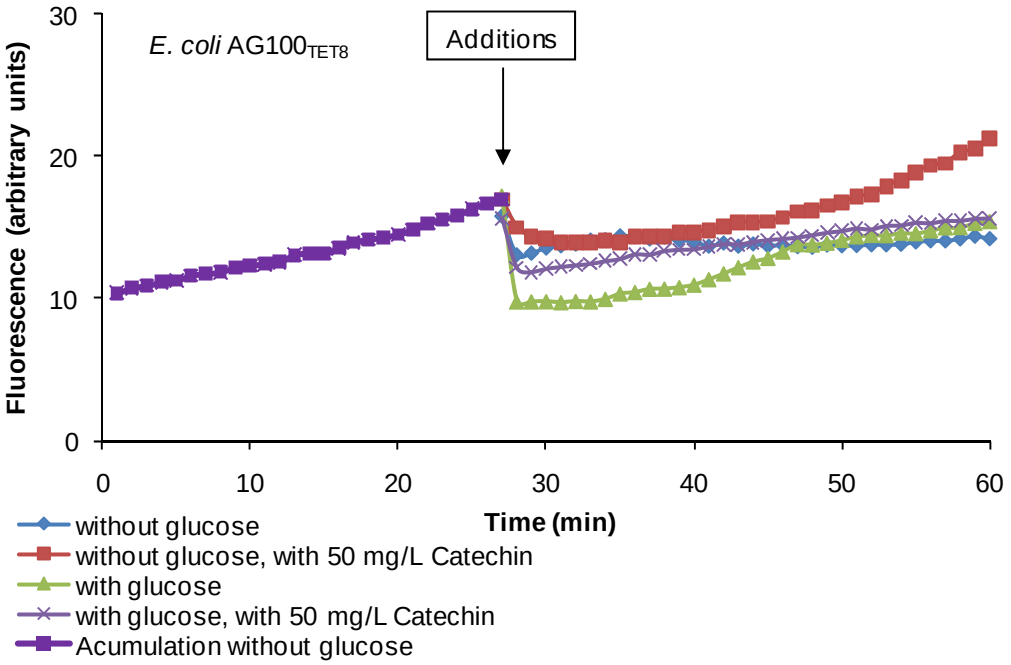


Figure 41 - Effect of catechin on the efflux of EB by *E. coli* AG100$_{TET8}$ in presence and absence of glucose.

Similar assays were done with *S. enteritidis* 5408$_{CIP}$ strain but increase in the accumulation of EB by the strain in the presence of any of the compounds was not observed.

3.4.2 Gram-positive bacteria

Because it was observed that some of the compounds reduced the MIC of OXA against MRSA COL strain (see Section 1.2.1 of the Results), the EB accumulation assay was employed to see if these compounds could affect accumulation/efflux of EB by that strain. The MRSA strain used was the MRSA COL adapted to 1600 mg/L of oxacillin since it was this strain for which the MIC of OXA was reduced by some of the isolated compounds. Furthermore, the use of the EB assay would also provide an understanding

of the physiological conditions that could modulate any noted effect by the compounds on accumulation/efflux of EB.

The compounds uvaol and MGDG increased accumulation of EB by the MRSA COL_{OXA} in a glucose dependent manner. However, the glucose effect is opposite for the two compounds. Whereas in the presence of MGDG the strain MRSA COL_{OXA} accumulates more EB in absence of glucose, in presence of uvaol accumulation takes place in the presence of glucose. The increase of EB accumulated by that strain is promoted by uvaol and is dependent upon metabolic energy. This is unusual and interesting because it suggests that, in order for uvaol to inhibit efflux, the penetration of this agent to the site of the efflux pump affected is regulated by an ATP type activity. This observation and its preliminary assumption are supported by a very recent report that shows that some agents that have activity against ATPases in turn reduce the energy needed for efflux (206). Access of uvaol to the plasma membrane sites where the efflux pump is situated is probably regulated by a putative ABC type influx transporter which is activated by the generation of ATP, a process primarily resulting from ATP synthase at a pH below 7 (206). Consequently, before uvaol has an opportunity to inhibit the efflux pump, it must first penetrate the cell envelope, and reach the plasma membrane situated efflux pump. This latter penetration is therefore postulated to be dependent upon metabolic energy. Further discussion on this new observation may be found in the Discussion section.

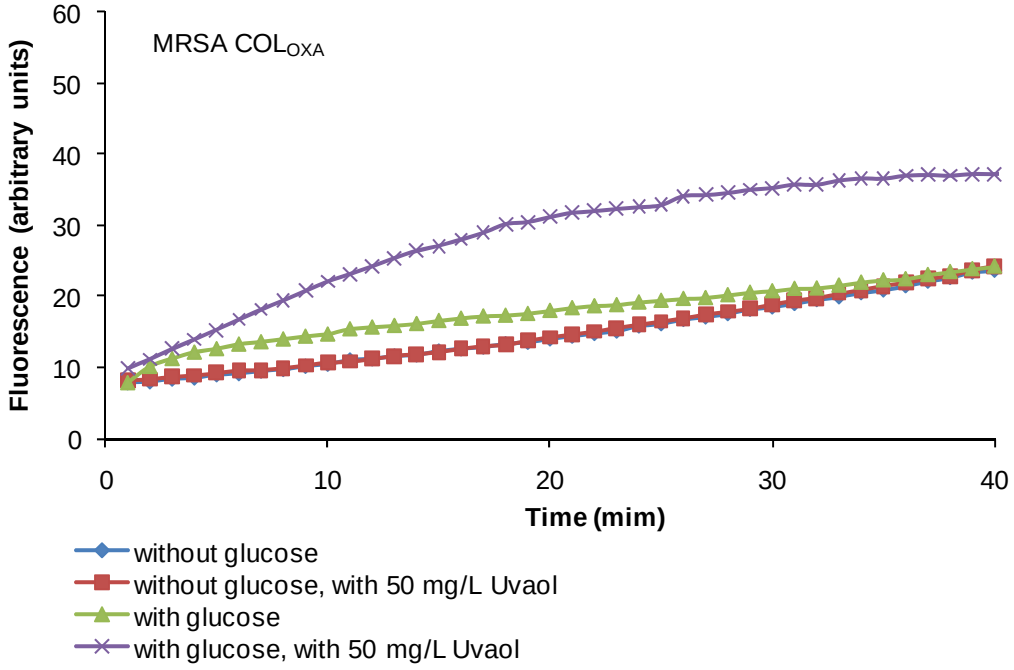


Figure 42 - Effect of uvaol on the accumulation of EB by MRSA COL$_{OXA}$ in presence and absence of glucose.

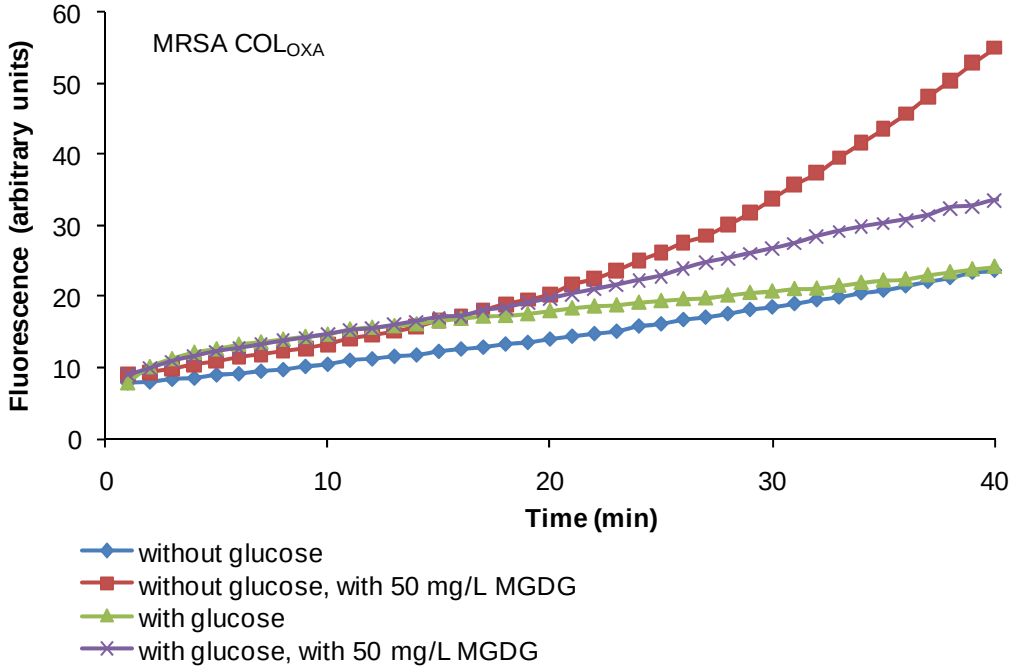


Figure 43 - Effect of MGDG on the accumulation of EB by MRSA COL$_{OXA}$ in presence and absence of glucose.

3.5 Activity *ex-vivo* against *S. aureus* strains

The methanolic extract of *C. edulis* was previously described as containing active compounds that increased the killing activity of macrophages infected with *M. tuberculosis* and *S. aureus* strains (175;176). As was the case for those previous studies (175;176), it was used the *S. aureus* strain model for the evaluation of the isolated compounds on the killing of these bacteria subsequent to their phagocytosed by non-killing human macrophages.

The results of this *ex vivo* assay, described in Section 4.4.4 of Materials and Methods, are presented as the percentage of CFUs retrieved from the cultures after 3 and 6 h of incubation in the presence and absence of the compounds. Results for *S. aureus* ATCC strain and MRSA are presented by Figure 44 and Table 18 and are the average of three independent experiments.

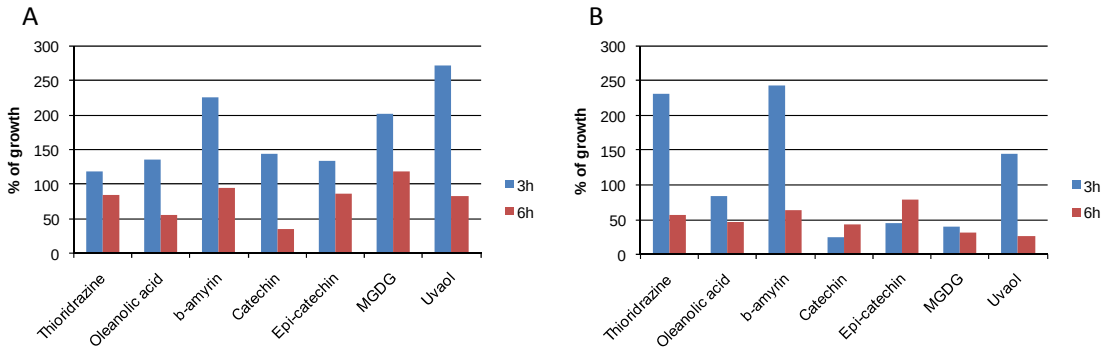


Figure 44 - Effect of the purified compounds from *C. edulis* on the increasing of the killing activity of macrophages infected with *S. aureus* strains.
A. *S. aureus* ATCC; **B**. MRSA. The values correspondent to the data showed in the graphs are presented by Table 18.

By the results presented it is shown that after three hours of the incubation of the macrophages infected with *S. aureus* ATCC strain did not increase its killing activity. However, the majority of the compounds enhanced the killing activity of the

macrophages after 6h. The compound catechin followed by oleanolic acid were the most active compounds against *S. aureus* ATCC strain, while uvaol and epicatechin were as active as the positive control thioridazine.

When the macrophages were infected with the MRSA clinical strain the compounds catechin, epicatechin, and MGDG increased the killing activity of the macrophages as evident from a decrease in CFUs. However, after 6h, the most active compounds were uvaol, catechin, oleanolic acid and MGDG whose activity is compared to the TZ that serves as a positive control.

Table 18 – Effect of the purified compounds from *C. edulis* on the increasing of the killing activity of macrophages infected with *S. aureus* strains.
Macrophages infected with *S. aureus* ATCC and MRSA were treated with 1 mg/L of compound and disrupted after 3 and 6 hours. The bacterial cells where plated and the CFU counted after 24h of incubation. Relative percentages of CFU ($CFU_{compound}$ / $CFU_{control}$ x 100) are presented in this table.

Relative percentage of CFU (%)				
Compound	***S. aureus* ATCC**		**MRSA**	
	3h	**6h**	**3h**	**6h**
control	100	100	100	100
TZ	119	85	232	57
Oleanolic acid	136	55	85	80
β-amyrin	226	95	242	64
Catechin	145	35	24	77
Epicatechin	135	86	45	78
MGDG 1mg/L	201	119	39	41
Uvaol 1mg/L	272	83	145	40

Similar assays with *M. tuberculosis* H37Rv are still in progress and the results are not presented on this dissertation.

3.6 Activity on Eukaryotic cell lines

Our previous studies, as well as those of others, have shown that some inhibitors of bacterial efflux pumps also inhibit the product of the *mdr1* gene, the P-gp (195;233). Moreover it was previously observed that the methanol extract of *C. edulis* that enhanced the killing activity of infected macrophages was also able to reduce the P-gp activity and increase the amount of rhodamine 123 that was accumulated by the cancer cells that over-express that protein (176). Because some of the purified compounds from *C. edulis* have been shown in this thesis to inhibit the efflux system of pathogenic bacteria, these latter studies were extended to examine the capability of the purified compounds for inhibition of the P-gp transporter that is responsible for the resistance of the mouse lymphoma cell transfected with the human *mdr1* gene.

3.6.1 Anti-proliferative assays

The anti-proliferative activity of the purified compounds on mouse lymphoma parental cells and human *mdr1* transfected mouse lymphoma cells is summarised in Table 19. The data presented identifies the concentration of the compound that produces a fifty percent reduction of the replication of the respective parental and *mdr1* transfected cells. Although all of the compounds reduced the proliferation of both parental and *mdr1* transfected cells, some compounds were more effective against the parental cells (oleanolic acid and uvaol) and others more effective against the *mdr1* transfected cells (MGDG and epicatechin). It should be noted that in this study the parental cells are cancer cells and therefore, compounds that reduce the replication of these cells also merit attention.

Table 19 - Antiproliferative activity (IC_{50}) of the compounds isolated from *C. edulis*

Compound	IC_{50} (mg/L)	
	PAR	MDR
β-amyrin	11	10
Oleanolic acid	10	21
Uvaol	7	13
MGDG	9	5
Catechin	10	12
Epicatechin	8	6
Procyanidin B5	nd	13

nd – not determined

3.6.2 Reversal of resistance in eukaryotic cells

Often, compounds that modulate resistance to antibiotics in bacterial cell are also shown to modulate resistance of MDR cancer cells to cytotoxic agents, and *vice versa*. For that reason the compounds isolated from the plant *C. edulis* were tested for their capacity to reverse or reduce the resistance of the human *mdr1* transfected mouse lymphoma cell line to agents to which it is resistant. Two techniques were used for this propose: the first one is based upon flow cytometry that measures the effects of an inhibitor of the transporter such as verapamil on the accumulation of rhodamine 123 (when the transporter is inhibited more rhodamine 123 is accumulated) and; the second technique is an adaptation of the semi-automated EB method, described in the previous sections for bacteria, that follows the increase in the accumulation of EB by the cells in the presence of the compound as compared to its unexposed control (234).

A) Flow cytometry assay

The effect of the isolated compounds on the inhibition of P-gp and consequent accumulation of rhodamine 123 inside the cells was measured in terms of fluorescence (Fl), by flow cytometry in presence and absence of the compounds. The activity is presented as a fluorescence activity ratio (FAR), which is equal to:

$$FAR = (Fl_{MDR\ treated} - Fl_{MDR\ control})/ (Fl_{PAR\ treated} - Fl_{PAR\ control}).$$

FAR values are summarized in Table 20. The antiproliferative assay indicates that IC_{50} (concentration of compound that inhibits cell proliferation in 50%) values smaller than 40 mg/L when the assay is conducted with the mouse lymphoma *mdr1* transfected, do not affect the viability of the cell. Therefore, concentrations of the compounds isolated from *C. edulis* that were equal and below this value were used for the evaluation of the compounds abilities to inhibit the P-gp transporter. Nevertheless, to insure that the concentrations employed were below that which affects cell viability, integrity of the cells were checked by comparing the values obtained with the flow cytometry assay and cells in the presence and absence of the agent. Under these conditions, the agents did not produce toxicity effects during the period the assays were conducted.

Base on the FAR values, the compound uvaol has high inhibitory activity against P-gp as shown by Figure 45.

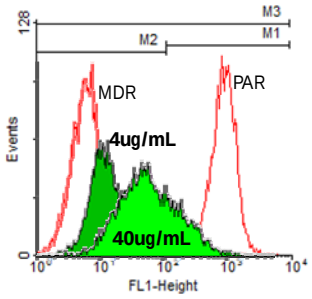


Figure 45 - Histogram of amount of rhodamine accumulated in the MDR cell line and parental cell line (red) and in the MDR cell line treated with 4 mg/mL and 40 mg/L of uvaol (green).

Oleanolic acid has a lower activity and only slightly increases the retention of rhodamine123. Whereas active inhibitory compounds showed a concentration dependent activity, β-amyrin, MGDG, catechin, epicatechin and procyanidin B5 were devoid of any inhibitory activity. These results are summarized by Table 20.

Table 20 - Fluorescence activity ratio (FAR) values for the isolated compounds at the two concentrations tested as well as the DMSO control.

Compound	Concentration (mg/L)	FAR
Verapamil	10	6.97
β-amyrin	4	n.d.
	40	1.26
Oleanolic acid	4	1.26
	40	**5.29**
Uvaol	4	**40.93**
	40	**76.07**
MGDG	4	1.16
	40	2.05
Catechin	4	2.50
	40	2.93
Epicatechin	4	1.16
	40	0.97
Procyanidin B5	4	0.89
	40	0.58
DMSO	4%	0.69

B) Semi-automated EB method

The activity of the compounds isolated from *C edulis* was also evaluated with the aid of the semi-automated EB method. As previously explained, a compound that inhibits an efflux system that extrudes the fluorescent substrate EB produces an increase of fluorescence as a consequence of the build up of EB concentrations within the cell. The use of two distinct methods that evaluate efflux reinforces the significance of inhibitory

activity noted for a given compound. Therefore, the flow cytometry evaluation of the isolated compounds was followed by the use of the semi-automated EB method, previously described for bacteria and now extended to the evaluation of agents that inhibit the P-gp transporter.

The results obtained from the use of the EB method are presented by Figure 46, Figure 47 and Figure 48.

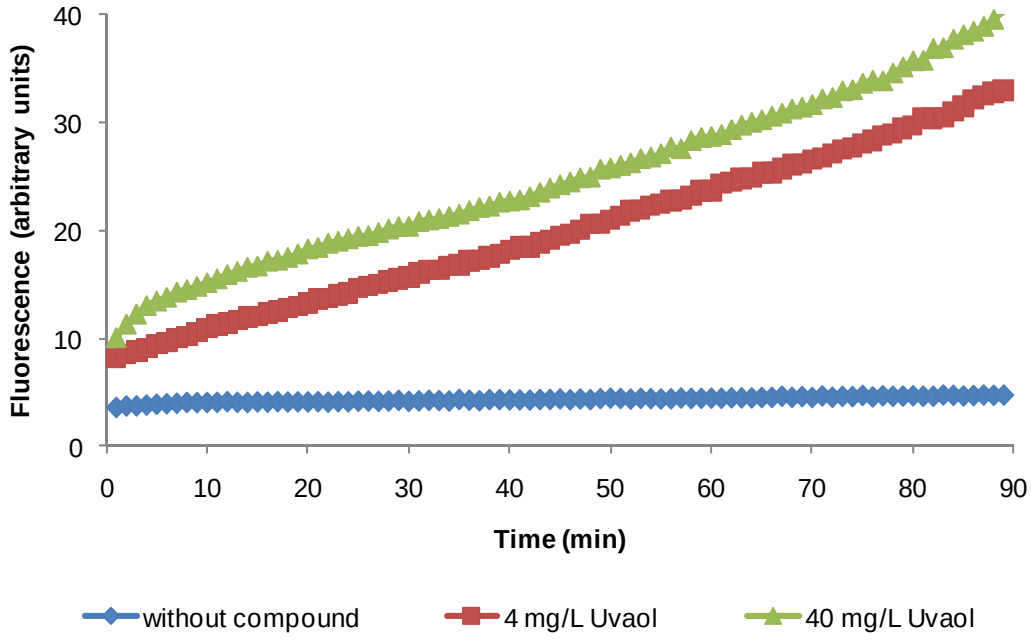


Figure 46 - Accumulation of EB in MDR mouse lymphoma cells in the presence of uvaol.

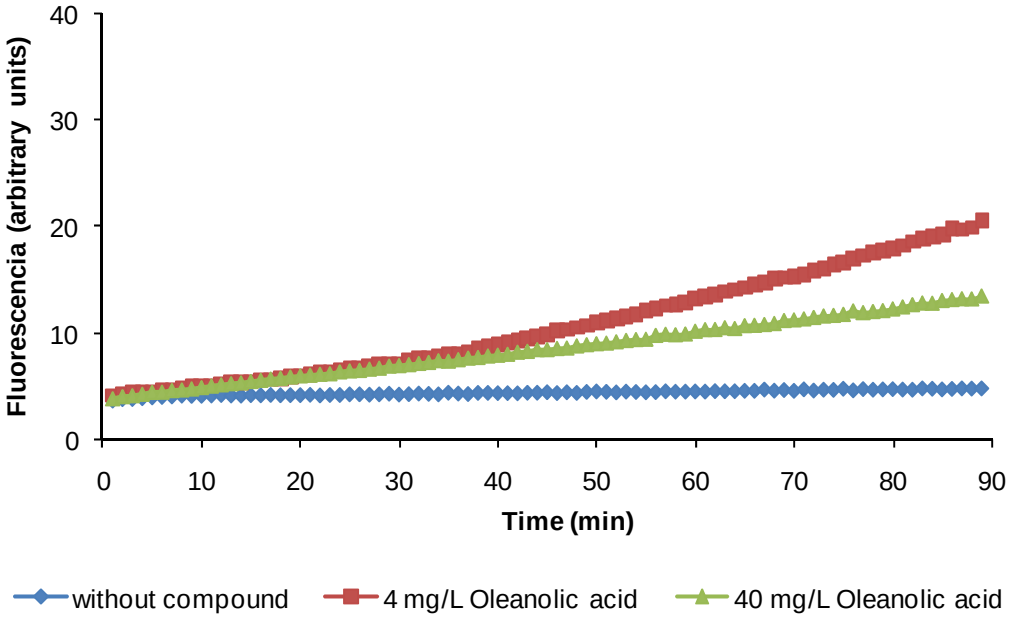


Figure 47 - Accumulation of EB in MDR mouse lymphoma cells in the presence of oleanolic acid.

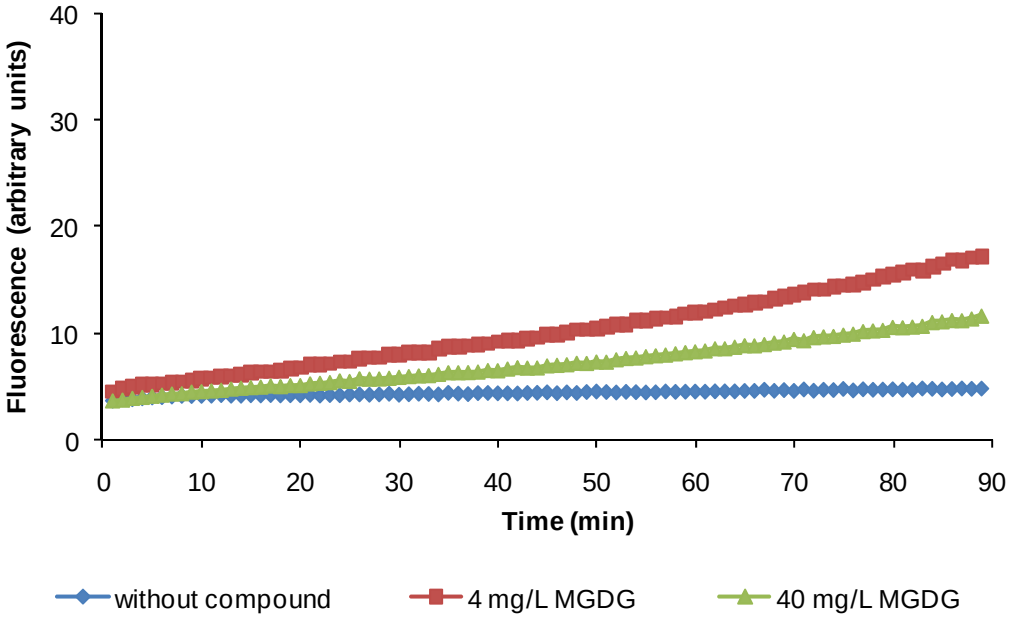


Figure 48 - Accumulation of EB in MDR mouse lymphoma cells in the presence of MGDG.

Once again uvaol was the most active inhibitor of P-gp followed by the oleanolic acid and MGDG. From the real-time data the relative final fluorescence (RFF) of the last time point (minute 90) of the assay was calculated (Table 21).

Table 21 - Relative fluorescence factor (RFF) values for the isolated compounds at the two concentrations tested.

Compound	Concentration (mg/L)	RFF
Verapamil	10	100
β-amyrin	4	4.9
	40	8.9
Oleanolic acid	4	15.8
	40	8.8
Uvaol	4	11.1
	40	18.4
MGDG	4	12.4
	40	6.9
Catechin	4	4.9
	40	4.6
Epicatechin	4	8.6
	40	9.9
Procyanidin B5	4	nd
	40	nd
DMSO	4%	<1

3.6.3 Checkerboard assay

Uvaol, the most effective compound that reversed resistance of the *mdr1* transfected mouse lymphoma cell to cytotoxic compound producing the greatest degree of inhibition of P-pg, was evaluated by an antiproliferative assay for synergistic properties when combined with chemotherapeutic doxorubicin. The proliferative assay employed was described earlier in Section 4.5.4 of Materials and Methods.

The interaction of any compound with the chemotherapeutic agent is evaluated based on the fractional inhibitory concentration index (FIX). The FIX value is obtained as the sum of the FIX values for each compound separately (29): FIX = FIX_{uvaol} + $FIX_{doxorubicin}$. The FIX value of each compound is the fractional IC_{50} of the combined drugs divided by their individual IC_{50} as follow:

$FIX_{(uvaol)} = IC_{50(uvaol+doxorubicin)} / IC_{50(uvaol)}$ and;

$FIX_{(doxorubicin)} = IC_{50\ (uvaol+doxorubicin)} / IC_{50doxorubicin}$.

If the FIX value is equal to 1 it represents an additive effect of the two drugs that is equal to the sum of the individual results for each of the drugs when used separately. If the FIX value is equal to any of the individual values it means that the result with the two drugs does not significantly differ from the result with the most effective drug alone. FIX values larger than 1 represent antagonism and mean that the result of the two drugs is significantly less than the additive response. Synergism corresponds to FIX values equal to or less than 0.5 (29).

The combination of uvaol and doxorubicin produced a FIX value of 0.49 and therefore we may conclude that the two compounds act synergistically.

V. DISCUSSION

1. Role of the environment in the membrane of Gram–negative bacteria

Therapy of the majority of bacterial infections may focus on different approaches dependent on the causative pathogen and site of infection. Successful infection is dependent upon the pathogen and the immune system of the host. Therapeutic failure due either to antibiotic resistance of the organism or to ineffective therapeutic modality (ex. dose, patient non-compliance) contributes to the continuation of infection. The severity of infection is dependent upon the virulence of the organism and the degree of immune function. Therefore, this thesis focused on one of the above aspects that contribute to the infective process, namely, antibiotic resistance and its modulation. It was studied the physiologic mechanisms that contribute to antibiotic resistance, the behaviour of bacteria under constant antibiotic stress and ensuing MDR resistance, the mechanisms by which MDR develops, and the role of distinct transient mechanisms that are invoked when the organism is under stress and when that stress is obviated. Lastly, because much is now known about the environmental conditions that affect the response of bacteria to stress, the thesis also included biochemical parameters that elicit responses of the organism as well as affect the degree of resistance to given antibiotics.

1.1 Growth, environment and OMP expression

The first part of Results section revealed important information relevant to the infection process and the role of the environment. If bacteria are to survive in an altered environment, they must adapt to the new conditions. Because the outer membrane is the organelle that is in contact with the environment, it is reasonable to suspect that it is this organelle which may first respond to altered environmental conditions that are noxious to the organism (19;23;24;33;34). Among the alterations of the outer membrane of adapting bacteria are reduction of the number of porins (196), over-expression of efflux pumps (19), changes in the lipid constitution of the membrane (23), among others. For

example, with respect to salmonella, its survival within the neutrophil post-phagocytosis is totally dependent upon changes of outer membrane structure (52). Because these changes are the result of differential gene expression such as the two step PmrA/B regulon, the organism escapes the deleterious effects of the neutrophil's lysosomal enzymes (52). Alterations of outer membrane composition such as down-regulation of OMPs that go into porin assembly, increased amount of LPS that render the organism impermeable (etc), are examples. Moreover, *in vitro* exposure to antibiotics such as polymyxin B and chlorpromazine alter the structure and protein composition of outer membrane of Gram-negative bacteria (235;236).

The first set of results described in this thesis involved the study of OMPs and conditions under which the organism is culture. When Gram-negative bacteria are grown in liquid media (TSB), the major protein that is differentially extracted from the outer membrane is a 55 kDa protein. When this same organism is grown in the agar (TSA), this 55 kDa protein is barely evident in the extract (Figure 17). This variation of expression of a major OMP may be a response to the hydrostatic pressure exerted by a liquid medium as opposed to the lesser pressure exerted by a solid medium. Previous work from Foulaki showed a similar 55 kDa protein which was the major protein extracted from the outer membrane of Salmonella (204). Immunization of rabbits with this protein led to afforded significant protection against experimental infection with Salmonella (204).

Salmonella are the major cause of food-borne infections (237). Acquisition of a salmonella infection takes place via two main sources: infection due to salmonella introduced by handlers of food such as salads, or from canned products that were contaminated with the organism during processing of the canned food (238). Conditions present in canned food that are designed to inhibit bacterial growth (239) include hydrostatic pressure (201) and low pH (240). Because the environment of canned food, with respect to hydrostatic pressure is similar to broth, and Salmonella that have contaminated the food during processing would be expected to express the 55 kDa protein that enhances the virulence of the organism. However, as of this writing nothing has been published that relates the virulence of a salmonella infection acquired from

canned foods or from contaminated water versus that acquired from contaminated salad due to the handling of the salad ingredients by a carrier.

Because salmonella grown in broth containing chlorpromazine do not express the 55 kDa protein (235), and because recent studies have shown that derivatives of this phenothiazine has antimicrobial activity *in vivo* against salmonella whereas *in vitro* the organism is very resistant (241;242), the ability of thioridazine to prevent a highly virulent salmonella infection can be due to the inhibition of expression of the virulent factor 55 kDa protein. This assumption may be highly relevant for the use of phenothiazines such as thioridazine for the therapy of MDR Gram-negative infections.

The mechanisms by which phenothiazines produce their *in vitro* and *in vivo* effects relevant to susceptibility of Gram-negatives to antibiotics will be discussed in Section 2.3.4 of Discussion.

1.2 Role of antibiotic-promoted stress

As shown in the previous sections, environmental conditions that bacteria face are of extreme importance to its survival and when in an intracellular environment (as for intracellular infections), if it is to survive, bacterium must initiate a response that protects it from intracellular mediated damages. Because macrophages that phagocytosed bacteria tend to concentrate antibiotics (194) to which the organism is susceptible while infecting the host, the organism may respond by expressing a number of outer membrane alterations that reduce its permeability to antibiotics. This decrease in permeability affords the organism time to accumulate mutations which render it even more resistant. With respect to intracellular infections by *M. tuberculosis*, isolation of the organism has shown that it presents a series of cell envelop changes that together render the organism more resistant to the antibiotic that had accumulated *in situ*. Among these alterations are decreased porin expression (243) and increased efflux pump expression (244).

With respect to salmonella, another example, immediately after its phagocytosis by the neutrophil, the two step PmrA/B regulon is activated and a cascade of as many as nine genes are activated, resulting in the synthesis of LPS (52). An increase of LPS decreases permeability of the organism to antibiotics (202), to biocides (52) and to antimicrobial peptides (52;245).

These situations are expected to occur during the infectious process, as well as during therapy. If therapy is to be successful, it is important to know exactly the resistance profile of the infectious agent. *In vitro* studies conducted in this dissertation aimed at developing an understanding of what happens *in vivo* when the infected patient is treated for a prolonged period of time with one concentration of antibiotic and the organism develops resistance to much higher concentrations of that antibiotic as well as to other antibiotic classes that have not been used in the therapy of the given infection.

Antibiotic therapy commonly results in the appearance of resistance of the infecting bacterium to the agent (246). Although a variety of mechanisms account for distinct forms of resistance (247), the mechanism now recognized as playing a major role in the resistance of clinical isolates is the over-expression of efflux pumps which extrude the antibiotic before it reaches its target (248). Because the tri-partite efflux pumps of Gram-negative bacteria, for reasons yet to be understood, have the capacity to recognize and extrude a wide variety of unrelated compounds such as antibiotics from different classes, biocides and other noxious agents like bile salts (35), their over-expression results in a multi-drug resistant phenotype which presents difficulties for the therapy of the MDR bacterial infection (249).

The mechanism by which these MDR efflux pumps are over-expressed has been studied in the laboratory; gradual and prolonged exposure of the bacteria to increasing antibiotic concentrations of the antibiotic that are just below its MIC promote the over-expression of individual efflux pumps that is accompanied with increasing resistance to the antibiotic as well as to other non-related antibiotics (37;65;202). Transfer of the now MDR phenotypic bacterium to drug free medium restores over time, initial susceptibility to the inducing antibiotic, as well as eliminates its MDR phenotypic status (37;65). Nevertheless, these studies do not entirely explain how MDR phenotypes develop in a clinical setting, since therapy does not involve progressive increases of

dose levels. Furthermore, because the level of resistance of the MDR clinical isolate, to a given antibiotic, may be hundreds of times greater than that of its wild-type reference strain, it is difficult to reconcile laboratory studies that induce high level resistance with continuous exposure to increasing concentrations of an antibiotic, to that high level present in MDR clinical isolates that have never been exposed beyond a level of drug compatible with therapeutic dosage. There must be more to the process by which MDR develops in a clinical setting and therefore, the studies to be described attempt to simulate the relationship between exposure of a given bacterium to an antibiotic under conditions expected to exist when a patient that is infected with this organism, is treated for a prolonged period of time with a constant dose of the antibiotic.

It has previously been shown that exposure of *E. coli* to stepwise increases in tetracycline concentrations increases resistance to TET (37) that is accompanied by increases in resistance to many other antibiotics and non-antibiotic agents, producing an MDR phenotype. This MDR phenotype is accompanied by significant increased activity of genes that code for transporter proteins (37). Similarly, exposure of isoniazid - sensitive *M. tuberculosis* to increasing concentrations of INH also increased the resistance of the organism to this antibiotic (205) but resistance to INH was not accompanied by resistance to any other drug employed for the therapy of pulmonary tuberculosis.

If it is assumed that events at the level of the bacterial cell envelope that result in increased efflux pump activity are independent of a chromosomal mutation that bestows high-level resistance of the bacterium to a given antibiotic, then prolonged exposure of that bacterium to increasing concentrations of the antibiotic to which it is resistant may induce the appearance of a MDR type efflux pump. In order to study the possibility that continuous exposure to an antibiotic results in the development of further increase of resistance to the given antibiotic as well as produce an MDR phenotype, methicillin-resistant *Staphylococcus aureus* (MRSA) COL strain whose resistance to 400 mg/L of OXA is due to the acquired *mecA* element (250) was exposed to stepwise increases of OXA and at each level of increased resistance the organism was examined for any changes in its susceptibility to other antibiotics and for evidence of efflux activity.

The results obtained in this study and described in Section 1.2.1 of Results demonstrate that when MRSA COL is placed under increasing antibiotic stress, regardless of the fact that it contains the *mecA* element and is highly resistant to OXA, as it adapts to increasing levels of OXA parallel increases of resistance to ERY take place. Because RES can eliminate the increased resistance to ERY, the MDR phenotype induced by increasing concentrations of OXA is, probably, due to an efflux pump. Accompanying the increased resistance to ERY were significant increases in resistance to other antibiotics, thus indicating the acquisition of an efflux pump-mediated MDR phenotype. To our knowledge, this was the first demonstration of how a MDR type efflux pump can develop due to continuous exposure to increasing concentrations of an antibiotic to which the organism is chromosomally highly resistant.

The second approach (section 1.2.2 of Results) was to maintain an already MDR strain under the same antibiotic concentration for prolonged periods of time, and simulate the situation when a patient infected with an MDR strain is maintained under the same antibiotic pressure during a long period of time. For this study, as described by Section 2.3.2 of Materials and Methods, a serial culture of an *E. coli* strain whose efflux pump had been over-expressed, in medium containing a constant amount of the antibiotic to which the strain had been induced to high level resistance, results in the restoration of the activity of genes that regulate and code for the efflux pump transporters, relative to those of the antibiotic susceptible *E. coli* AG100. Accompanying this restoration is the continued increase of resistance to the antibiotic even though the bacterium had not been exposed to higher concentrations of the antibiotic to which it had been made resistant. This strain also displayed progressively increased resistance to compounds that target cell envelope constituents, gyrase and ribosomes, supporting the assumption that a large number of mutated targets resulted from continuous sub-culture in medium containing a constant concentration of an antibiotic. The inability of the *E. coli* $AG100_{TET10}$ strain to revert in drug-free medium to the initial susceptibility to tetracycline or to the antibiotics that contributed to its MDR status, as did its *E. coli* $AG100_{TET8}$ parent (65), supports the contention derived from phenotypic array studies that indeed mutations had taken place.

Bacteria obey the second law of thermodynamics - conservation of energy. The maintenance of an over-expressed efflux pump system must consume a large, yet undefined, amount of energy given the dependence on metabolic energy as the main source of protons contributing to the proton motive force (54). To the bacterium, one may suppose that after exposure to an environment that remains noxiously constant, the genetic system of the organism responds by activation of a mutator system (251) that results in the accumulation of mutations that render the organism multi-drug resistant. Although as long as the organism remains in that environment its survival is assured, if the environment returns to that initially present, the organism cannot compete with its counterpart which has its relevant targets fully functional.

The study therefore demonstrates "evolution of a bacterial strain" within a laboratory environment, and, may parallel what takes place within a patient who is infected with a bacterium and is treated for a prolonged period of time with a constant dose of a given antibiotic, ultimately resulting in the increased resistance of the bacterium to that given antibiotic as well as to other non-related antibiotics. Lastly, this study demonstrates, for the first time, that an over-expressed efflux pump provides the opportunity by which other mechanisms of resistance may ensue.

1.2.1 Correlation between the two studies

Both of the studies described above showed that exposure of a bacterium to increasing concentrations of antibiotic invokes a phenotypic adaptation that not only increases the resistance of the bacterium to the antibiotic to which it is exposed, but also results in an MDR phenotype. Serial exposure of a strain that is already resistant to the given antibiotic also results in further increases of resistance when the strain is exposed to further increases of antibiotic. Therefore, generalisations may be made between the two sets of studies: 1) Continued exposure of an already resistant strain to the same antibiotic whose concentration remains constant, yields ever increasing resistance (the $AG100_{TET8}$ example). 2) Continued exposure of an already resistant strain to a given

antibiotic whose concentration is continuously increased yields further increases of resistance to the antibiotic (the MRSA example). 3) Both examples yield MDR phenotypes that are mediated by an increased expression of the main efflux pump of the bacterium. 4) When the medium remains constantly noxious, the organism receives protection by the development of mutations. The latter renders the bacterium less fit if co-cultured in drug-free medium with its wild-type counterpart. Interestingly, a recent study demonstrated the presence of mutations which did not affect the fitness of the organism but which insured its survival if the medium were to contain fluoroquinolones (252). This is an example of pre-adaptation which may be an explanation for the some of the observations made in this thesis.

The response of an already resistant bacterial strain to further increases of concentration of the same antibiotic is highly relevant to the clinical observation that prolonged therapy of the patient with the same dose of antibiotic also yields further increased resistance to the agent.

The above studies show that at first, the induced MDR phenotype is subject to reversal by common inhibitors of efflux pumps. However, after further serial passages in high concentrations of the antibiotic, the strain remains resistant and cannot revert to wild type phenotype. These observations are related to those that demonstrate that whereas some MDR clinical isolates may yield a reduced resistance to a given antibiotic when an EPI is added to the medium other strains are not affected by the EPI. It can be assumed that the former case represents the early response to prolonged therapy with the same antibiotic either at the same or greater dose levels whereas the latter represents a much later adaptive response.

During long term therapy such as that used for pulmonary infections produced by *M. tuberculosis*, the prolonged and sustained pressure of high concentrations of antibiotic actually contributes to the selection of spontaneous mutants. This reality was reproduced in the laboratory (253) as well as in the studies described in this Section. Adaptation of bacteria develops via a series of mechanisms each one of which, at the time of exposure, when invoked, assists the bacterium to survive. The studies described in this section, coupled to others (254;255) also suggests a controlled series of adaptive responses each one of which minimizes the amount of energy consumed. Among the

earliest responses is that of over-expression of an efflux pump system. The maintenance of this system is energy dependent and therefore, energy that would otherwise go for physiological processes such as replication, are channelled for the maintenance of efflux. That this hypothesis is correct is supported by the observation that whenever efflux pumps are over-expressed the organism replicates more slowly (205;206). When the noxious environment remains "eternal", the organism invokes a "mutator" system that produces mutations of key targets normally and more readily reached by noxious agents-namely those of the cell envelope. Hence, as expected by this "mutator" hypothesis (251), prolonged exposure to the same concentration of an antibiotic ("eternal noxious environment"), results in mutations of β-lactam targets as shown in this thesis. However, there is the possibility that mutations of genes such as those that code for penicillin binding proteins or gyrase need not take place if the sole mutation is restricted to a site of the 16S ribosome unit involved translation. Such a mutation may cause a shift in the reading of the mRNA as is the case for streptomycin dependency (256) and yield altered targets. Nevertheless, it remains for future studies, the elucidation of "a mutator gene" system that is activated when the organism faces an "eternally noxious environment".

The concepts that result from the studies described above involve conservation of energy. Because efflux pump systems of Gram-negative bacteria are dependent upon protons generated by metabolism, and metabolism that yields energy is regulated by enzymes with distinct optimal pH, the next section of this Discussion interprets the results obtained from experiments where the efflux pumps of bacteria are modulated by metabolic energy and pH.

2. Energy and pH roles on efflux by Gram - negatives

The cell membrane is the most dynamic structure of the cell. Its main function is to act as a permeability barrier that regulates the passage of substances into and out of the cell. Group translocation system, more commonly known as the phosphotransferase system (PTS), in *E. coli*, is primarily used for the transport of sugars. Like binding protein-dependent transport systems, they are composed of several distinct components. However, group translocation systems specific for one sugar may share some of their components with other group transport systems. In *E. coli*, glucose may be transported by a group translocation process that involves the phosphotransferase system. The actual carrier in the membrane is a protein channel fairly specific for glucose. Glucose specifically enters the channel from the outside, but in order to exit into the cytoplasm, it must first be phosphorylated by the PTS. The PTS derives energy from the metabolic intermediate phosphoenol pyruvate (PEP). PEP is hydrolyzed to pyruvate and glucose is phosphorylated to form glucose-phosphate during the process. Thus, by the expenditure of a single molecule of high energy phosphate, glucose is transported and changed to glucose-phosphate (81;257).

Bacteria do not have intracellular organelles for energy producing processes such as respiration or photosynthesis as do eukaryotes. Instead, the plasma membrane of the cell envelope carries out these functions. The cytoplasmic side of the plasma membrane is the site of electron transport systems (ETS) used to produce energy (ATP) and it is the location of the ATP synthase. The equilibrium for the synthesis or hydrolysis of ATP is pH dependent: at pH below 7 the reaction favours the synthesis of ATP; at pH above 7 it favours the hydrolysis of ATP (258).

The electron transport system establishes a pH gradient across of the membrane as a consequence of protons (H^+) that are generated and not used for the formation of H_20. The protons accumulate on the surface of the cell and are distributed unevenly-with higher concentrations in the pits present on the outer cell surface such as those that exist in salmonella (54). The concentration of protons on the surface is affected by the pH of the medium such that at low pH their dissociation into the bulk medium is extremely

low whereas at pH above 7, the dissociation into the bulk medium is increased accordingly (54). Therefore, the proton gradient that is formed from the energy producing metabolic activities of the cell creates the proton motive force (PMF) and it is this PMF which is responsible for the transfer of protons to the periplasmic space of Gram-negative bacteria that are then used for the activation of PMF dependent transport processes such as those involved in the extrusion of noxious agents such as antibiotics, biocides and toxins. Under normal circumstances, severe alteration of the pH of the medium (environment) does not alter the PMF of bacteria (259;260). However, the situation involving alteration of pH and presentation of a noxious agent is different due to the energy requirements of the transporter. Consequently, when the PMF force is under duress due to high pH, the protons available to the periplasm of the Gram-negative bacterium are limited inasmuch as when the proton binds to the transporter and activates it, it is translocated to the cytoplasmic side of the plasma membrane. This translocation decreases the pH of the cytoplasm medial to the plasma membrane and therefore the synthesis of ATP is assured. This newly synthesised ATP is now available for ATP binding transporters that can now assist in the extrusion of the noxious agent from the periplasm of the cell (206).

A familiar example of energy-producing and energy-consuming functions of the bacterial membrane, related to the establishment and use of PMF and the production of ATP, is described for the plasma membrane of *E. coli* by Figure 49.

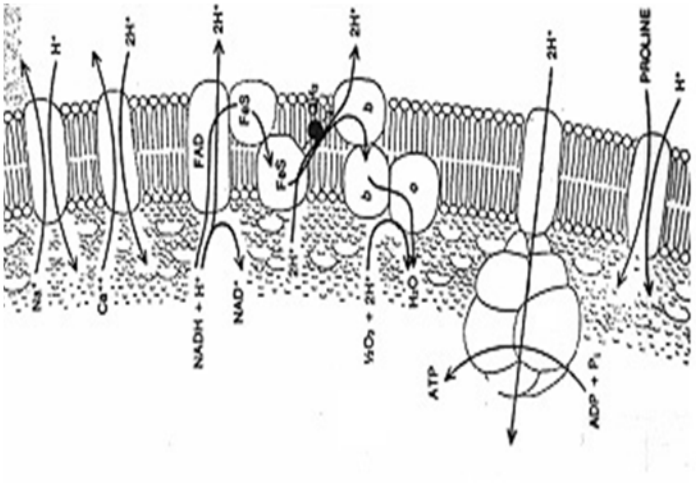


Figure 49 - The plasma membrane of *Escherichia coli.*

The membrane in cross-section reveals various transport systems, the respiratory electron transport system, and the membrane-bound ATPase enzyme. At certain points in the electron transport process, the

electrons pass "coupling sites" and this results in the translocation of protons from the inside to the outside of the membrane, thus establishing the PMF on the membrane. The PMF is used to do work or conserve energy: active transport (e.g. lactose symport; calcium and sodium antiport) and ATP synthesis Adapted from (81)

Production of ATP can also occur at a substrate level phosphorylation in which ATP is made during the conversion of an organic molecule from one form to another. Energy released during the conversion is partially conserved during the synthesis of the high energy bond of ATP. It occurs during glycolysis, intermediary metabolism, fermentations and respiration (the TCA (tricarboxylic acid) cycle) processes.

As an example, the net equation for producing ATP after glycolysis and the TCA cycle in *E. coli* is as follows:

$$\text{Glucose} + 6\ O_2 \rightarrow 6\ CO_2 + 10\ NADH_2 + 2\ FADH_2 + 4\ ATP$$

Two $NADH_2$ and 2 ATP are formed during glycolysis, while 8 $NADH_2$, 2 $FADH_2$ and 2 ATP are formed during the TCA cycle. In *E. coli*, 2 ATP are produced for each pair of electrons that are introduced into the ETS by $NADH_2$. One ATP is produced from a pair of electrons introduced by $FADH_2$ (a prosthetic group). Hence, the total equation can be rewritten:

$$\text{Glucose} + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O + 26\ ATP\ (81)$$

The penetration of hydrophilic compounds through the cell envelope takes place via porins ultimately reaching the periplasm of the Gram-negative bacterium. As noted before, the plasma membrane is an important organelle and contains proteins that are involved in the resistance of cells to noxious agents. Its lipid composition forms a barrier to hydrophilic molecules which, when noxious to the cell and its concentration builds up in the periplasm, are recognised by efflux pumps and extruded to the surface of the cell (environment).

For the study of efflux pump activity, the semi-automated EB method was used as described in the Method Section of this thesis. The method relies on the absence of detected fluorescence by the fluorochrome EB in the medium and the detection of

fluorescence when the fluorochrome is accumulated in the periplasm of the cell. Accumulation of EB is due to the periplasmic concentration of EB that exceeds the capacity of the efflux pump to extrude the agent (187). Accumulation of EB is also accented by the presence of efflux pump inhibitors (187), modulation by pH (206), agents that interfere with accessibility to protons (e.g. CCCP) (187), or by compounds that successfully compete with EB for extrusion by the efflux pump system (206). Therefore, the study that involves different conditions that influence the response of the cell to given noxious agents provides an understanding of the mechanisms involved in the adaptive response to a given noxious agent resulting in increased resistance to that agent.

Bacteria contain multiple efflux pumps that are involved in the extrusion of a noxious agent. This multiplicity provides pre-adaptation to the presence of a large gamut of agents that are normally not present in the habitat of the organism (206). As of this writing, only one study has shown that, in the absence of a given efflux pump of a Gram-negative bacterium, the organism can over-express one or more other efflux pumps when challenged with a noxious agent such as tetracycline (64). However, as shown by the studies of Viveiros et al, the over-expressed efflux pump is not nearly as efficient as the main efflux pump that has been deleted, and it is more susceptible to the majority of the antibiotics tested (37). The involvement of efflux pumps that complement the main efflux pump of *E. coli* has been demonstrated by others (261).

During the infectious process, bacteria have to survive in different environments of the host that are toxic to the organism (262;263). As an example food-borne bacteria traverse the digestive tract that differs widely with respect to pH. During this passage, the organism is threatened by toxic bile salts in the duodenum where the pH is acidic and by the same bile salts in the colon where the pH is near neutral. An aspect of the thesis studied the effects of pH on the efflux system of Gram-negative bacteria. Moreover, the role of metabolic energy in the extrusion of a noxious agent at different pH was studied and this afforded the understanding of conditions that regulate the preferential activity of the different kinds of efflux system of bacteria.

2.1 Role of glucose

The first assays performed for the assessment of the accumulation and efflux of EB by *E. coli* AG100 and *E. coli* $AG100_{TET8}$ were in the presence and absence of glucose, as previously described (187). Glucose plays an important role in the efflux mechanism as noted previously (187) but this work was conducted at pH 7. The question of efflux at varying pH with and without metabolic energy and the results obtained from this study are presented in Section 2.4 of Results. Glucose, as an energy source will be used preferentially for ATP synthesis via glycolysis and tricarboxylic acid (TCA) cycle, as described earlier in this Discussion Section. This ATP will be used preferentially by transporters from the ABC superfamily. Similar glucose-activation of H^+-ATPase was previously shown by Pereira and co-authors in *Saccharomyces cerevisiae* (258). In this study, one specific phospholipase and one specific kinase of yeast are involved in the glucose-activation of ATPase. However, the role of these enzymes in the glucose activated-ATPase of bacteria is still not defined.

In the study of the role of efflux modulators or modifiers, or compounds that interfere with the integrity and stability of the plasma membrane, glucose is important because it helps to clarify the action of the compound. Because the study showed that the effects of glucose on accumulation and efflux of EB were pH modulated, the role of glucose will be discussed in more detailed in the following section that deals with varying pH and agents that modify or modulate accumulation and efflux of EB.

2.2 Modulation of accumulation and efflux of EB by pH.

Environmental conditions play major roles in the physiology of the cell envelope as note in the section dealing with protons and the PMF. As noted earlier, bacteria face widely different environments with respect to pH and presence of noxious agents. Because EB is a noxious agent and is recognized and extruded by efflux pump systems

of bacteria (187;234;264;265), the use of EB provides a general understanding of how a given bacterium deals with noxious agents in its habitat or environment.

Previously, it was shown that exposure of *Salmonella* to mild pH activates the PmrA/B two component regulatory system which not only allows the organism to survive the low pH of the phagolysosome but also increases its resistance to antibiotics (266). The activation of the PmrA/B system is not solely by low pH since its activation has now been shown to take place at pH 8 (267). Other authors also observed that exposure of *E. coli* to low pH activates a wide spectrum of genes, some of which code for cell envelop proteins (65;268). These studies suggest that low pH readily activates genes of a Gram-negative bacterium which render the organism resistant. Although the question of whether pH-induced resistance of a Gram-negative involves the activation of genes that regulate and code for efflux pump constituents remains to be answered. Does the pH have a direct effect on the RND efflux pump family of *E. coli*?

It is known that for the RND family:

- The necessary energy to power extrusion of a compound is obtained directly from the trans-membrane proton gradient (211;212);

- Maintenance of this gradient presumes the entrance of the protons from the periplasmic space through the pump, energizing it and the agent which is believed to be concentrated within the outer leaflet of the inner membrane is, in turn, extruded (269);

- The proton is then released to the medial side of the plasma membrane (269);

- The fusion proteins are believed to physically assist the extrusion of the agent (269).

The PMF of the cell membrane is, in part, established by protons generated following the hydrolysis of ATP catalyzed by plasma membrane bound ATPases (56;270-273) and by oxidative metabolism (54). The generation of these protons takes place at sites medial to the inner membrane of the Gram-negative bacterium. After, they are exported

to the periplasm by a variety of transport processes and most of which are then exported to the surface of the cell. The distribution of protons between the periplasm and the cell surface results in a proton gradient that is greatest at the surface of the cell and least in its periplasm.

This distribution establishes a relative negative charged periplasmic space and positive charged surface of the cell and results in an electrochemical gradient. The resulting transmembrane difference in the electrochemical potential of hydrogen ions was at first seen to be the driving force behind the energy consuming enzymes and ATP synthase. It was defined as the proton motive force (274).

Because of the largesse of the bulk water phase, the dissemination of protons from the surface of the cell would quickly eliminate the pH gradient across the cell envelope, and hence, the PMF and the energy it provides for driving efflux pumps would be eliminated (274). PMF is maintained by the bacterium when challenged by changes in the pH of the environment (259;260), and the concept was extended to include the distribution of protons on the surface of the cell which due to the components of the outer cell envelop, would be selectively concentrated and result in a pH of the medium immediately surrounding the surface of the cell that would be much lower than the pH of the bulk medium (55). This surface distribution of protons therefore assists the bacterial cell in maintaining a PMF that would not be significantly affected by the pH of the medium (52).

The results presented in this thesis demonstrate that the pH of the milieu modulates the over-all activity of the intrinsic and over-expressed efflux pump system of *E. coli*. pH modulation of genes that code for ion transporters of *E. coli* has been previously demonstrated by others (275). However, this is the first demonstration that pH is shown to modulate the accumulation and extrusion of an efflux pump substrate such as EB.

The effect of pH on the cell envelope, its constituents, genes that regulate growth and metabolism has been reported and reviewed in detail (275). As also shown in the current study, low and high pH reduces optimum growth demonstrable at pH 7. However, low pH is not a problem for *E. coli* inasmuch as its survival, regardless of a slower growth rate, is not significantly affected as evident from the success of an orally consumed *E.*

coli reaching and successfully colonizing the colon. The survival of the organism is dependent upon its ability to extrude noxious agents present in the digestive system of the host. Clearly, the ability to extrude a noxious agent is intrinsically present in wild-type *E. coli*. The extrusion capability when over-expressed, make therapy of MDR *E. coli* infections problematic.

The selection of pH affords the distinction between an efflux pump system immediately dependent on the PMF, such as a RND efflux pump, and one that is dependent upon metabolic energy and inhibited by verapamil. As shown in this thesis, extrusion of EB can be prevented at pH 8 by verapamil, an agent that is known to inhibit ABC transporters (276). Because the *msbA* gene of *E. coli* is similar to the ABC transporter gene *efrAB* of *E. faecalis* (277;278), the glucose dependent efflux at pH 8 noted in our study may be conducted by the putative MsbA transporter. Further studies have to be performed in order to identify the ABC transporter in this study. Nevertheless, an ABC type transporter system that has the capability to extrude the efflux pump substrate EB has now been demonstrated for the first time in *E. coli*.

Consequently, it is widely held that the pH of the medium should not affect the activity of an RND efflux pump even though there is no evidence in support of this conclusion. However, work done with yeast showed that acidification of the medium during cellular growth triggers a decrease in cytosolic pH and this situation also leads to ATPase activation (258). If this observation can be also considered as true for *E. coli*, as it seems to be from the results presented on section 2.2 of the Results, the low pH (acidic) at the cytoplasmic side of the plasma membrane that results from the translocation of protons from the periplasm to the cytoplasm by the RND type transporter, promotes the synthesis of ATP. The synthesis of ATP therefore removes protons from the cytoplasm and the pH at the cytoplasmic side of the plasma membrane increases. This process not only maintains a relatively stable PMF but it also provides the ATP that is used by the ABC transporters that are then preferentially used by the cell for transport of nutrients into the cell. The hydrolysis of ATP by the ATP binding transporters is thus an ATPase like function that has been identified in yeast and which is surmised in this thesis to take place in a Gram-negative bacterium. This is the first time that the entire energy-proton-

pH cycle has been described in a Gram-negative bacterium that is coupled to the pH of the medium (environment).

The study of this dissertation supports the contention that the survival of a bacterium as it makes its way through the digestive system and eventual colonization of the colon, is assured by the activity of two types of efflux pump systems: one that operates directly from PMF derived energy and the other from a putative ABC transporter. It is supposed that when the bacterium reaches the duodenum, the toxicity presented by high concentrations of bile salts must be obviated and this is accomplished by the extrusion of these toxic substances by an RND efflux pump such as the AcrAB of *E. coli*. Because the results demonstrate that the intrinsic efflux pump system of wild-type *E. coli* is perfectly capable of extruding EB in glucose-free saline at pH 5, and this extrusion can be eliminated by an un-coupler of the PMF, it should be this PMF dependent pump system which protects the bacterium while passing through the duodenum. There is no need for an over-expressed PMF dependent efflux pump system. When the organism reaches the colon, bile salts, as well as many secreted agents produced by the normal flora, are present and are toxic to the organism. The pH of the colon is near 7 and because of the scarcity of protons in this bulk medium the concentration of surface bound protons must be affected, and is probably much lower than that at pH 5.

If the RND pump is to operate under these conditions, the PMF must be maintained and the protons needed to activate the RND efflux pump must be available in the periplasm (269). Subsequent to the extrusion of the noxious agent the protons are released to the cytoplasm. Two general situations are expected from the activity of the RND efflux pump at pH 7 or higher:

1) The consumption of protons from the periplasm reduces the PMF unless protons are replaced from the surface of the cell. The process of replacement is probably limited and inadequate for the maintenance of an RND efflux system under these conditions;

2) The release of protons to the medial side of the cytoplasm membrane will decrease the pH and the synthesis of ATP by ATP-synthase is favoured (187;279). The utilization of protons for the synthesis of ATP insures that the pH gradient between the

periplasm and cytoplasmic side of the plasma membrane is maintained. The ATP generated is then bound by the ABC transporter, hydrolyzed and the energy from this process activates the ABC transporter to extrude the noxious agent.

Metabolic energy also contributes to this process. In the toxic environment of the colon, it is supposed that the organism receives protection from the ABC type efflux pump system that relies primarily on metabolic energy, energy that is derived from the richness and practically unlimited supply of carbohydrates continuously replenished by the digestive processes of the human host.

These observations are very important at clinical level since one must make a distinction between the process of an infection caused by *Salmonella* or *E. coli* strains and its colonisation of the colon that is made possible by the collaboration of PMF dependent efflux and ABC transporters from an infection by their MDR counterparts. When these MDR infections are efflux mediated, therapy which is normally ineffective may be improved by the co-administration of non-toxic compounds that inhibit the efflux pump machinery and hence, the previously inactive antibiotic can now reach its intended target. The following section therefore discusses the results obtained in Section 2.3 of Results that demonstrated the effects of agents on the accumulation and efflux of EB by intrinsic and over-expressed efflux pumps.

2.3 Role of efflux modulators

The demonstration of pH modulated efflux pump activity is extremely significant for the design of agents that are to serve as efflux modulators. If the agent is to be an effective adjuvant to antibiotic therapy for the management of a food-borne infection caused by an efflux mediated MDR Gram-negative bacterium such as an *E. coli* strain, it must be active against the ABC transporter that is protecting the bacterium from the toxic agents of the colon, and during antibiotic therapy. Efflux modulators whose effectiveness is shown against RND type efflux pump systems may not be effective in the environment in which the offensive bacterium resides. Consequently, an efflux

modulator that is to serve as an adjunct to antibiotic therapy aimed at an efflux mediated MDR coliform infection should be evaluated for activity under physiological conditions, namely at a pH that favours the activity of RND and ABC type efflux pump systems. In the following discussions, the results obtained in this thesis will present along new interpretive lines, considerations of efflux pumps that had not been previously considered.

2.3.1 CCCP

CCCP is a un-coupler that is known to destroy the PMF of the cell envelope by binding the surface bound protons of the bacterium (258). As per prior discussions, the pH of the medium is now known to be the major controller of surface bound protons of Gram-negative and Gram-positive bacteria (54). Consequently, the question that was asked in this thesis was: Does CCCP has differential quantitative effects on the accumulation of EB when assessed by the semi-automated EB method at varying pH? As per the results obtained and described in Section 2.3.1 of Results, at pH 5 the concentration of CCCP needed to significantly increase the retention of EB is considerably greater than at pH 8. The interpretation of these results is that at pH 5 the concentration of protons on the surface of the cell is greater and exceeds the proton binding capacity of low concentrations of CCCP that are very effective at pH 8.

The effectiveness of the over-expressed efflux pump is dependent upon the concentration of protons on the surface of the cell. At pH 8 due to the lower concentration of surface protons the available protons for the over-expressed pump is limited and rapidly depleted from the periplasm. If CCCP is then added, it produces a synergistic effect on the retention of EB. From the data presented in the CCCP section, it is readily seen that lower concentrations of CCCP are more effective in causing the retention of EB by over-expressed efflux pump cells at pH 8 than wild type cells containing the intrinsic level of efflux pump activity.

2.3.2 PAβN

PAβN has been used as an EPI by many authors. However it was shown previously, at pH 7, that this agent causes accumulation of EB in a concentration dependent manner but does not influence efflux (187). Therefore the following question was asked: Is PAβN an EPI or competes with EB for a site of the efflux pump? To answer this question an assumption was tested: If PAβN is an EPI, the activity of the efflux pump at pH 5 should be reduced as a consequence of its binding to the transporter component of the pump (264;269;280;281). The data presented in the PAβN Section of the Results show that PAβN has no effect on the efflux of EB by the *E. coli* strain at pH 5, but causes an increase in its accumulation that is concentration dependent. These results support the previous contention that PAβN is not an EPI but a competitor with EB (280) for access to the substrate "active" site of the pump. In other words, efflux systems of *E. coli* extrude PAβN preferentially to EB. The preferential extrusion of PAβN is surmised from the effects of increasing concentrations of PAβN on the accumulation of EB when EB is maintained at 1 mg/L. Therefore, relative to this EB concentration, a K_M for PAβN was calculated and yielded a value of 4.21 mg/L. It should be noted that if the PAβN is added after accumulation of EB, and the assay is allowed to take place for much longer periods, the effect of PAβN on accumulation will be noted (data not shown). The reason for this renewed accumulation of EB is due to the penetration of EB that is taking place continuously during the assay whereas PAβN is extruded preferentially. The approach used in this PAβN assay can be used for other efflux pump substrates that compete with EB and for which a similar K_M may be calculated at pH 5 and compared to the K_M of other substrates. In this manner the relative binding of various efflux pump substrates to a given efflux pump transporter may be determined. More detailed kinetic and computational docking studies should be done to understand more clearly the mode of action of PAβN. Nevertheless, the results obtained are very important in the understanding of the mode of action of efflux modulators that could be used in therapy of infections caused by MDR strains. The results show that the commonly called EPIs – efflux pump inhibitors – do not have to inhibit directly the pump. In fact, it seems that the some of those compounds act in systems that interfere with the membrane organization as PMF or energy dependent pathways and by that way

cause less efflux of the antimicrobial agent. It was demonstrated that for the study of new "helper compounds" (20) its activity should be tested by a wide range of experiments including not only accumulation but also efflux assays.

2.3.3 Verapamil

Verapamil, known as an inhibitor of the ABC transporters was used in the assay in order to distinguish the role of the two main types of general efflux systems, at different pH. Since verapamil is an inhibitor of the ABC type of efflux pumps that is dependent upon immediate metabolic energy, and its inhibition is an indirect result of inhibiting access to calcium, when efflux is mainly due to an RND transporter, such as the AcrAB pump, verapamil should not alter the efflux of EB and no increase in EB accumulation should be observed. Conversely, if an efflux pump of the ABC family has an important role on the efflux of EB by the strain, then an effect of verapamil should be expected. The results obtained and described by the Verapamil Section of Results, indicate that the inhibitory effect of verapamil was mainly observed at pH 8 in a concentration dependent manner, indicating that, at pH 8 the extrusion of EB by *E. coli* is taking place by a metabolic energy dependent system. The genes *efrA* and *efrB* are responsible for the coding of the ABC transporter of *Enterococcus faecalis*, EfrAB, and have a homologue gene in *E. coli*, the *msbA* (278). The MsbA, which acts as a flippase and inserts the de novo synthesised Lipid A into the nascent LPS layer of the outer cell envelope (282;283), probably has a dual role, and can extrude EB at pH 8. But this remains to be proven.

As previously observed in the preceding sections, at pH 8 ATPase preferentially hydrolysis ATP and contributes to the replenishment of protons and maintenance of the PMF. This idea is supported by the fact that at this pH the efflux that is dependent on an energy source is highly affected by verapamil Therefore transporters from the ABC superfamily are supposed to be the main extruders of noxious agents at pH 8.

Because verapamil is also known to limit the access of calcium to calcium dependent ATPases (108;277) and because calcium is an important ion in cell signalling (bacteria included) (220;258), the role of phenothiazines, inhibitors of calcium and potassium transporters (154), and calcium itself were also studied and the results obtained are discussed in the following sections.

2.3.4 Phenothiazines

Phenothiazines are calcium channel inhibitors that, at sub-inhibitory concentrations, were shown to reduce *in vitro* minimum inhibitory concentrations of some antibiotics against strains of *E. coli* (37), *S. aureus* (119), *M, tuberculosis* (186), etc. The effect of sub-inhibitory concentrations of TZ against Salmonella was also followed during 24h (267). Growth curves of different strains were followed in presence and absence of TZ and when this compound was present, the initial lag phase was increased in a concentration dependent manner (increase of TZ concentration promotes and increases lag phase duration). After that period of inhibition, adaptation takes place and growth of Salmonella strains is similar to that of unexposed controls. rtRT-PCR revealed that genes that regulate the expression of RND pumps were over-expressed just before the strain began to grow in the presence of TZ (267). When the TZ exposed strain attained full growth, only the *acrB* transporter gene was over-expressed. Interestingly, exposure of a salmonella strain to TZ in the semi-automated EB assay demonstrates a rapid accumulation of EB that is followed 30 to 60 minutes later by efflux (267). Because there is no metabolic energy in the assay, and because salmonella has a great ability to use its lipid metabolic pathway for generating energy (267), the role of a lipid such as palmitic acid was examined by the authors. As expected, the addition of this fatty acid resulted in the reduction of accumulation of EB (267) and therefore its efflux and those results support the proposal that a full examination of fatty acids (short *versus* long chained) should take place in order to establish the source of lipid derived energy. Moreover, one may conceive that by adding a lipid as a source of metabolic energy, genes that are activated may now be assessed by rtRT-PCR.

Therefore, the results obtained in this dissertation and presented in section 2.3.4 of Results showed that TZ and CPZ promote accumulation of EB and inhibit its efflux by *E. coli* AG100 but not by *E. coli* $AG100_{TET8}$, and glucose decreases the activity of the phenothiazines. The inhibition of efflux was observed at pH 7 and 8. At pH 5 no inhibition was observed regardless of an energy source. These results again suggest that, for the parental strain, at pH 8, a putative ABC transporter plays an important role in efflux that is affected by TZ. However, in the adapted strain, because the RND systems are over-expressed, this influence is not that significant.

The results obtained in the thesis relate TZ to genetic aspects that regulate efflux pumps as well as demonstrate that TZ itself increases accumulation and inhibits efflux at pH 7 and 8 while having little effect at pH below 7. One may surmise that TZ targets at least two different systems that together provide protection against a noxious agent. Because phenothiazines affect access to calcium (154), and calcium is important for signalling (220) and activating genetic systems (220), the role of calcium alone and in combination with CPZ, at varying pH with and without metabolic energy was evaluated with the aid of the semi-automated EB method and is discussed in the following section.

2.4 Role of Calcium

Calcium is needed for a wide variety of metabolic and energy deriving pathways within the cell. Central to these pathways are ATPases that hydrolyse ATP and furnish protons for the activation of ABC type transporters. Although the medium employed in our study does not contain calcium, calcium is nevertheless present within the cell and is recycled as needed. The results presented in our study suggest that there are two general types of transporter systems in *E. coli*; one system that is dependent upon metabolic energy for the extrusion of the universal substrate EB and is evident at pH 8, and another general system that is readily demonstrable at pH 5 and which consists of 8 or more efflux pumps that accompany the main efflux pump of this organism, namely, the

AcrAB-TolC pump (65). These latter efflux pumps are dependent upon protons present in the periplasm for their activation, as discussed previously.

The role of calcium was investigated in this thesis and described in Section 2.4 of the Results. Briefly, the addition of EDTA, a chelator of calcium, increases accumulation of EB and decreases its efflux. The addition of calcium inhibits the EDTA responses as well as the responses to CPZ. The addition of EDTA and CPZ has additive effects on the accumulation and efflux of EB. These results suggest that the activity of CPZ on accumulation and efflux is due to it having an effect on calcium. Because there is no calcium in the medium and there is no replication of cells during the assay period, the calcium that is being affected must be surface bound calcium that had been retained from the culture of the bacterium in complete MHB medium.

Previous observations of ATPase activation made in yeast showed that extracellular calcium appeared to be essential for the CCCP-induced activation of ATPase, because pre-incubation of cells with EGTA (a calcium chelator equivalent to EDTA) completely inhibited this activation process. On the other hand, when an equimolecular calcium concentration was added together with EGTA, the CCCP-induced activation of the ATPase took place (258).

The increase of EB accumulated and inhibition of EB efflux by CPZ at pH 8 and not at pH 5 is interpreted to result from an interference with access to calcium by ATPases that provide protons at pH 8. Because at pH 8 hydrolysis of ATP is favoured and contributes protons for the activation of ABC type transporters, CPZ is, at this time, suspected of having its effects on accumulation/efflux of EB by indirectly affecting ATPase activity.

In conclusion, a phenothiazine such as CPZ or TZ has a variety of effects on the efflux machinery of the bacterium. These effects involve adaptive responses that are mediated by the activation of global and local regulator genes, stress genes and genes that code for the transporter component of the efflux pump AcrAB (267). In addition, the phenothiazines inhibit access to calcium more or less in the same manner they have been shown for eukaryotes (154;194). Because the EB assay is of short duration, one may say that the phenothiazine effects, noted via this assay, represent the earliest

response of the bacterium to the noxious phenothiazine. This early response allows the organism to survive while the genetic responses to the phenothiazine prepare the organism for long term survival in the presence of the agent.

3. Search for new active compounds against resistance

As a consequence of new concepts introduced in the previous sections, a search for new compounds that modulate efflux has been undertaken. In this respect, because plants have been shown to contain such agents and previous studies indicated that one particular plant *Carpobrotus edulis* had properties that affected efflux, the isolation of active compounds from this plant was undertaken in this thesis. The isolation and characterisation of isolated compounds from plant sources is encouraged given the fact that such plants have a long history of medicinal use that suggest the presence of efflux pump modulators (example: plants that alleviated gastric reflux contain inhibitors of efflux pumps that secrete H^+ and cause gastritis, etc. (284)). Because the therapy of MDR infections that are mediated by efflux pumps may eventually include agents that inhibit efflux activity of the clinical isolate, the compounds isolated from *C. edulis* were evaluated for activity against efflux by MDR bacteria that over express an efflux pump. Moreover, because medicinal use of some plants for cancers and other malignancies is widely reported and result in the isolation of compounds that inhibit the P-gp transporter of MDR cancer cells, the activity of the isolated compounds on the over-expressed P-gp transporter of MDR cancer cells was also investigated.

Because of the results obtained in this thesis, attention has been focused on a variety of physiological factors that define conditions under which MDR bacteria and MDR cancer exist. Among these factors is pH. If a better understanding of the infection process and its relationship to efflux is to be gained, pH is an important and fundamental tool. However, the approach is first to conduct screening of isolated agents for effects on efflux by MDR bacteria and MDR cancer at pH 7 simply because all assays that evaluate the effects of an agent on efflux are conducted at this pH plus, if the isolated compound had been previously evaluated, the use of pH 7 provides a comparison between the data that is obtained by different methods versus those used in this work, such as the semi-automated EB method results. Active compounds may then be examined at varying pH in order to relate the activity at sites of infection or site of cancer that differ with respect to pH (examples: infection of the stomach by *H. pylori* or gastric MDR carcinoma, both of which take place at acidic pH).

Because the methanol extract of the plant *C. edulis* was shown to enhance the killing activity of infected macrophages and inhibit the ABC transporter in human *mdr1* transfected mouse lymphoma cells, this extract was investigated for its active compounds responsible for the reported activities. Moreover, this plant has also been described for many uses in traditional medicine. Consequently, the two main characteristics of a plant that support the isolation and evaluation of compound, namely, traditional medicinal use of the plant and activity of the plant extract are satisfied by *C. edulis*. The discussion of the results obtained for the purification and study of the activity of the isolated compounds are described below.

3.1 Purification and identification of the compounds

Methanol, as a semi-polar solvent, is appropriate for the extraction of constituents of a wide range of polarity. Moreover, previous activity of this plant was reported for the methanolic extract (175;176). Methanol was then chosen for the first solvent of the extraction. Extensive percolation with this solvent is a simple and effective method for the extraction because such extracts contain a wide scale of compounds (small molecular, hydrophobic, hydrophilic, polypeptides, etc). The fresh aerial parts (leaves) of *C. edulis* collected in November and January were extracted with methanol and the resulting aqueous was used in solvent-solvent partition.

Solvent-solvent partition on the crude extract is a high capacity, economic technique, which is commonly used in natural product chemistry as the first separation step. The *C. edulis* crude extract was partitioned with hexane in order to obtain the fraction with the most apolar compounds of the plant leaves. This was followed by successive separations of the methanolic extract with chloroform and ethyl acetate, in an increasing order of polarity.

Preparative-scale classical adsorption chromatography on silica is also a valuable method in the preliminary purification. This method has a very high capacity and is

cheap enough to be economic in the separation of samples in large amounts. After preliminary purification, a multistep procedure of combined chromatographic methods was followed for the isolation of active compounds. All of these chromatographic steps were monitored by using TLC, which is a simple and fast method. The TLC behaviour of the compounds helped in the choice of adequate solvent systems for the next isolation steps. In the end 5 more apolar compounds from the hexane/chloroform fraction and 3 more polar compounds from the ethylacetate fraction were obtained.

The compounds isolated were identified by NMR as 3 flavonoids: catechin, epicatechin and procyanidin B5; 3 triterpenes: uvaol, oleanolic acid and β-amyrin, and a galactolipid: monogalactosyldiacylglycerol (MGDG). All of these compounds were isolated for the first time from *C. edulis* and for the Aizoaceae family. However, these compounds have also been isolated and characterised from other plants (222-229).

Phenolic compounds such like catechin, epicatechin and its derivatives, mainly found in green tea, have been shown to act as anti-oxidants and provide protection from congestive heart failure (285), as anti-atherosclerotic (286), as chemopreventives (287), as anti-inflammatory agents (288) or as inhibitors of the gastric H^+, K^+-ATPase (289). Therefore these compounds could be the responsible for the successful use of *C. edulis* in traditional medicine for stomach problems and inflammatory pathologies (167;169;170). The results obtained and described in Section 3.6.2 of the Results show that epicatechin has no inhibitory effect on P-gp. These results are in agreement with others (290). However, some inhibitory effect has been reported with chemical derivatives of this compound (290). Procyanidin B5 dimmers were, also, demonstrated as potential chemopreventive agents against breast cancer by suppressing *in situ* estrogen biosynthesis (291).These flavonoids and related metabolites have been reported to exhibit mild activity towards mycobacteria, inhibiting its growth (148). Data from the literature also identify simple phenolic compounds such as epicatechin as antimicrobial compounds via a mechanism that disrupts the cell envelope (292). Catechin was also identified as an antimicrobial with MIC between 2 and 78 mg/L against a wide range of Gram-negative bacteria and 10 and 20 mg/L against Gram-positive (293). In earlier investigations, catechin has been described to potentiate the action of streptomycin against *M. tuberculosis* infection in mice and decreases the

incidence of pulmonary tuberculosis fourfold, and that, this effect is probably due to their inhibitory effect on fatty acid and mycolic acid biosynthesis (150). This is an important result since *C. edulis* juice is used in the traditional medicine against TB infections. Other flavonoids were even shown to inhibit mycobacterial efflux pumps and potentiate the action of INH, but the mechanism is yet to be determined (150), or inhibit the efflux activity of NorA from MRSA strains (28).

Other three of the isolated compounds belong to the class of the triterpenes. This class of compounds was also described to exhibit antimycobacterial activity. Uvaol, oleanolic acid and β-amyrin had an MIC of 8 mg/L (148;294), 28.7 mg/L (148;295) and 12.2 mg/L (148;296) respectively, against *M. tuberculosis* determined by microplate alamar blue assay. By the same method oleanolic acid had an MIC of 25 mg/L against *M. tuberculosis* H37Rv and 50 mg/L against *M. tuberculosis* H37Rv strains individually resistant to streptomycin, isoniazid, rifampicin and ethambutol (297). These compounds and others from oleane and ursane skeleton triterpenes were also described with anti-ulcer activity and other pharmacological properties such as anti-inflammatory, anti-allergic, anti-nociceptive, anti-tumor and anti-viral activities (298). Antiproliferative and apoptosis-inducing effects of oleanolic acid on colon cancer cells were also reported (299). In 1995 Jiu Liu reported different properties of oleanolic acid and its use in human therapies such in liver failure and disorders, anti-inflammatory or anti-tumor activities, among others and no toxic effects were observed in rats or in humans. Those clinical trials also led to the patenting in Japan of the oleanolic acid as addictive to health drinks, hair tonics or topical used preparations for prevention of skin cancer (300).

During bibliographic search for this thesis some biological activities were found for the galactolipid monogalactosyldiacylglycerol; anti-algal, anti-viral, anti-tumor and anti-inflammatory activities reported for glycoglycerolipids isolated from cyanobacteria (301). This compound, together with digalactosyldiacylglycerol, represents *ca.* 75% of the total membrane lipids in plant leaves. They have special importance in membranes where photosynthesis occur and are not commonly found in animals (301).

3.2 Activity of *C. edulis* compounds against bacteria

During this first screening for *in vitro* activity of the compounds against Gram-negative, Gram-positive and Mycobacteria, it was possible to observe that none of the compounds were active against Gram-negative bacteria but some of them were very active against certain Gram-positive bacteria and had moderate activity against Mycobacteria.

The results for the MRSA COL_{OXA} strain and *S. aureus* HPV 107 are of significant importance. Susceptibility/resistance of given bacteria is defined by critical concentrations and regression analysis of Kirby-Bauer data (29). The application of this approach for defining susceptibility/resistance is beyond the scope of this thesis. However, if we employ the MIC results in terms of mg/L, some comparison can be made between the activity of the compounds isolated against given bacteria and that by conventional antibiotics. Oleanolic acid can be considered to exhibit high activity against the *E. faecalis* strain inasmuch as its MIC 6 mg/L comparing to the susceptibility of this strain to common antibiotics (189). Similarly, results obtained to the *M. tuberculosis* H37Rv strain are good and in correlation with data from the literature to the oleanolic acid (297).

Previous results showed that the methanolic extract could reduce or reverse the activity of antibiotics against specific bacteria (175). Therefore the compounds isolated were evaluated for that property. None of the compounds reduced the resistance of *S. enteritidis* 104_{CIP} and MRSA strains to antibiotics to which they were resistant. This suggests that these compounds did not affect the efflux system of these organisms. However, some of the compounds reduced or reversed resistance to some antibiotics in other strains. Among these compounds, uvaol was the compound that had the greatest ability to reduce resistance of MDR Gram-negative and MDR Gram-positive strains to antibiotics to which they were initially resistant. These MDR bacterial strains owed their MDR phenotypes to over-expressed efflux pump systems. Uvaol also reversed resistance of MDR cancer cells; therefore it is this compound that has promise as a modulator of efflux by MDR bacteria and MDR cancer cells and should be furthered studied.

However, when tested for the modulation of activity against bacteria by the semi-automated EB method, uvaol did not increase the accumulation of EB inside Gram-negative strains (*E. coli* AG100 and *E. coli* $AG100_{TET8}$). However, it did increase accumulation of EB by the MRSA strain (*S. aureus* COL_{OXA}). This increased accumulation was influenced by glucose. This glucose influence was demonstrable by the rates of accumulation; whereas in the absence of glucose the rate of accumulation was steady per unit period of time; in the presence of glucose accumulation was much steeper. These results suggest that uvaol may penetrate the bacterium via a glucose controlled pathway. Once it penetrates it may then act on the efflux pump system.

MGDG was the other compound that promotes increases of accumulation of EB by the MRSA COL_{OXA} strain but only in the absence of glucose. This may be due to a glucose dependent efflux pump system that extrudes the MGDG and that accumulation of EB is very much similar to the effects of PAβN, namely, MGDG competes with EB. However complementary studies should be done to understand its mechanism of action.

Oleanolic acid, a compound with a similar skeleton to uvaol, was, together with catechin and epicatechin, modestly active in the modulation of accumulation and efflux of EB by *E. coli* strains. Oleanolic acid is known as an inhibitor of protein kinase, while flavonoids were shown to inhibit the transport of glucose. These two mechanisms prevent the derivation of energy needed for efflux pump activity, therefore accumulation takes place. In the presence of glucose, the capacity for inhibition by the compounds is not very significant and therefore less accumulation is observed. However as the effects were not considerably higher as the ones observed previously for other compounds such as verapamil or phenothiazines whose sites of action are more directly related with the efflux pumps itself, it can be thought that for inhibition of efflux activity, the compounds should inhibit key physiological pathways that are more proximal to the efflux pumps.

The compounds were then tested for their *ex-vivo* activity against the model strains *S. aureus* ATCC and MRSA. Again, uvaol was the compound that increases the killing activity of the macrophages when infected with either one of the two strains. Oleanolic acid, catechin and MGDG were also active but only when infected with the ATCC strain (first two) or the MRSA (MGDG). At this time we cannot say that the

enhancement of killing by non-killing macrophages is due to accumulation of the compounds to levels compatible with *in vitro* bactericidal levels (152) or by direct interaction with the phagolysosomal system of the macrophage itself (194).

The activity of uvaol is again the most promising for future studies in *Mycobacterium tuberculosis* infection of macrophages. This compound apparently has activity against a wide range of efflux pump families.

3.3 Anticancer activity

From the results obtained, although all of the compounds had anti-proliferative activity against parental (PAR) and its *mdr1* transfected progeny, only MGDG had major activity against the MDR cell line while uvaol and oleanolic acid were more effective against the PAR cell. The remainder of the compounds, although effective, did not discriminate among the two cell lines with respect to activity. Nevertheless it must be remembered that both cell lines are cancer cells and therefore the discrimination between the cancer cell lines by some of the compounds has clinical significance. Previous authors showed that the MGDG compound could decrease the biosynthesis of cholesterol by inhibiting the human enzyme lanosterol synthase (228). This result, together with the lipophilicity of MGDG, can explain that the compound MGDG interacts with the MDR cell line in a more specific manner, and hence is more effective against the more sensitive MDR cell line.

Uvaol exhibited a significant effect on the inhibition of the P-gp that resulted in the accumulation of rhodamine 123 inside the MDR mouse lymphoma cells. This compound also showed a concentration dependent activity with an increase of the FAR values for the higher concentrations. Oleanolic acid has very weak activity and β-amyrin, MGDG, catechin, epicatechin and procyanidin B5 were not effective. These results were confirmed by the ones obtained by the semi-automated EB method adapted for the evaluation of activity against the P-gp transporter of these cancer cell lines.

Uvaol also promotes an increase in concentration of EB; the oleanolic acid is less effective. By this technique, EB accumulation promoted by MGDG was also observed.

Of the compounds isolated from *C. edulis*, uvaol was the most effective in inhibiting the extrusion of rhodamine 123 and EB by the MDR mouse lymphoma cells as evident from the accumulation of these P-gp substrates, demonstrated by two distinctly different methods, flow cytometry and the semi-automated EB method, respectively. Because this compound does not produce toxicity at the concentrations employed, it may have potential as an adjuvant for the therapy of MDR cancer that is refractory to therapy.

The structural difference between uvaol and β-amyrin or oleanolic acid is the methyl group at position C-29 (Figure 51 – 1, 2 and 3) and seems to play an important role in the activity of this class of compounds. Further studies should be conducted in order to study in more detail the structure-activity relationship. Furthermore, members of the triterpenes group may serve as lead compounds for the synthesis of new compounds that may prove even more effective as inhibitors of P-gp than those isolated from *C. edulis*; hence, they may serve as adjuvants in cancer chemotherapy.

3.4 Structure activity relationship

During this dissertation work, a group of compounds were tested for their activity as modulators of efflux. Figure 50 shows the structure of the compounds used in this work and that were previous characterized as efflux modulators or EPIs against MDR bacteria and cancer. Figure 51 shows the structure of the active compounds obtained in this thesis from the plant *C. edulis*. The compounds oleanolic acid, uvaol, catechin, epicatechin and MGDG presented activity against bacterial efflux pumps, showed by the reduction of antibiotic MIC whose resistance was acquired by over-expression of EP. When reduction of MIC was observed, the compound uvaol was the one that always showed activity. Compound uvaol also significantly inhibits the P-gp pump of the tested cancer cell line, followed by oleanolic acid whose activity was much lower.

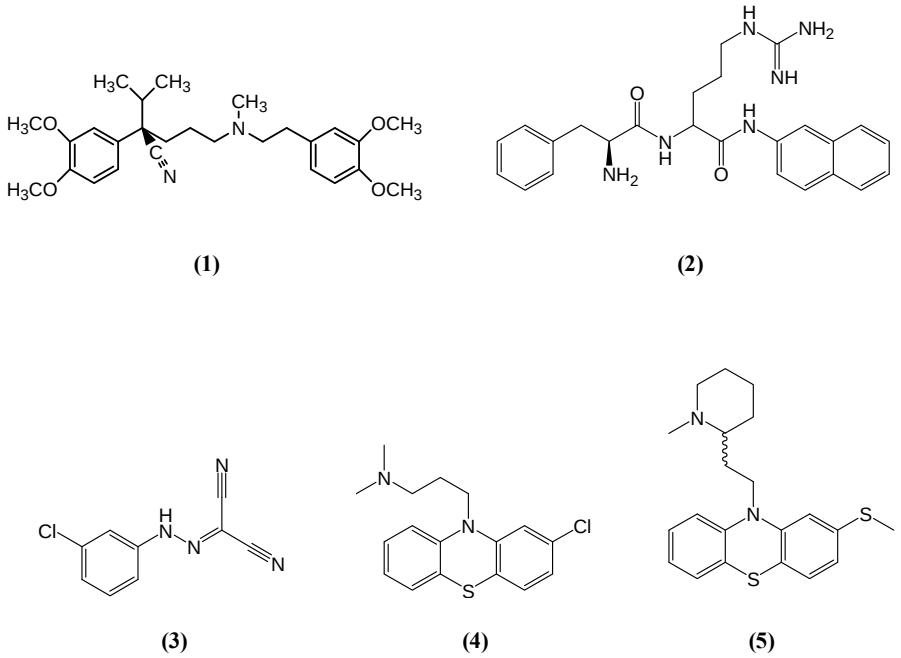


Figure 50 – Structures of the commonly used efflux modulators.
(1) Verapamil; (2) PAβN; (3) CCCP; (4) chlorpromazine; and (5) thioridazine.

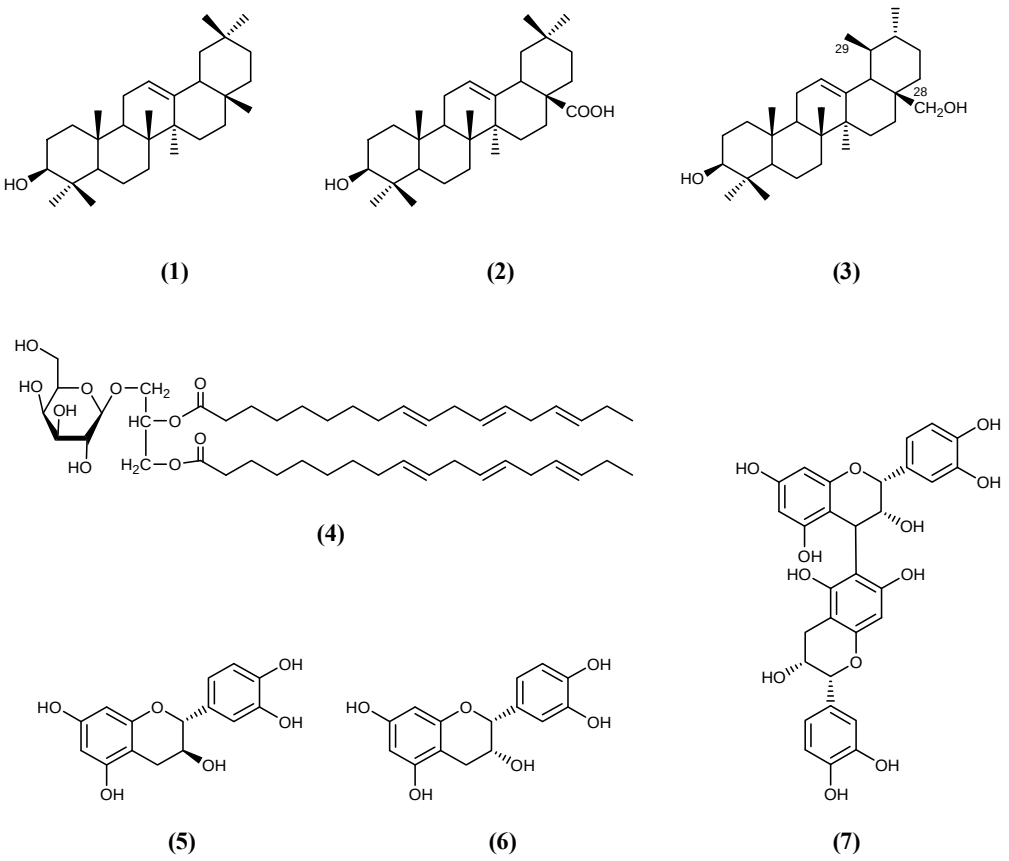


Figure 51 - Structures of the isolated compounds from the methanolic extract of *C. edulis*
(1) β-amyrin; (2) oleanolic acid; (3) uvaol; (4) monogalactosyldiacylglycerol (MGDG); (5) catechin; (6) epicatechin; and (7) procyanidin B5.

In this work it is not possible to make a detailed study about structure activity relationship (QSAR) of the tested compounds. For this kind of study, a greater number of compounds from the same structural family are needed, in other words with the same structural core, or exactly with the same activity assay, in order of a possible correlation between the structure (2D and 3D) and the activity observed. However, from the structures presented in the previous figures and others that can be found in the literature, no inference of structure to activity is at this time possible. The wide variety of EPs, together with the differences on the cellular envelope of Gram-positive and Gram-negative bacteria, Mycobacteria and all kinds of Eukaryotic cells, makes this "job" very difficult. However, compounds that influence the energy systems of the cell, specifically those that block common constituents of the majority of efflux pumps or interact with the membrane of the cells, can be promising agents for the use as adjuvants in therapy. It is also important that the referred compounds specifically interact with bacterial cells, if for use against bacterial infections or the cancer cells, if adjuvants in chemotherapy, but not active on healthy human cells.

The presented work gave an important contribution to the study of the mechanisms of efflux, as well as for the discovery/synthesis of new compounds that could fill the requests cited previously and then, be used in therapy for reversion of multi-drug resistance.

VI. FINAL REMARKS AND FUTURE PERSPECTIVES

From the work conducted for this thesis, important conclusions can be taken according to the physiology and modulation of efflux and the activity of *C. edulis* isolated compounds. The conclusions of this dissertation are summarized below.

1. Physiology and modulation of efflux

- The cell envelope of bacteria is fundamental to their survival and response to the changes in the environment;
- *In vitro* antibiotic pressure to drug resistance strains results in the acquisition of an MDR phenotype mimic what happens *in vivo* during chemotherapy;
- Efflux of EB by Gram-negative bacteria, namely *E. coli* is dependent on the pH of the environment of the cell (media);
- The efflux response is independent on the pH of growth of the bacteria;
- Energy dependent efflux mechanisms vary upon the pH and the conjunction of pH and glucose is an important tool in the study and understanding of the physiology and mechanisms of efflux;
- Efflux pumps belonging to the ABC superfamily have an important role in efflux at pH 8 as shown by the assays with CCCP, Verapamil, EDTA, CPZ and Calcium;
- PMF is essential for RND family mediated efflux as per the results obtained at pH 5;
- CCCP is an important molecule to use at different pH in order to distinguish the role of protons on the efflux and consequently the PMF due to its capacity of

binding protons. Together with pH, it helps to distinguish which should be the main types of efflux transporters that are working at the different conditions;

- PAβN modulates efflux of EB by competing with EB for the site of extrusion of the pump and a K_M was determined;

- A proposal of regulation of the efflux mediated by pH and energy is presented in the following figures:

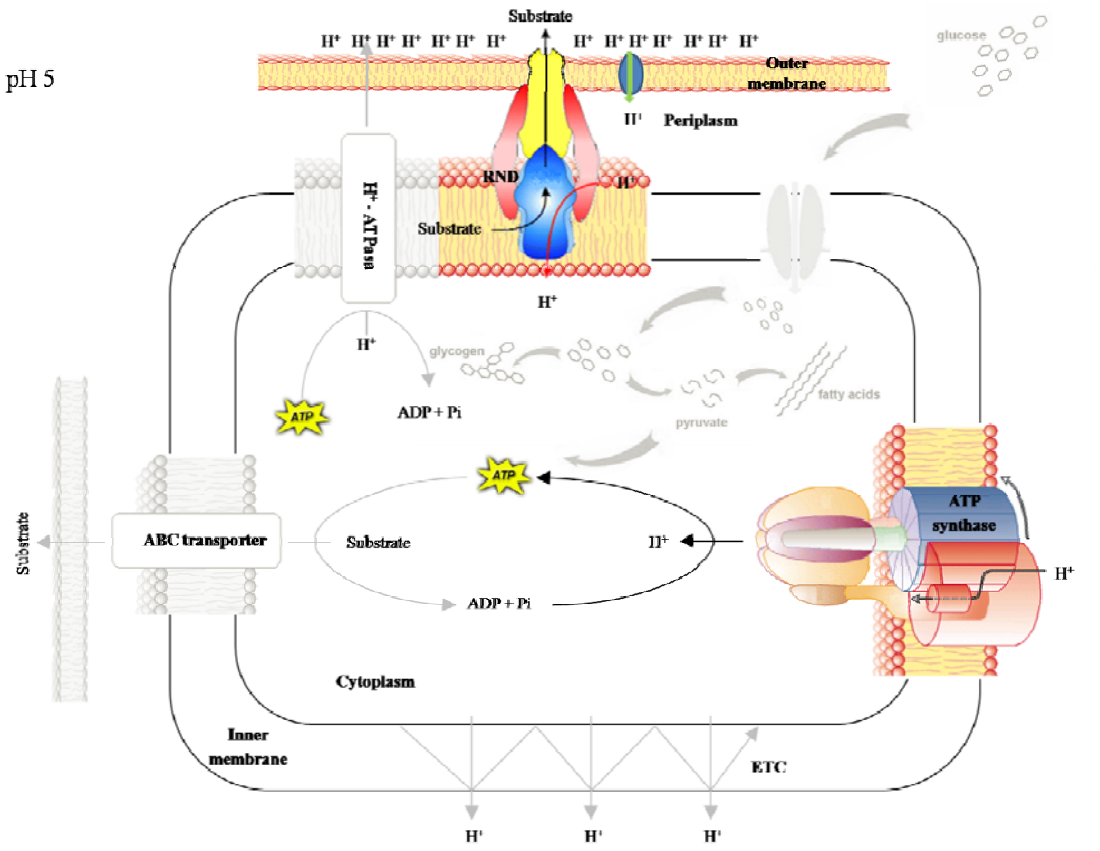


Figure 52 - Representation of the proposed efflux mechanisms at pH 5.
The relevant mechanisms that occur at pH 5 and influence the efflux activity of bacteria are colored while grey pathways, unless present, have a minor contribution for the efflux at this pH. At pH 5 the essential aspects of efflux are carried out by the RND efflux pump system of Gram-negative bacteria. This system, as noted, is dependent upon the establishing and maintenance of the PMF which results from the metabolism (catabolism) of glucose and the production of H^+ that are handled by two related mechanisms: production of ATP and transport to surface of cell. The equilibrium may be presented as follows: $2H^+ + pi + ADP \rightarrow ATP / 2H^+ \rightarrow surface$. The balance is affected by efflux pump activity.

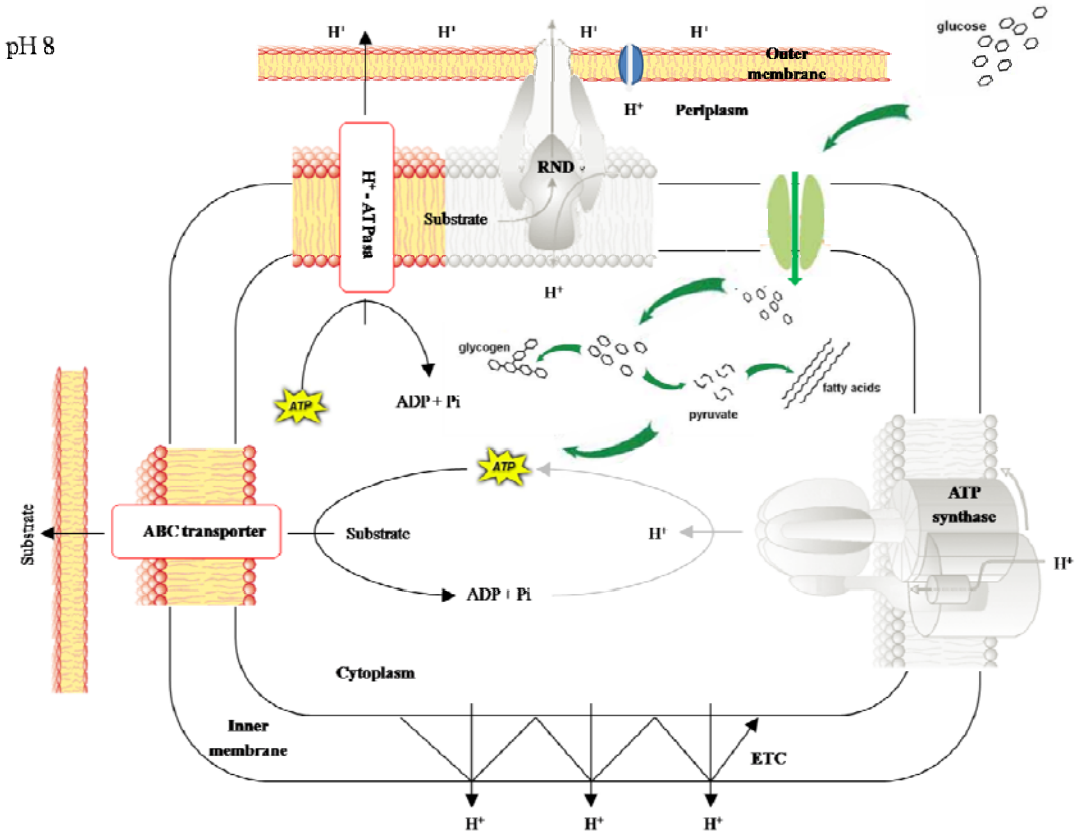


Figure 53 - Representation of the proposed efflux mechanisms at pH 8.
The relevant mechanisms that occur at pH 8 and influence the efflux activity of bacteria are colored while grey pathways, unless present, have a minor contribution for the efflux at this pH. At pH 8 the essential aspects of efflux are carried out by the ABC transporter system of Gram-negative bacteria. This system, as noted, is dependent upon glucose as source of energy (ATP) and much less dependent upon PMF than at pH 5. ATPase and ETC (electron transporter chain) play an important role in pumping out the protons to maintain the membrane PMF.

- Some studies on docking of efflux pumps and efflux modulator compounds should be done considering the conditions used in the assays described in this thesis to allow a better understanding of the possible interactions;

- Similar work should be done with Gram-positive strains. The results of such kind of assay should also improve the knowledge about the role of the different structures of the Gram-negative and Gram-positive cell envelope on efflux regulation;

- The term EPI, efflux pump inhibitor, is not adequate in the majority of the situations when a compound interfere with the efflux of an agent. Some

compounds seem to modulate or inhibit efflux through other processes than direct inhibition of the pump.

2. Activity of *C. edulis* isolated compounds

- This was the first time that compounds β-amyrin, oleanolic acid, uvaol, MGDG, catechin, epicatechin and procyanidin B5 were isolated from the plant *C. edulis* and from the Aizoaceae family.

- The majority of the compounds isolated from *C. edulis* are active as antimicrobial agent or efflux pump inhibitor. Oleanolic acid presented in this study a high antibacterial activity against a higher number of bacterial strains. Its activity against *E. aerogenes* is of great significance so that it is suggested to be studied, in the future, in more detail how oleanolic acid interferes with the growth of this strain;

- The triterpene uvaol was the most active compound as efflux modulator in bacteria and cancer cells. In the future, its activity on bacterial efflux pumps could be studied under different conditions such as pH, glucose availability and influence on the efflux of EB with the addiction of the compound after a period of EB accumulation, as described in the previous section in order to understand its mechanism of action;

- Triterpenes could be the family of compounds responsible for the activity of *C. edulis* in the reversal of resistance in the studied cancer cell line. However, their activity could be dependent on the substituent of the molecule which facilitates the interaction of the compound with the efflux pump;

- Promising studies could be done in the structure / activity relation of triterpene molecules in order to use them as efflux modulators in cancer chemotherapy and therapy of infections with MDR strains;

- The use of some of this compound as adjuvants in TB therapy is also promising. However, the next step of the work should be the use of MDR *M. tuberculosis* strains as test its modulation of resistance in presence of the compounds, namely oleanolic acid and uvaol. At the time of the writing of this thesis additional work to verify this premises was ongoing.

- *C. edulis* plant also seems to be a promising plant to search of more effective compounds. Its availability and not specially requirements in the growth is an advantage to the use of this plant in extraction of active compounds in sufficient amount.

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