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Reversing antibacterial resistance of *Staphylococcus aureus*: the use of phytochemicals as antibiotic-adjuvants and resistance-modifying agents towards more effective therapies

Dissertation for the degree of Doctor in Chemical and Biological Engineering

Ana Cristina Ralha de Abreu

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Supervisor

Prof. Manuel José Vieira Simões
LEPABE - Laboratory for Process Engineering, Environment, Biological and Energy, Department of Chemical Engineering, Faculty of Engineering of University of Porto (FEUP)

Co-Supervisors

Prof. Young Hae Choi

NPL – Natural Products Laboratory, Institute of Biology of Leiden (IBL), University of Leiden, The Netherlands

Prof. Maria José Félix Saavedra

CECAV – Animal and Veterinary Research Centre Department of Veterinary Sciences University of Trás-os-Montes and Alto Douro

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“Penso nisto, não como quem pensa, mas como quem respira.
E olho para as flores e sorrio...
Não sei se elas me compreendem
Nem se eu as compreendo a elas,
Mas sei que a verdade está nelas e em mim
E na nossa comum divindade
De nos deixarmos ir e viver pela Terra…”

“Mas as coisas não têm nome nem personalidade:
Existem, e o céu é grande e a terra larga,
E o nosso coração do tamanho de um punho fechado...

Bendito seja eu por tudo quanto não sei.
Gozo tudo isso como quem sabe que há o sol.”

Alberto Caeiro, “O guardador de rebanhos”
(Fernando Pessoa)

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THESIS OUTPUTS

Most of the results of this thesis were published/submitted in the following publications:

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Abreu A. C., Simões, M. (2016) Evaluation and classification of antibiotic-potentiating activity promoted by resistance-modifying agents (RMA) against drug-resistant *Staphylococcus aureus. Submitted*.

Abreu A. C., Paulet, D., Coqueiro, A., Malheiro, J., Borges, A., Saavedra, M. J., Choi, Y. H., Simões, M. (2016) Antibiotic adjuvants from *Buxus sempervirens* to promote effective treatment of drug-resistant *Staphylococcus aureus* including biofilms. *RSC Advances;* **6**: 95000-95009.

Abreu A. C., Saavedra, M. J., Simões, L. C., Simões, M. (2016) Combinatorial approaches with selected phytochemicals to increase antibiotic efficacy against *Staphylococcus aureus* biofilms. *Biofouling*; **32**: 1103-1114.

Borges, A., **Abreu, A. C.**, Dias, C. Saavedra, M. J., Borges, F., Simões, M. (2016) New perspectives on the use of phytochemicals as an emergent strategy to control bacterial infections including biofilms. *Molecules*; **21**: Article ID 877.

Abreu, A. C., Serra, S., Borges, A., Salgado, A., Saavedra, M. J., McBain, A. J., Simões, M. (2015) Combinatorial activity of flavonoids with antibiotics against drug-resistant *Staphylococcus aureus*. *Microb Drug Res*; **21**: 600-609.

Abreu, A. C., Serra, S., Borges, A., Salgado, A., Saavedra, M. J., Simões, M. (2014) Evaluation of the best method to assess antibiotic potentiation by phytochemicals against *Staphylococcus aureus*. *Diagn Microbiol Infect Dis*; **9**: 125-134.

Abreu, A. C., Tavares, R. R., Borges, A., Mergulhão, F., Simões, M. (2013) Current and emergent strategies for disinfection of hospital environments. *J Antimicrob Chemother*; **68**: 2718-2732.

Abreu, A. C., McBain, A. J., Simões, M. (2012) Plants as sources of new antimicrobials and resistance-modifying agents. *Nat Prod Rep*; **29**: 1007-1021.

➢ Chapters in books

Abreu, A. C., Borges, A., Malheiro, J., Simões, M. (2013) Resurgence of the interest in plants as sources of medicines and resistance-modifying agents. In: Mendez-Vilas A, ed. *Microbial pathogens and strategies for combating them: science, technology and education.* Formatex Research Center, Microbiology Book Series – 2013 Edition, Badajoz, Spain. Vol. 1, pp. 32-41.

➢ Oral communications in international conferences

Abreu, A. C., Simões, M., Choi, Y. H. (2016) Important interactions between phytochemicals from *Cytisus striatus*: from their role in plant defense system to their application as antibiotic-potentiating compounds against resistant *Staphylococcus aureus*. *IV International Conference on Antimicrobial Research (ICAR).* Malaga, Spain.

Abreu, A. C., Simões, M., Choi, Y. H. (2016) A global perspective about isoflavonoids: from their role in defence of Leguminosae plants to their application in clinical setting as antibiotic potentiators against MRSA. *IV International Conference on Antimicrobial Research (ICAR).* Malaga, Spain.

Abreu, A. C., Simões, M. (2013) Therapeutic effects of alkaloids and flavonoids as antibiotics potentiators against *Staphylococcus aureus*. *International Conference on Natural Products Utilization: From Plants to Pharmacy Shelf*. pp. 79. Conference Abstracts Book. Bansko, Bulgaria.

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Abreu A. C., Simões, M. (2016). Combinatorial approaches with selected phytochemicals to increase antibiotic efficacy against *Staphylococcus aureus* biofilms. *Biofilms7.* Porto, Portugal.

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Abreu, A. C., Simões, M. (2013) Enhancement of antibiotics activity when combined with selected flavonoids against drug resistant *Staphylococcus aureus. 1st Symposium on Medicinal Chemistry of University of Minho.* Braga, Portugal.

Abreu, A. C., Simões, M. (2013) Antimicrobial and synergistic activities of several plant extracts with antibiotics against *Staphylococcus aureus*. *International Conference on Natural Products Utilization: From Plants to Pharmacy Shelf*. pp. 79. Conference Abstracts Book. Bansko, Bulgaria.

Abreu, A. C., Borges, A., Saavedra, M. J., Simões, M. (2012) Phenyl isothiocyanate for biofilm prevention and control. *II International Conference on Antimicrobial Research (ICAR).* Lisbon, Portugal.

Abreu, A. C., Borges, A., Saavedra, M. J., Simões, M. (2012) Synergy effects between antibiotics and alkaloids against *Staphylococcus aureus*. *II International Conference on Antimicrobial Research (ICAR).* Lisbon, Portugal.

ETHICS STATEMENT

The research was carried out mostly at Faculty of Engineering of University of Porto (FEUP) at the Laboratory for Process Engineering, Environment, Biotechnology and Energy (LEPABE, Porto, Portugal), at Institute of Biology of Leiden (IBL, The Netherlands) and at Hospital Erasmus MC-Sofia (Rotterdam, The Netherlands) and it was developed between 2012 and 2016. All the experiments and data analysis presented in this thesis were performed by the PhD student. The contributions of each author for each publication are described in this section.

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> **Authors' contributions:** ACA performed the experiments, analysed the data and wrote the manuscript; AC and ARS helped with a part of the experimental work. MS provided the plant and WJBW provided MRSA isolates of several origins. All the authors participated in writing and giving feedback on the manuscript.

Abreu A. C., Simões, M. (2016) Evaluation and classification of antibiotic-potentiating activity promoted by resistance-modifying agents (RMA) against *Staphylococcus aureus*. *Submitted*.

> **Authors' contributions:** ACA did the experimental work and wrote the manuscript. All the authors participated in writing and giving feedback on the manuscript.

Abreu A. C., Paulet, D., Coqueiro, A., Malheiro, J., Borges, A., Saavedra, M. J., Choi, Y. H., Simões, M. (2016) Antibiotic adjuvants from *Buxus sempervirens* to promote effective treatment of drug-resistant *Staphylococcus aureus* including biofilms. *RSC Advances;* **6**: 95000-95009.

> **Authors' contributions**: ACA performed the experimental work with the plant extracts and wrote the manuscript; DP, ACA and AC did the experimental work focusing on *B. sempervirens* biofilm testing and fraccionation. MS and MJS provided the plants; MJS provided the MRSA isolate from Centro Hospitalar de Trás-os-Montes e Alto Douro (CHTMAD, Vila Real, Portugal). MS and YHC analysed the data. All the authors participated in writing and giving feedback on the manuscript.

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Borges, A., **Abreu, A. C.**, Dias, C. Saavedra, M. J., Borges, F., Simões, M. (2016) New perspectives on the use of phytochemicals as an emergent strategy to control bacterial infections including biofilms. *Molecules*; **21**: Article ID 877.

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> **Authors' contributions**: ACA developed the experimental work and wrote the manuscript. SS and AS supervised the experiments with animal cells. AB helped with the experimental work and the writing. MJS provided the MRSA isolates from CHTMAD (Portugal). All the authors participated in writing and giving feedback on the manuscript.

Abreu, A. C., Serra, S., Borges, A., Salgado, A., Saavedra, M. J., Simões, M. (2014) Evaluation of the best method to assess antibiotic potentiation by phytochemicals against *Staphylococcus aureus*. *Diagn Microbiol Infect Dis*; **9**: 125-134.

> **Authors' contributions**: ACA developed the experimental work and wrote the manuscript. SS and AS supervised the experiments with animal cells. AB helped with the experimental work and the writing. MJS provided the MRSA isolates from CHTMAD (Portugal). All the authors participated in writing and giving feedback on the manuscript.

Abreu, A. C., Tavares, R. R., Borges, A., Mergulhão, F., Simões, M. (2013) Current and emergent strategies for disinfection of hospital environments. *J Antimicrob Chemother*; **68**: 2718-2732.

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Abreu, A. C., McBain, A. J., Simões, M. (2012) Plants as sources of new antimicrobials and resistance-modifying agents. *Nat Prod Rep*; **29**: 1007-1021.

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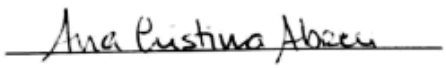
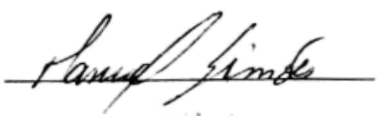


Ana Cristina Abreu

(PhD candidate)



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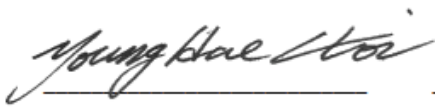

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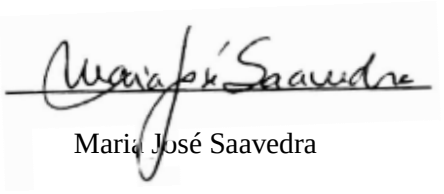

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ABSTRACT

Two major circumstances have accentuated the quest for new antibacterial agents and alternative therapies in the last decades. Primarily, because microbes, due to their incredible adaptability, seem to have at least equal chances for survival as scientists and pharmaceutical industries develop methods to kill them (Berdy, 2012). Multidrug-resistant (MDR) bacteria are responsible for a large number of nosocomial but also community-acquired infections and are spreading all over the globe (González-Lamothe et al., 2009). Additionally, the limitation of our current arsenal of effective antibiotics accompanied by the lack of new antimicrobial alternatives are prompting the beginning of the "post-antibiotic era", which threats all the achievements of modern medicine.

The use of plants as therapies in traditional medicine is as old as mankind. Ethnopharmacologists, botanists, microbiologists, phytochemists and other natural product researchers have also been searching plants hoping to find new, strong and viable antibiotics. However, it can be rapidly established that this effort of finding individual active antibiotics in plants has been pointless, since the spectrum of activity of purified components is often non-specific (thus toxic) or very narrow, and for sure weaker than compounds from other sources such as fungi and bacteria (Tegos et al., 2002). However, plants can still fight most of their infections successfully, which proves that plant defense mechanisms are still not well understood.

Plants do not produce single strong antibacterial compounds as their main defense mechanism, but hundreds of structurally different chemicals with a wide range of activity (González-Lamothe et al., 2009). Some of them are antimicrobial and act synergistically between each other to produce an enhanced effect against the pathogen. Others are non-antimicrobials, but can improve solubility, absorption and stability of the active compounds. At last but not least, some phytochemicals have been associated with an antibiotic adjuvant activity, especially due to the inhibition of the resistance mechanisms from plant pathogens.

The present thesis has the main purpose of finding phytochemicals with such an adjuvant or regulatory activity that could be used in cotherapy with antibiotics to promote the effective treatment of *Staphylococcus aureus*, including multi-drug resistant strains, such as methicillin-resistant *S. aureus* (MRSA). Finding antibiotic coadjuvants capable to inhibit the bacterial resistance mechanisms would be a valuable mid-term solution, until new classes of antibiotics are discovered.

Firstly, four methods detecting antibiotic potentiation were optimized and compared, in terms of efficacy and simplicity. In this task, ten phytochemicals (five alkaloids and five flavonoids)

were combined with five antibiotics (ampicillin, oxacillin, ciprofloxacin, erythromycin and tetracycline) against *S. aureus* strains. The alkaloids reserpine, quinine, pyrrolidine and the flavonoids quercetin and morin at subinhibitory concentrations were found to reduce the minimal inhibitory concentrations (MIC) of ciprofloxacin, erythromycin and tetracycline by several times. A modified and optimized disk diffusion method was highlighted as the simplest and easiest method to perform and detect antibiotic potentiation. However, it is recommended to use this method together with at least other, the microdilution checkerboard, in order to overcome the limitations of each method and obtain conclusions more trustable.

Additionally, since many reports have shown that staphylococcal infections were associated with biofilm formation, which can be much more difficult to eradicate, biofilm control and prevention abilities of reserpine, quinine, pyrrolidine, quercetin and morin were evaluated. The phytochemicals were also studied for their ability to avoid bacterial adaptation to ciprofloxacin, when exposed for long periods to it, and to inhibit NorA efflux pumps [by measuring the accumulation of ethidium bromide (EtBr) in the NorA overexpressing SA1199B strain]. Morin, pyrrolidine and quercetin (0.5 g l^{-1}) had significant effects in biofilm prevention and/or control when applied alone and combined with antibiotics. Synergy was found especially in combinations with ciprofloxacin against biofilms of SA1199B. This strain growing with subinhibitory concentrations of ciprofloxacin for 15 days developed increased tolerance to this antibiotic. However, this was successfully reversed by quinine (0.1 g l^{-1}) and morin (0.5 g l^{-1}). Besides reserpine (0.1 g l^{-1}), quercetin (0.5 g l^{-1}) also showed significant NorA efflux pump inhibition. Cytotoxicity on lung fibroblast cell lines was evaluated as well.

Further, 29 plants from different families were assessed for their antibacterial activity, and also as adjuvants in antibiotic therapy, against *S. aureus* and MRSA strains. The methanolic extracts of *Eucalyptus globulus*, *Castanea sativa*, *Agrimonia eupatoria* and *Fraxinus excelsior* showed antibacterial activity with MIC values of 0.125-0.5, 0.5-1.0, 1.0-2.0, and 2.0-4.0 g l^{-1}, respectively. Non-antibacterial plants were assessed in combination with the five antibiotics by the modified disk diffusion method. Methanolic extracts of *Cytisus striatus*, *Acacia dealbata*, *Prunus* spp. plants, *Centaurea nigra*, *Eupatorium cannabium* and *Buxus sempervirens* showed a potentiating effect mostly of ciprofloxacin, erythromycin and tetracycline. *C. striatus* (0.5 g l^{-1}) and *B. sempervirens* (1 g l^{-1}) were highlighted for their antibiotic-potentiating activities and selected for further studies for the identification and elucidation of the active compounds.

Cytisus striatus leaves methanolic extract showed to selectively potentiate ciprofloxacin and erythromycin by 4 to 8-times against MRSA strains belonging to clonal complex CC8, including the common ST239 found in Asia and USA300 originated in North America. To look for secondary metabolites that could act as antibiotic adjuvants in this plant, a Nuclear Magnetic

Resonance (NMR)-based metabolomics approach was used. Luteolin was found to have antibacterial activity (with MIC between 30 and 120 mg l^{-1}) and three other phytochemicals, genistein, 2'-hydroxygenistein and apigenin, were found to be potentially implicated in antibacterial-potentiation. Daidzein, also found in the plant, showed 4-fold potentiation of ciprofloxacin against *S. aureus* SA1199B. This study reveals the high synergy between metabolites from this plant, showing a great potential for their application in clinical therapy of MRSA infection. Additionally, it was observed that both genistein and daidzein (60 mg l^{-1}) were able to increase accumulation of EtBr in SA1199B cells, showing a NorA efflux pump inhibition. Genistein showed the same activity on MRSA strains. Daidzein showed the best eradication of biofilms when exposed for 1 h [reduction of the number of colony-forming units (CFU) cm^{-2} of 62%] and 24 h (CFU cm^{-2} reduction of 63%) on SA1199B cells.

To understand the mode of action of isoflavonoids and to study their structure-activity relationship (SAR), 22 isoflavonoids were assessed as antibiotic adjuvants. Three isoflavonoids showed antibacterial activity – neobavaisoflavone (MIC = 20 mg l^{-1}), corylifol A (MIC = 0.06 - 20 mg l^{-1}) and orobol (MIC = 60 - 120 mg l^{-1}). Genistein, tectorigenin and biochanin A were highlighted for reducing the MIC of ciprofloxacin and erythromycin by 2-8 times against MRSA. The hydroxyl group in position 5 seems to be correlated with the activity of the three isoflavonoids since the same compounds lacking this group did not show antibiotic-potentiation. The inhibition of topoisomerases or of other processes in the cell such as transport could explain the activity obtained by these compounds. The possible inhibition of Erm methyltransferases by these isoflavonoids, which could explain the potentiation observed with erythromycin on ST239 MRSA strains, was not confirmed. In total, seven isoflavonoids demonstrated potentiation of ciprofloxacin against the NorA overexpresser SA1199B strain: genistein, biochanin A, tectorigenin, daidzein calycosin, irigenin and irisflorentin. A NorA efflux pump inhibitory activity of the isoflavonoids was tested by EtBr accumulation in the presence of these compounds. The results support that NorA inhibition is a possible mechanism for this coadjuvant activity by the seven isoflavonoids. Additionally, the effect of the isoflavonoids was assessed on biofilms and, besides daidzein, irisflorentin was also highlighted for promoting 83% of CFU cm^{-2} reduction within 1 h of exposure, and 54% within 24 h of exposure.

Buxus sempervirens methanolic extract was also studied for its high ability to increase the efficacy of several antibiotics, even for β-lactams. This extract was applied against biofilms of *S. aureus* strains, being able to cause 88% reduction of 24 h-old biofilms of *S. aureus* CECT 976 within 1 h of exposure. Further phytochemical investigation of *B. sempervirens* active fraction allowed to identify betulinic acid as a major component together with oleanane and ursane type terpenoids. Studies with this and other five similar terpenoids allowed to highlight oleanolic acid and ursolic acid, with MICs of 62.5 and 15.6 mg l^{-1}, respectively, against *S. aureus* CECT 976

and synergy when combined with tetracycline and/or erythromycin by checkerboard technique. Additionally, oleanolic acid at ½ MIC, MIC and 2 × MIC promoted biofilm reductions of 70, 81 and 85%, respectively.

In conclusion, this study allowed to assess the potential of 29 plant extracts from different species and 41 commercial phytochemicals to be used in co-therapies against *S. aureus* and MRSA and decrease bacterial resistance to antibiotics. The concept of restoring and enhancing the therapeutic value of antimicrobials is a formidable challenge. This thesis greatly contributed to highlight the richness and superiority of plants as therapeutic sources of valuable compounds. Understanding their complex defense systems can provide new insights that could be used in our own fight against human pathogens.

Keywords: multidrug resistance, *Staphylococcus aureus*, MRSA, biofilm, phytochemicals, metabolomics, resistance-modifying agents, antibiotic potentiation

RESUMO

Nas últimas décadas, dois fatores principais têm despoletado a procura por novos agentes antibacterianos e terapias clínicas alternativas. Primeiro, o facto dos microrganismos, devido à sua capacidade incrível de adaptação, terem tantas hipóteses de sobreviver como têm os cientistas de os matar (Berdy, 2012). As bactérias multirresistentes são responsáveis por uma enorme quantidade de doenças a nível hospitalar e da comunidade, e estão a disseminar-se pelo mundo todo. Adicionalmente, a limitação do nosso arsenal de antibióticos eficazes, acompanhada pela falta de novas alternativas terapêuticas, desencadearam o início da chamada "era pós-antibióticos", que revela ser uma ameaça a todas as conquistas e descobertas trazidas pela era moderna da medicina.

As plantas são usadas como terapia na medicina tradicional desde os princípios da humanidade. Inúmeros etnofarmacólogos, botânicos, microbiologistas, fitoquímicos e outros investigadores na área dos produtos naturais têm procurado incessantemente por novas plantas e compostos, com a esperança de encontrar antibióticos novos, viáveis e eficazes. No entanto, todo este esforço para encontrar antibióticos ativos em plantas tem-se mostrado ineficaz, uma vez que a maioria dos compostos isolados de plantas apresenta espetros de atividade não-específicos (sendo, portanto, tóxicos) e é, no geral, menos eficaz e potente comparando com compostos de outras proveniências, como por exemplo de bactérias e fungos. As plantas sobrevivem, contudo, à maioria das infeções, o que reforça a ideia de que os seus mecanismos de defesa não são, de todo, compreendidos.

É importante realçar que as plantas não produzem antibióticos individuais e potentes como mecanismo de defesa principal, produzindo ao revés centenas de compostos estruturalmente diferentes que apresentam uma gama de atividade ilimitada. Alguns compostos têm mostrado atividades antimicrobianas e parecem atuar de forma sinérgica entre eles de forma a produzir um efeito global mais intenso e eficaz. Outros compostos não-antimicrobianos aparentam ter uma função mais reguladora, promovendo o aumento da solubilidade, absorção e estabilidade química dos compostos mais ativos. Por último, outros compostos têm sido associados a uma atividade antibacteriana adjuvante, principalmente por serem capazes de inibir ativamente alguns mecanismos de resistência por parte de organismos patogénicos.

A presente dissertação teve como principal objetivo encontrar fitoquímicos que apresentem uma atividade adjuvante na defesa antibacteriana ou uma atividade reguladora/auxiliar, que possam ser usados em coterapia com antibióticos comerciais de forma a promover o tratamento eficaz de *Staphylococcus aureus*, inclusive de estirpes multirresistentes, como é o caso de *S. aureus* resistente à meticilina (MRSA). Estes compostos adjuvantes capazes de inibir os

mecanismos de resistência das bactérias podem ser considerados como uma solução intermediária, até que novas classes de antibióticos sejam descobertas.

Inicialmente, pretendeu-se otimizar quatro métodos distintos para a deteção de atividades potenciadoras dos antibióticos, e compará-los em termos de eficácia e simplicidade. Para este efeito, 10 fitoquímicos foram combinados com cinco antibióticos (ampicilina, oxacilina, ciprofloxacina, eritromicina e tetraciclina) para o tratamento de *S. aureus*. Três alcalóides (reserpina, quinina e pirrolidina) e dois flavonóides (morina e quercetina) destacaram-se dos restantes fitoquímicos e reduziram a concentração mínima inibitória (MIC) da ciprofloxacina, eritromicina e tetraciclina. O método de difusão em disco, como se encontra otimizado e descrito nesta dissertação, foi considerado o mais fácil e simples para detetar a potenciação de antibióticos promovido por extratos de plantas. No entanto, o uso conjunto deste método com pelo menos outro, particularmente o do *checkerboard*, permite ultrapassar as limitações de cada método e obter conclusões mais fiáveis.

Adicionalmente, uma vez que a maioria das infeções desenvolvidas por *Staphylococcus aureus* encontra-se associada à formação de biofilmes, as melhores combinações entre fitoquímicos e antibióticos foram testadas em biofilmes de *S. aureus*, que são à partida mais resistentes ao tratamento que células suspensas. Averiguou-se de igual forma se os fitoquímicos seriam eficazes em prevenir o desenvolvimento/aumento de tolerância das estirpes bacterianas à ciprofloxacina e em inibir bombas de efluxo dos antibióticos (medindo para este efeito a acumulação de brometo de etídio na estirpe SA1199B, que sobre-expressa a bomba de efluxo NorA). A morina, pirrolidina e quercetina (0.5 g l^{-1}), aplicadas em concentrações sub-inibitórias, mostraram ter um efeito positivo na prevenção da formação de biofilmes e na remoção ou inativação de biofilmes previamente formados, quer quando aplicadas individualmente, quer em combinação com os antibióticos. A combinação de alguns fitoquímicos com a ciprofloxacina foi considerada sinérgica na remoção ou controlo dos biofilmes produzidos pela estirpe de *S. aureus* SA1199B. Esta estirpe desenvolveu uma tolerância acrescida a este antibiótico ao ser incubada em concentrações sub-inibitórias do mesmo por 15 dias. No entanto, este processo foi revertido em parte na presença da quinina (0.1 g l^{-1}) e morina (0.5 g l^{-1}). Adicionalmente, a reserpina (0.1 g l^{-1}) e a quercetina (0.5 g l^{-1}) demonstraram serem capazes de inibir a bomba de efluxo NorA. A citotoxicidade destes fitoquímicos foi também avaliada em linhas celulares de fibroblastos.

Após esta etapa inicial, 29 plantas de diversas famílias foram selecionadas e testadas quanto à sua atividade antibacteriana e como adjuvantes de terapia antibiótica no tratamento de *S. aureus* e MRSA. Os extratos metanólicos das folhas de *Eucalyptus globulus, Castanea sativa, Agrimonia eupatoria* e *Fraxinus excelsior* apresentaram atividade antibacteriana, com MICs de 0.125-0.5, 0.5-1.0, 1.0-2.0, e 2.0-4.0 g l^{-1}, respetivamente. Adicionalmente, os extratos metanólicos das

folhas de *Cytisus striatus*, *Acacia dealbata*, três plantas de espécie *Prunus*, *Centaurea nigra*, *Eupatorium cannabium* e *Buxus sempervirens* demonstraram uma atividade de potenciação quando combinadas com os antibióticos, especialmente com a ciprofloxacina, eritromicina e tetraciclina. Destas plantas, duas destacaram-se pelas suas atividades potenciadoras, *C. striatus* (0.5 g l^{-1}) e *B. sempervirens* (1 g l^{-1}), e foram selecionadas para serem estudadas em mais pormenor, para a identificação e elucidação dos compostos ativos presentes nas mesmas.

O extrato metanólico das folhas de *C. striatus* potenciou 4 a 8 vezes a atividade da ciprofloxacina e eritromicina no tratamento de estirpes MRSA pertencentes ao complexo clonal CC8 (ST239 e USA300, provenientes da Ásia e América do Norte, respetivamente). De forma a identificar os metabolitos secundários da planta responsáveis por esta atividade, foi realizado um estudo metabolómico acoplado à técnica de Ressonância Magnética Nuclear (NMR). A luteolina foi isolada desta planta e apresenta atividade antibacteriana, com MICs compreendidas entre 30 e 120 mg l^{-1}, e outros três compostos, genisteina, 2'-hidroxigenisteina e apigenina, foram associados a uma atividade potenciadora antibacteriana. Outro isoflavonóide também isolado desta planta, a daidzeina, foi eficaz em potenciar a ciprofloxacina no tratamento de *S. aureus* SA1199B. Este estudo revelou e identificou com sucesso relações de sinergismo entre vários metabolitos desta planta, mostrando o potencial para aplicação deste tipo de sistemas em infeções provocadas por MRSA. Adicionalmente, observou-se que quer a genisteina como a daidzeina (60 mg l^{-1}) aumentaram a acumulação de brometo de etídio em células SA1199B, podendo estar desta forma a causar inibição das bombas de efluxo NorA. A genisteina apresentou a mesma atividade nas estirpes MRSA. A daidzeina exibiu a melhor erradicação do biofilme formado por SA1199B, quando exposta por 1 hora (remoção de biofilme de 62%) e por 24 horas (remoção de biofilme de 63%).

De forma a entender o modo de ação dos isoflavonóides e estudar a relação atividade-estrutura (SAR) dos mesmos, 22 isoflavonóides foram testados como potenciadores de antibióticos. Três isoflavonóides demonstraram possuir atividade antibacteriana: neobavaisoflavona (MIC = 20 mg l^{-1}), corilifol A (MIC = 0.06 - 20 mg l^{-1}) e orobol (MIC = 60 - 120 mg l^{-1}). A genisteina, tectorigenina e biochanina A potenciaram 2-8 vezes a atividade da ciprofloxacina e eritromicina no tratamento de MRSA. O grupo hidróxilo na posição 5 da estrutura dos isoflavonóides parece estar relacionado com esta atividade, uma vez que isoflavonóides idênticos, mas sem o referido grupo hidróxilo, não demonstraram atividade potenciadora. A inibição de topoisomerases e outros processos celulares, como o transporte, poderia explicar a potenciação de ambos os antibióticos observada. Adicionalmente, a inibição de Erm metiltransferases pelos isoflavonóides foi também testada, o que poderia explicar a potenciação da eritromicina em estirpes ST239. No entanto, o resultado foi negativo. Para além dos isoflavonóides previamente referidos, calicosina, irigenina e irisflorentina potenciaram de igual forma a atividade da ciprofloxacina quando aplicados à

estirpe SA1199B. Através da medição da acumulação de brometo de etídio nestas células, foi possível confirmar que estes isoflavonóides inibem esta bomba de efluxo NorA, explicando deste modo a atividade potenciadora da ciprofloxacina. Paralelamente, a eficácia destes isoflavonóides no controlo de biofilmes foi testada, sendo de destacar a irisflorentina, para além da daidzeina, que apresenta percentagens de remoção do número de CFU cm^{-2} de 83% após 1 hora de exposição, e de 54% após 24 horas de exposição.

O extrato metanólico de *Buxus sempervirens* foi igualmente investigado por ter promovido a atividade de vários antibióticos, incluindo β-lactâmicos. Desta forma, este extrato foi testado em biofilmes de *S. aureus*, promovendo uma redução de 88% de biofilmes da estirpe CECT 976 quando exposto por 1 hora. Um composto maioritário, o ácido betunílico, foi idendificado na fração ativa do extrato de *B. sempervirens*, assim como terpenóides do tipo *oleanane* e *ursane*. O ácido betulínico e outros cinco terpenóides estruturalmente semelhantes foram testados, realçando-se o ácido oleanólico e o ácido ursólico por apresentarem atividade antibacteriana (MIC de 62.5 e 15.6 mg l^{-1}, respetivamente, em *S. aureus* CECT 976) e sinergia quando combinados com a tetraciclina e/ou eritromicina pelo método *checkerboard*. Adicionalmente, o ácido oleanólico promoveu reduções de biofilme de 70, 81 e 85% quando aplicado a ½ MIC, MIC e 2 × MIC, respetivamente.

Como conclusão principal, poderá dizer-se que este estudo permitiu avaliar o potencial de 29 extratos de plantas de espécies diferentes e 41 fitoquímicos comerciais para serem aplicados em coterapia com os antibióticos, de forma a promover um tratamento mais eficaz de infeções de *S. aureus* e MRSA e diminuir a resistência bacteriana aos antibióticos. É de realçar que o conceito de restaurar o valor terapêutico dos antibióticos apresenta um potencial enorme. Esta dissertação contribui desta forma para engrandecer o potencial das plantas como fonte de grande diversidade de compostos medicinais e terapêuticos. O estudo destes sistemas de defesa complexos das plantas pode ser uma mais-valia na nossa própria luta contra agentes patogénicos humanos.

Palavras-chave: resistência a múltiplas drogas, *Staphylococcus aureus*, MRSA, biofilme, fitoquímicos, metabolómica, agentes modificadores de resistência, potenciação de antibióticos

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LIST OF ABBREVIATIONS

ABC	ATP binding cassette
AHL	*N*-Acyl homoserine lactones
AIs	Autoinducers
ATCC	American Type Culture Collection
BUOH	Butanol
CA-MRSA	Community-acquired methicillin-resistant *Staphylococcus aureus*
CC	Clonal Complex
CECT	Spanish Type Culture Collection
CFU	Colony-forming units
CHTMAD	Centro Hospitalar de Trás-os-Montes e Alto Douro
COSY	Correlation spectroscopy
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
EMRSA	Epidemic methicillin-resistant *Staphylococcus aureus*
EN	European Standard
EPI	Efflux pump inhibitor
EPS	Extracellular polymeric substances
ESβL	Extended-spectrum β-lactamases
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
EtBr	Ethidium bromide
EtOAc	Ethyl acetate
EUCAST	European Committee for Antimicrobial Susceptibility Testing
FBS	Foetal bovine serum
FDA	US Food and Drug Administration
FID	Free Induction Decay
GAIN	Generating Antibiotics Incentives Now

GC-MS	Gas Chromatography–Mass Spectrometry
GRAS	Generally regarded as safe
HAI	Hospital-acquired infections
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum coherence
IC_{50}	Half maximal inhibitory concentration
ISO	International Organization for Standardization
LC-MS	Liquid Chromatography–Mass Spectrometry
LPS	Lipopolysaccharide
MATE	Multiple antibiotic and toxin extrusion
MDR	Multidrug resistance
MeOH	Methanol
MFI	Maximal fluorescence intensity
MFS	Major facilitator superfamily
MH	Mueller–Hinton
MIC	Minimum inhibitory concentration
MLSB	Macrolide-lincosamide-streptogramin type B
MPLC	Medium pressure liquid chromatography
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyl-methoxy-phenyl)-2(4-sulfophenyl)-2H tetrazolium)
MVDA	Multivariate Data Analysis
NMR	Nuclear Magnetic Resonance
OD	Optical density
OM	Outer membrane
OMP	Outer membrane protein
OPLS	Orthogonal projection to latent structures
P	Statistical significance level

PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PC	Principal components
PCA	Principal component analysis
PFAF	Plants For A Future
PLS	Partial least-squares
PQS	*Pseudomonas* quinolone signal
PRSA	Penicillin-resistant *Staphylococcus aureus*
PRSP	Penicillin-resistant *Streptococcus pneumonia*
PVL	Panton-Valentin Leukocidin
qRT-PCR	Quantitative real-time reverse transcription polymerase chain reaction
QS	Quorum-sensing
R&D	Research and Development
RF	Relative Fluorescence
RMA	Resistance-modifying agent
RND	Resistance-nodulation division
RP-HPLC	Semi-preparative reverse-phase high performance liquid chromatography
SAR	Structure-Activity Relationship
SCC	Staphylococcal chromosome cassette
SMR	Small Multidrug Resistance
SPSS	Statistical Package for the Social Sciences
ST	Sequence Type
VRE	Vancomycin-resistant enterococci
VRSA	Vancomycin-resistant *Staphylococcus aureus*
VISA	Vancomycin-intermediate *Staphylococcus aureus*
TEM	Transmission Electron Microscopy
TLC	Thin Layer Chromatography
WHO	World Health Organization

Chapter 1

WORK OUTLINE

This chapter aims to describe the context and background of this thesis, the main purposes and the motivations to develop this work. Furthermore, it serves as a guideline to the overall work, describing the main elements constituting this thesis and their organization along the five main chapters.

1.1. BACKGROUND AND PROJECT PRESENTATION

The development of antibiotic-resistant microbes is the consequence of decades of constant selective pressure from human applications of antibiotics, *via* underuse, overuse, and misuse. The worldwide spread of MDR bacteria is increasingly drawing the attention of global surveillance authorities and media, being undoubtedly rated as a major threat in the 21st century. Each year over 13 million deaths in the world are attributed to the emergence of new infectious diseases or to the re-emergence of old pathogens with new resistance determinants (Kourtesi et al., 2013). This situation affects everyone, independent of age, gender or country in which they live, and threatens to undermine all recent notable achievements of modern medicine. The harsh prediction for a close future is that the treatment of certain types of severe bacterial infections will experiment a therapeutic dead end. There is a rising clamor for new medicines to combat infectious diseases and lethal pathogens resistant to existing treatments, but few promising therapies are in the biopharmaceutical R&D pipeline. The situation raises fear of a "post-antibiotic" age of super-resistant superbugs, a prediction each day made more real.

Natural products, mainly, though not exclusively of microbial origin, have become one of the most important sources of lead antimicrobials for the pharmaceutical industry (Clardy et al., 2006; Piddock et al., 2010). Since the beginning of mankind, plants were undoubtedly the most important source of therapeutic remedies with an enormous range of applications. The earliest records of natural products were depicted from Mesopotamia (2600 B.C.) and included oils from cypress (*Cupressus sempervirens)* and myrrh (*Commiphora* species), which are still used today to treat coughs, colds and inflammation (Dias et al., 2012). Plants have been the source of numerous drugs of clinical application as, for example, glucoside inhibitors of sodium/potassium ATPase, which are used to treat cardiac arrhythmias and certain kinds of heart failure, and the antimalarial drugs quinine and artemisinin (Lewis, 2013). Plants have been also studied with the expectation of finding novel leads for antibiotics. However, though resistant to many microorganisms, plants have not yielded promising antimicrobial leads so far. A likely explanation for this is that plants use a combination of strategies to deal with infections rather than individual metabolites.

Plants produce an array of structurally diverse secondary metabolites that seem to be involved in defense mechanisms against a wide range of microorganisms including fungi, yeasts and bacteria (Gibbons et al., 2004; Siebra et al., 2016). However, when isolated, these antimicrobial metabolites exhibit only weak or moderate activities. In fact, their MICs are typically in the range of 100-1000 mg l^{-1}, that is, several orders of magnitude weaker than those of typical antibiotics produced by bacteria and fungi (MICs of 0.01-10 mg l^{-1}) (Tegos et al., 2002), and accounts for the absence of plant-derived antimicrobials in clinical applications (Cowan, 1999). It is thus clear

that if plants have a highly efficient defensive system to deal with microbial threats. This capability makes it of interest for further studies, as there might be lesson to learn that can be lead to treat human infectious diseases. Millions of years of evolution have resulted in plant defense systems that are not easily violated by the development of microbial resistance. Plants have different levels of defense against microorganisms: constitutive chemical defense, direct inducible chemical defense (phytoanticipins) and gene-level inducible chemical defense (phytoalexins). These most likely complement each other. The defense strategy involves not only various secondary metabolites, but also other very diverse molecules ranging from proteins to H_2O_2 and oxygen radicals. This could explain why the classical approach consisting in bioassay guided fractionation to find antibiotics from plants has not yet resulted in any lead for antibiotics as this approach fails to reveal interactions between compounds, such as synergism, and the isolated single compounds might not be very active in *in vitro* tests (Tegos et al., 2002). Synergistic interactions between various metabolites could enhance the activity of weakly antimicrobial agents that are not in contact either due to location or in time in normal circumstances, but are released or produced when needed in the site of an infection. The defense strategies may thus involve the synergistic activity of two or more compounds, which could act via different mechanisms and/or targets (Lila and Raskin, 2005). A great number of plant metabolites have been reported to inhibit several MDR efflux pumps for Gram-positive bacteria (Cushnie and Lamb, 2005; Gibbons et al., 2004; Gibbons et al., 2003a; Jin et al., 2011; Kang et al., 2011; Marquez, 2005; Miller et al., 2001; Stavri et al., 2007), implicated in bacterial resistance to several antibiotic classes, or to inhibit protein-binding proteins (PBP) 2a (Shimizu et al., 2001; Shiota et al., 2004), among others. Synergy could also be due to physico-chemical interactions such as the opening of membranes or an increase in the solubility of the active antimicrobial compounds.

The fact that the only 50 years of use of antibiotics has already led to wide spread resistance, shows that more sophisticated systems are required to effectively treat infectious diseases. Improving the clinical performance of various antibiotics by employing active molecules capable of restoring antibiotic susceptibility in MDR pathogens represents a novel and promising approach to deal with multidrug resistance. These molecules have been termed resistance-modifying agents (RMAs). The design of such combinations is a promising alternative taking into account the scarcity of new antibacterials available and active. The effort to discover plant EPIs has been delayed by the fact that, until now, this process consisted mostly on bioassay-guided isolations, which can be time-consuming and laborious (Fiamegos et al., 2011). However, new perspectives and methodologies such multivariate data analysis coupled to metabolomics studies have been promising and open new and exciting perspectives in the field.

1.2. MAIN OBJECTIVES

Plants offer an untapped source of such adjuvant compounds, which represents an increasingly active research topic for the design of modified or combination therapeutics. The goal of this thesis was to test whether it was possible to take advantage of the plant's powerful defense system, presumably based on complex synergistic interactions, to potentiate the activity of antimicrobial activity of known antibiotics. The purpose is to reverse drug resistance on *Staphylococcus aureus*, which is an important cause of serious, invasive and life threatening infections worldwide and remains a leading cause of community and nosocomial bacteraemia (Lawes et al., 2012).

Firstly, it was aimed to evaluate, compare and optimize the best method to detect antibiotic potentiation against *S. aureus* strains, including efflux pump overexpressing and methicillin-resistant *S. aureus* (MRSA) strains. Thus, four methods (checkerboard, etest, disk diffusion method, time-kill assay) were assessed to detect potentiation of antibiotics promoted by phytochemicals. The ten phytochemicals chosen for this part of the study belonged to two classes, alkaloids and flavonoids. One of the tested compounds is reserpine, that is recognized as an EPI. The antibiotics chosen (ampicillin and oxacillin - β-lactam, ciprofloxacin – fluoroquinolone, erythromycin – macrolide, and tetracycline) have more limited application nowadays due to increased bacterial tolerance.

Since several of the tested phytochemicals were effective antibiotic potentiators and considering that staphylococcal infections are mostly associated with biofilm formation, combinations of selected phytochemicals with antibiotics were tested on the prevention and control of *S. aureus* biofilms. The phytochemicals were also studied for their ability to avoid antibiotic adaptation within long exposure times and to inhibit antibiotic efflux pumps. Cytotoxicity of lung fibroblast cell lines was evaluated as well.

After the selection and optimization of the methods to detect antibiotic potentiation, the ability of a considerable range of plants belonging to different families was screened for their antibacterial activity and antibiotic-potentiating ability with the five antibiotics. From this initial screening with 29 plant methanolic extracts, two plants were highlighted and selected for further phytochemical elucidation, for showing interesting antibiotic-potentiating activities: *Cytisus striatus* and *Buxus sempervirens*.

Cytisus striatus, commonly known as Portuguese broom, is particularly abundant in the Iberian Peninsula. As mentioned before, the classical bioassay guided fractionation is rarely efficient when synergistic interactions are responsible for a given bioactivity. While antimicrobial compounds may be identified, detecting synergistic interactions would imply testing an almost infinite number of combinations. A metabolomics approach seemed to be promising. The starting

point was evaluating the antibiotic-potentiating activity of different types of extracts of this plant and then submit them to NMR-metabolomics to identify the signals related to that activity. The next step was to isolate and identify the compounds responsible for these signals and to test them for potentiating antimicrobial activity. In particular, MRSA strains belonging to CC8, including ST239 and USA300 strains, were used.

In view of the promising results obtained with isoflavonoids as antibiotic potentiators, twenty-two isoflavonoids were evaluated for their antibacterial and synergistic effects with ciprofloxacin and erythromycin against *S. aureus* strains. The most studied phytoalexins from Fabaceae plants are isoflavonoids (Jeandet et al., 2013). The aim was to understand the structure-activity relationship of isoflavonoids as antibiotic adjuvants. The effect of the isoflavonoids was also evaluated on the accumulation of EtBr in *S. aureus* using both fluorometric and flow cytometry methods, looking for MDR efflux pumps inhibition. Likewise, the hypothesis of inhibition of Erm methyltransferases by the isoflavonoids was assessed. Additionally, the effect of the isoflavonoids on the eradication of *S. aureus* biofilms was evaluated, either when applied alone or in combination with antibiotics.

Buxus sempervirens was also selected for its potentiating activity and applied against *S. aureus* biofilms. It was intended to proceed with phytochemical investigation of *B. sempervirens* and to identify the main metabolites responsible for the activity. The triterpenoid found in this plant was assessed, along with other five similar triterpenoids, regarding its antibacterial, antibiotic-potentiation and biofilm eradication activities (within 1 and 24 h of exposure).

1.3. THESIS ORGANIZATION

This thesis is organized in five chapters comprised by: work outline (Chapter 1), literature review (Chapter 2), materials and methods (Chapter 3), results and discussion (Chapter 4) and concluding remarks (Chapter 5). A brief introduction to each chapter is made in FIGURE 1.1.

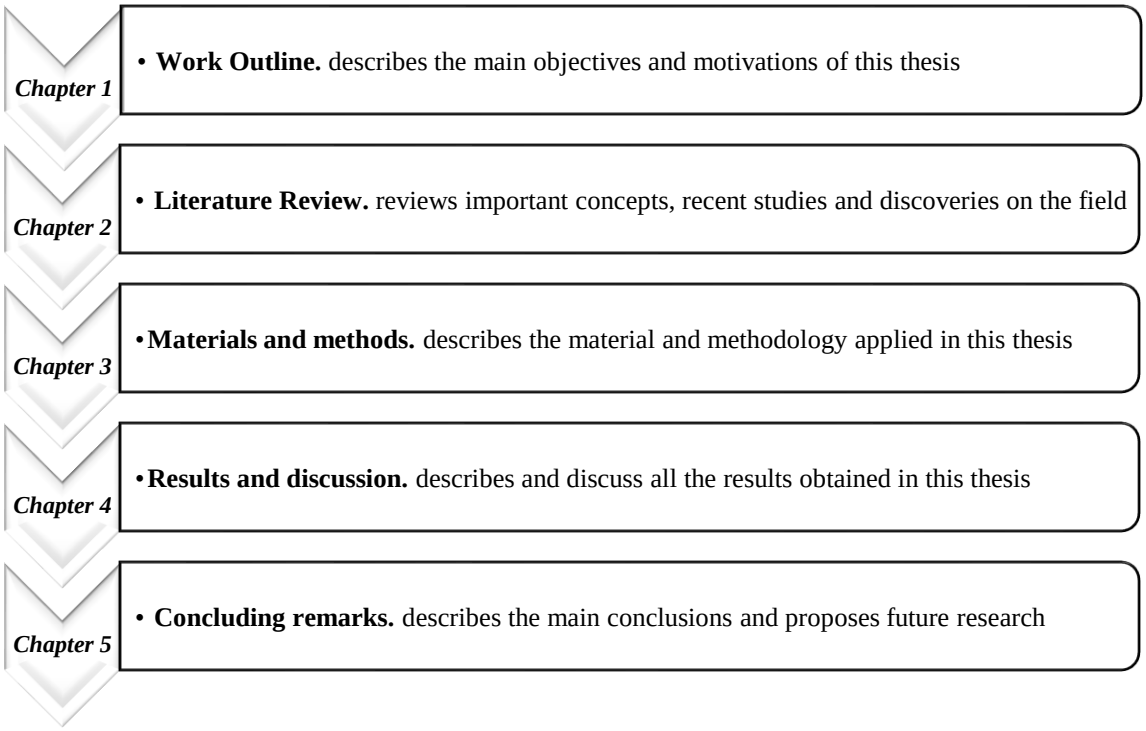


FIGURE 1.1 | Scheme of the organization of this thesis according to each chapter.

Chapter 1 describes the main objectives, context and motivations for the development of this work and serves as a guideline to the overall work presented in the further chapters.

In *Chapter 2* a review of the literature is provided. Plant secondary metabolites (phytochemicals) have already demonstrated their potential as antibacterials when used alone and as synergists or potentiators of other antibacterial agents. Phytochemicals frequently act through different mechanisms than conventional antibiotics and could, therefore be of use in the treatment of resistant bacteria. The therapeutic utility of these products, however, remains to be clinically proven. The aim of this chapter is to review the advances in *in vitro* and *in vivo* studies on the potential chemotherapeutic value of phytochemical products and plant extracts as RMAs to restore the efficacy of antibiotics against resistant pathogenic bacteria. The mode of action of RMAs on the potentiation of antibiotics is also described. Important chemical and biological concepts and explanations are given.

In *Chapter 3*, the materials and methods used to perform all the experimental work are fully described. The material of biological and chemical sources tested in this thesis is characterized and some important technical or mechanistic details are described. A detailed chronological description of the project is given by tasks according to FIGURE 1.2. In *Chapter 4*, the results are showed, analysed and discussed. The organization of this chapter is done with the same logic sequence comprised by these 6 tasks. Important remarks and conclusions after each step of this work are also performed.

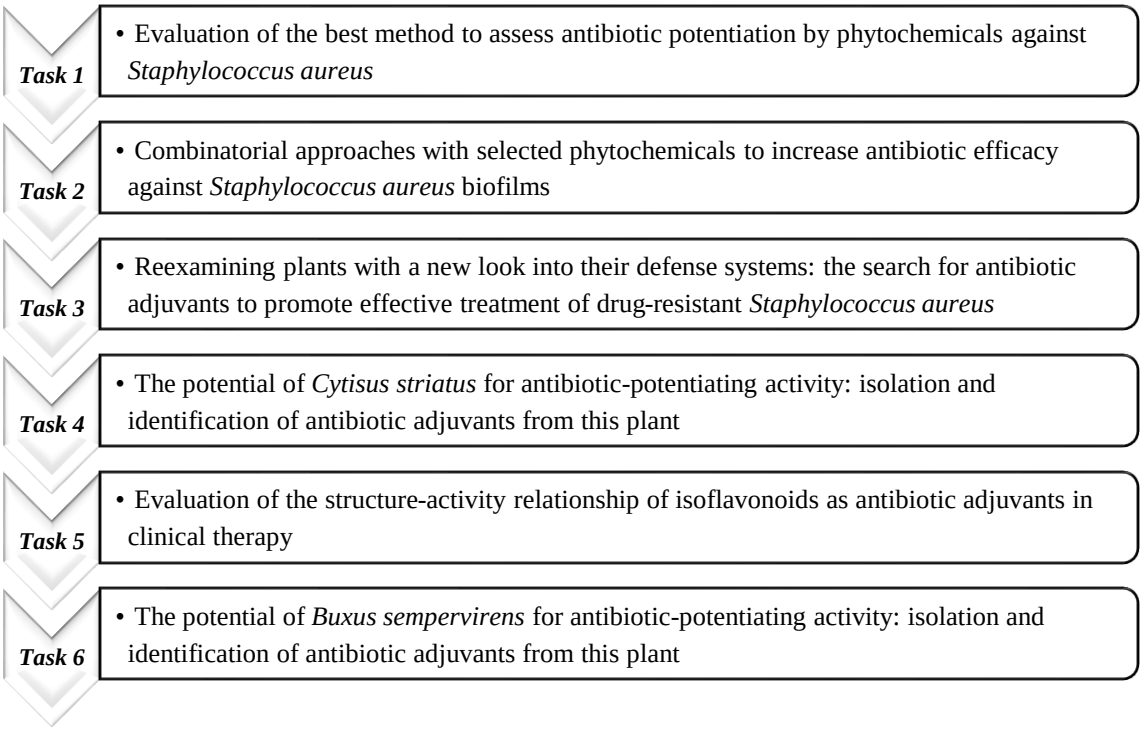


FIGURE 1.2 | Sequence of the six main experimental tasks developed in this project.

Briefly, in *Task 1*, four methods to measure antibiotic potentiation are optimized using combinations between ten phytochemicals and five antibiotics against ten *S. aureus* strains, including 3 MRSA strains. Comparison between methods is performed and the best method is selected. In *Task 2,* the best phytochemical-antibiotic combinations previously obtained were then applied against biofilms and other mechanistic details concerning the mode of action of the phytochemicals are revealed. In *Task 3*, 29 plant methanolic extracts were screened for antibacterial and antibiotic potentiation activities against *S. aureus* strains. *Cytisus striatus* was selected for further phytochemical characterization and elucidation using a NMR-based metabolomics study (*Task 4*). Isolated compounds form this plant were characterized for their biological activities. Isoflavonoids proved to be important compounds of this plant; thus, 22

different isoflavonoids were also evaluated for antibacterial and antibiotic-potentiation activities and as efflux pump inhibitors; a structure-activity relationship (SAR) study was performed, and finally their activity against biofilms was assessed (*Task 5*). In *Task 6, Buxus sempervirens* was also selected among the 29 plants for further investigation and tested for biofilm control. Further fractionation of this plant methanolic extract allowed to identify one major triterpenoid as bioactive compound. The antibacterial and antibiotic-potentiating activities of this and other five similar triterpenoids was assessed. The most promising triterpenoid was selected for further studies against biofilms.

Finally, *Chapter 5* presents an overview of all the developed work, with emphasis on the main conclusions obtained within this project. Perspectives and suggestions for further research are also identified and described.

Chapter 2

LITERATURE REVIEW

This chapter provides a review of important aspects that constitute the background of this thesis. First, the current concern about the emergency of MDR pathogens accompanied by the lack of effective antibacterial options is analysed, and so are the possible strategies to address this problem. Further, an insight to the rich variety of interesting secondary metabolites from plants and on their biological role against microorganisms is performed. Several studies have provided clear evidence that plant-derived products can be used to improve the therapeutic efficacy of antibiotics. A special emphasis will be performed on non-antimicrobial compounds, which are able to act synergistically with antimicrobials in order to promote effective defense. The biotechnological values of these antibiotic adjuvants as potential candidates in cotherapies with antibiotics for the fight against MDR bacteria is highlighted. Several mechanisms have been proposed to explain the mode of action of these compounds and are discussed in this section. This chapter therefore reviews data on the combinatorial interaction of plant-derived products as RMAs with antibiotics for the treatment of infectious diseases. Additionally, *in vivo* and toxicity data, when available, are mentioned.

This chapter contains information that was published in:

- **Abreu, A. C.**, McBain, A. J., Simões, M. (2012) Plants as sources of new antimicrobials and resistance-modifying agents. *Nat Prod Rep*; **29**: 1007-1021.
- **Abreu, A. C.**, Tavares, R. R., Borges, A., Mergulhão, F., Simões, M. (2013) Current and emergent strategies for disinfection of hospital environments. *J Antimicrob Chemother*; **68**: 2718-2732.
- **Abreu, A. C.**, Borges, A., Malheiro, J., Simões, M. (2013) Resurgence of the interest in plants as sources of medicines and resistance-modifying agents. In: Mendez-Vilas A, ed. Microbial pathogens and strategies for combating them: science, technology and education. Formatex Research Center, Microbiology Book Series – 2013, Badajoz, Spain. Vol. 1, pp. 32-41.
- Borges A., **Abreu, A. C**., Dias, C. Borges, F. Saavedra, M. J., Simões, M. (2016) New perspectives on the use of phytochemicals as an emergent strategy to control bacterial infections including biofilms. *Molecules*; **21**: Article ID 877.

2.1. THE BEGINNING OF THE "POST-ANTIBIOTIC ERA"

Antibiotics have proven to be powerful drugs for the control of infectious diseases and remain one of the most significant discoveries in modern medicine. Their extensive and unrestricted use has, however, imposed a selective pressure upon bacteria, leading to the development of antimicrobial resistance (Čižman, 2003; Oluwatuyi et al., 2004; Russell, 2003; Schelz et al., 2010; Stewart, 2002). The capacity of bacteria to acquire and transmit genetic determinants of resistance is a conserved evolution strategy and has exacerbated the worldwide resistance problem. Antibiotic resistance is recognized by the World Health Organization (WHO) as the greatest threat in the treatment of infectious diseases (Conly, 2004; Nascimento et al., 2000; Oluwatuyi et al., 2004; Walker and Levy, 2001; Wright, 2005).

In order to control the occurrence and spread of resistant strains, the WHO has promoted a complex action plan, based on the slogan *"No action today, no cure tomorrow"* that includes strategic actions for mitigation, prevention and control (WHO, 2011). The plan is based on the accomplishment of several objectives: the prudent use of antibacterial drugs (with the correct drug at the right dosage and for the appropriate duration) across all relevant sectors; enhanced infection control and environmental hygienic practices to reduce the transmission of resistant strains and the strengthening of surveillance systems to monitor antibiotic use and resistant bacteria in human and animal health, including the food chain; and the encouragement to the discovery of new active agents (Conly, 2004; Dötsch et al., 2009; Pandra et al., 2010; WHO, 2011). However, this campaign was not enough to prevent the beginning of the "post-antibiotic era", as it was stated by WHO in the beginning of 2014. Among several studies, a recent study in the United States estimated an annual inappropriate antibiotic prescription up to 30%, supporting the need for establishing a goal for outpatient antibiotic stewardship (Fleming-Dutra et al., 2016). Additionally, 44% of outpatient antibiotic prescriptions are written to treat patients with acute respiratory conditions, where half of these prescriptions are unnecessary (The Pew Charitable Trusts, 2016).

The quest for new antimicrobials to overcome resistance problems has long been a top research priority for the pharmaceutical industry (Alekshun and Levy, 2007; Oluwatuyi et al., 2004; Schelz et al., 2010). However, as shown in FIGURE 2.1, in the past thirty years only two novel classes of antibiotics have entered the market, the oxazolidinones and the cyclic lipopeptides, both of which are used against Gram-positive bacterial infections (WHO, 2011). No new anti-Gram-negative drugs have been developed.

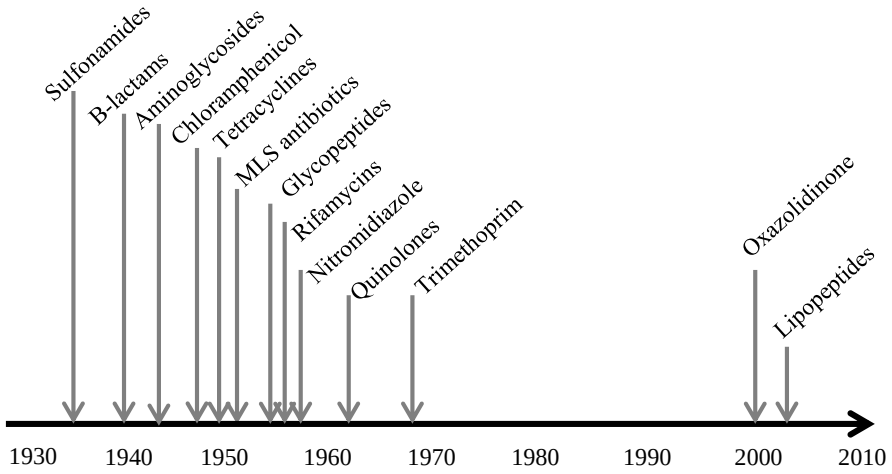


FIGURE 2.1 | History of antibacterial drug approval and introduction in the market. MLS: Macrolides/Lincosamides/Streptogramins antibiotics. Figure adapted from Powers (2004).

The low rate of emergence of new drug classes since the 1960s is evident when one examines the history of the Food and Drug Administration (FDA) approval for antibacterial agents shown by FIGURE 2.2, in which most of these approved drugs (75%) are from two classes, β-lactams and quinolones, and the success of most of these has been compromised due to the emergence of resistance (Alekshun and Levy, 2007; Chan et al., 2011; Gibbons, 2008). Only six new antibiotics have been approved over the last six years (Ventola, 2015).

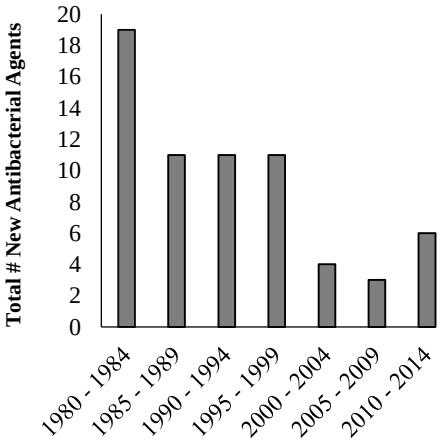


FIGURE 2.2 | Number of new antibacterial agents approved by FDA from 1980 to 2014. Data obtained from Ventola (2015).

All other similar efforts to find a new antibiotic failed, which indicates that there is an extremely low probability of discovering a new drug (Lewis, 2013). A successful antibiotic must satisfy a perplexing number of demands: it should be able to cross bacterial cell membranes at high rates, avoid bacterial efflux pumps, not be a target for bacterial modifying or hydrolyzing

enzymes, reach its target at a sufficiently high concentration, have a broad spectrum of antibacterial activity, have little or no toxicity and minimal side effects in humans (Hughes, 2003). There is no perfect antibiotic, and even if such antibiotic is discovered, it would be essential to restrict its use to very specific situations. Anti-infective therapies are difficult to develop, and clinical trials are complex and costly. As of March 2016, only 37 new antibiotics were in development, and just 13 in Phase III clinical trials - way less than some 500 oncology therapies in the pipeline (The Pew Charitable Trusts, 2016). In addition to serious scientific and clinical challenges, there are difficult financial issues to overcome since effective antimicrobial treatments face tight policies that limit use to prevent resistance. Increasingly, more rigorous clinical trials and safety checks have been required before a drug is introduced to the market. Despite this legislation is aimed to protect the consumer; certain antimicrobials prescribed today would most likely not meet such standards (Gill et al., 2015). Government incentives to fill the gap are likely to help over time. For example, the new drugs Dalvance® and Oritavancin® (approved by the FDA in 2014), which are both administered intravenously to combat skin infections caused by Gram-positive bacteria including MRSA, are a result of the American Generating Antibiotics Incentives Now (GAIN) incentive (Gill et al., 2015). Recently, a new antibiotic was discovered, teixobactin, produced by a hitherto undescribed soil microorganism (provisionally named *Eleftheria terrae*) (Ling et al., 2015). Teixobactin has activity against Gram-positive organisms (such as MRSA and *Streptococcus pneumoniae*) and mycobacteria (such as *Mycobacterium tuberculosis)* and a novel mode of action inhibiting peptidoglycan biosynthesis (Piddock, 2015). However, clinical trials and full toxicology tests of this compound still need to be carried out.

2.2. GLOBAL EPIDEMIC PROPORTIONS OF MDR BACTERIA

Resistance of pathogenic microorganisms to individual antibiotics is a great problem by itself. However, the emergence of MDR strains represents an increasing complication in the treatment of bacterial infections (Dötsch et al., 2009; Guz et al., 2000; Schelz et al., 2010). The number of hospital-acquired infections (HAI) has been growing exponentially worldwide since 1980s, especially due to the emergence and widespread of MDR bacteria. Approximately 50% of HAI worldwide are caused by MDR microorganisms (Hoffman, 2001). HAI are among the major causes of death and increased morbidity among hospitalized patients with a minimum of 175,000 deaths every year in industrialized countries (Cotter et al., 2011; Joshi et al., 2010; Rutala and Weber, 2001; Zollfrank et al., 2012). Several investigations showed that over 60% of worldwide HAI have been linked to the attachment of different pathogens on medical implants and devices, such as venous and urinary catheters, arthroprostheses, fracture-fixation devices, heart valves, among others (Costerton et al., 2005; Cozad and Jones, 2003; Griffith et al., 2000; Otter et al.,

2011; Pinto et al., 2011; Weber et al., 2010). As a direct consequence, the replacement of implants, which involves significant costs and suffering for patients, often remains the only efficient therapy (Wach et al., 2008). Additionally, it has been demonstrated that the increased incidence of HAI is related to cross-infections from patient to patient or hospital staff to patient and to the presence of pathogenic microorganisms that are selected and maintained within the hospital environment (Burts et al., 2009; Madigan et al., 2004; Madkour et al., 2009; Rutala and Weber, 2001; Sexton et al., 2011; Talon, 1999).

Traditional antibiotics are increasingly suffering from the emergence of MDR bacteria, which made it imperative to search for alternative treatments (Fiamegos et al., 2011). Among the most problematic MDR bacteria are vancomycin-resistant enterococci (VRE), MRSA, vancomycin-resistant *S. aureus* (VRSA) and bacteria producing extended-spectrum β-lactamases (ESβL) such as *Pseudomonas aeruginosa, Helicobacter pylori, Acinetobacter baumannii, Escherichia coli* and *Klebsiella pneumonia* (Alekshun and Levy, 2007; Gibbons et al., 2003b; Zahin et al., 2010). Other important MDR pathogens include *Legionella pneumophila*, penicillin-resistant *S. pneumonia* (PRSP), and *Shigella* and *Salmonella* species (Conly, 2004; Spížek et al., 2010). Additionally, tuberculosis caused by *M. tuberculosis*, continues to be a major problem, with estimated 2 million deaths worldwide each year (WHO, 2015). The reemergence of tuberculosis is partially associated with the development of mycobacterial resistance against two of the most important tuberculosis drugs, rifampicin and isoniazid, without any new drug being commercialized since 1964 (Spížek et al., 2010; WHO, 2015).

2.2.1. A focus on *Staphylococcus aureus*

Staphylococcus aureus is notorious for its ability to become resistant to antibiotics. Around 90 – 95% of *S. aureus* strains worldwide are resistant to penicillin and, in most of Asian countries, 70 – 80% are also methicillin resistant (Hemaiswarya et al., 2008). The emergence of antibiotic resistance in *S. aureus* can be visualised as a series of waves as shown in FIGURE 2.3.

Infections that are caused by antibiotic-resistant strains of *S. aureus* have reached epidemic proportions globally. MRSA merits special attention since it is responsible for a high-level of HAIs (Gibbons, 2008; Gould et al., 2010; Oluwatuyi et al., 2004; Pinho et al., 2001; Smith et al., 2007a; Stapleton et al., 2004). In fact, few agents can treat infections caused by this bacterium since many MRSA strains are resistant not only to almost all kinds of β-lactams but also to macrolides, quinolones and even to aminoglycosides (Gould et al., 2010; Shibata et al., 2005).

The first MRSA clinical isolates were ST250 and members of CC8 and circulated in Europe before the 1970s but had largely disappeared by the 1980s (Chambers and DeLeo, 2010).

However, other highly successful clones emerged, including the ST247 Iberian or Epidemic MRSA (EMRSA)-5 clone. MRSA strains have generally been found to be members of a subset of *S. aureus* CCs, including CC1, CC5, CC8, CC22, CC30 and CC45. Between 2005 and 2011, overall rates of invasive MRSA dropped 31%; the largest declines (around 54%) were observed in HAIs (Ventola, 2015). However, during the past decade, rates of community-associated (CA)-MRSA infections have increased rapidly among the general population, being associated with fulminant and lethal infections and worse clinical outcomes (Chambers and DeLeo, 2010).

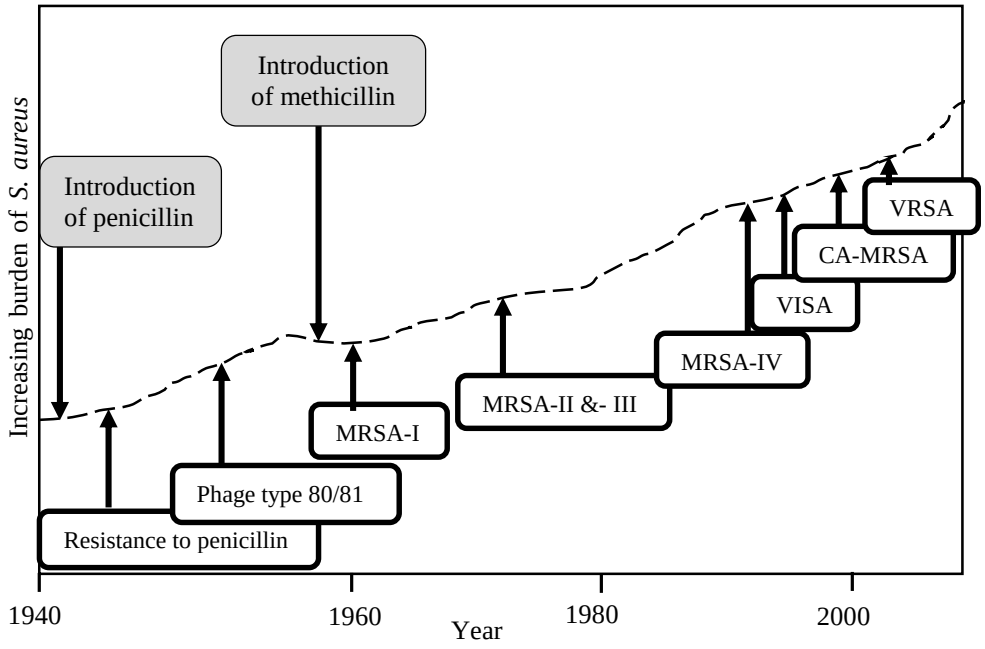


FIGURE 2.3 | Timeline of the emergence of antibiotic resistance in *Staphylococcus aureus.* Wave 1: began shortly after the introduction of penicillin into clinical practice in the 1940s. The first resistant strains from phage type 80/81 were penicillin-resistant and Panton-Valentine leukocidin (PVL)-positive; Wave 2: began almost immediately following the introduction of methicillin into clinical practice with the isolation of MRSA-I, which contained staphylococcal chromosome cassette (SCC) *mec*I; Wave 3: began in the mid to late 1970s with the emergence of MRSA-II and MRSA-III, containing SCC*mec*II and SCC*mec*III, respectively. The increase in vancomycin use for the treatment of MRSA infections led to the emergence of vancomycin-intermediate *S. aureus* (VISA) strains; Wave 4: began in the mid to late 1990s and marks the emergence of CA-MRSA strains [containing SCC*mec*IV (MRSA-IV) and various virulence factors]. VRSA strains, first identified in 2002, have been isolated exclusively in health care settings. To date, eight SCC*mec* allotypes, designated SCC*mec*I–SCC*mec*VIII have been described, along with numerous subtypes. Figure adapted from Chambers and DeLeo (2010).

2.3. AN INSIGHT INTO THE MECHANISMS OF BACTERIAL RESISTANCE TO ANTIBIOTICS

The term 'antibiotic resistome' was been proposed for the collection of all antibiotic resistance genes in microorganisms, including those from pathogenic and non-pathogenic bacteria (Wright, 2007). This resistome has suffered several alterations over time, mainly in the last years with the introduction of new resistance genes.

Bacteria evolve rapidly causing the failure of antimicrobial treatments, not only by mutation and rapid multiplication, but also by transfer of genetic material, which can result in strains with beneficial changes (Thomas and Nielsen, 2005; Gilbert et al., 2002; Simões et al., 2008). The horizontal gene transfer (FIGURE 2.4), is the process of exchange and acquisition of new genetic material and may occur via three main mechanisms: transduction (from one bacterium to another by a bacteriophage), transformation (by uptake from the external environment) or conjugation (by direct contact between two bacterial cells). It contributes to the rapid horizontal dissemination of resistance determinants. The localization of resistance determinants on mobile elements, such as genomic islands, transposons, plasmids, or phages, greatly enhances their dispersion (McCallum et al., 2010). Although most frequent in bacteria, this transfer also occurs naturally in viruses, plants, insects, and even between bacteria and fungi (Cruz and Davies, 2000).

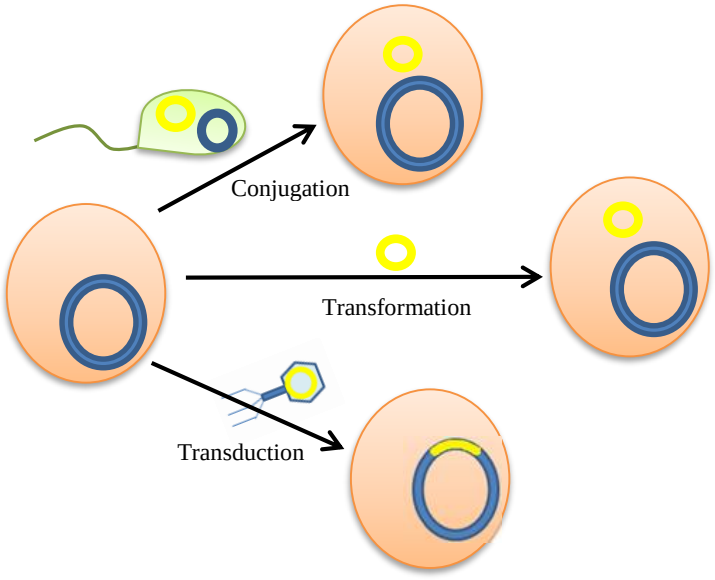


FIGURE 2.4 | Scheme explaining the main horizontal dissemination mechanisms of resistance determinants in bacteria: conjugation, transformation and transduction. Figure adapted from von Wintersdorff et al. (2016).

The major biochemical mechanisms of bacterial resistance to antimicrobials are demonstrated in FIGURE 2.5 and include drug inactivation, target modification, alteration in the accessibility to the target through drug efflux and decreased uptake (Brehm-Stecher and Johnson, 2003; Dantas et al., 2008; Kumar and Schweizer, 2005; Russell, 2003; Wright, 2005). All of these mechanisms require new genetic programming by the cell in response to the presence of antibiotics (Wright, 2005).

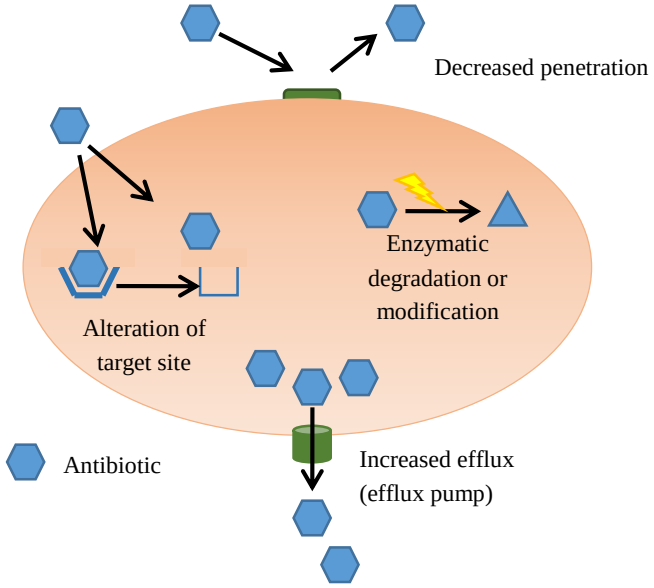


FIGURE 2.5 | Main mechanisms explaining the resistance of bacterial cells to antibiotics. Active drug efflux systems from the cell via membrane-associated pumping proteins that effectively remove toxic compounds from cells; mutations resulting in altered cell permeability; enzymatic degradation of antimicrobials by the synthesis of modifying- or inactivating-enzymes that selectively target and destroy these compounds; alteration/ modification of the target site, e.g., through mutation of key binding elements such as ribosomal RNA.

There is an amazing diversity of antibiotic resistance mechanisms within each of these four categories and a single bacterial strain may possess several types of resistance mechanisms. Resistance becomes a clinical problem when the frequency of the resistant variant threatens the effectiveness of empirical drug therapy. The selection of resistant determinants in the patient is determined by the level of exposure of the pathogen population to an antibiotic, as well as by the pharmacokinetic and pharmacodynamic properties of the antibiotic (Andersson and Hughes, 2010). Relevant examples of bacterial resistance mechanisms are briefly described in this section.

2.3.1. Inactivation of antibiotics

Enzymatic degradation or modification of antimicrobials is an important mechanism of bacterial resistance (Pandra et al., 2010; Wagner and Ulrich-Merzenich, 2009). There are several strategies of antibiotic inactivation. Hydrolysis, group transfer, and redox enzymes are involved in this type of resistance. β-lactam antibiotics are the largest antibiotic family used in medicine. However, their use is readily compromised partly because of the production of β-lactamases, such as TEM, leading to clavulanate resistance, or SHV-1 (Demanèche et al., 2008; Yap et al., 2014). The resistance to β-lactams by the overproduction of β-lactamases that partially hydrolyze methicillin and related penicillins is probably the most widespread example of antibiotic inactivation involving enzymes (Shibata et al., 2003). Several generations of drugs with enhanced activity have been developed to counter antibiotic resistance to β-lactamases such as 3rd and 4th generation cephalosporins, carbapenems or quinolones (Bax et al., 2000; Hoffman, 2001; WHO, 2011). Some of them, e.g. the isoxazolyl penicillins, imipenem and meropenem, are more resistant to enzymatic inactivation (Chopra et al., 1997). However, ESBLs have been reported to confer resistance to newer generations of cephalosporins in *E. coli*, *Enterobacter* and *Klebsiella* species (Hoffman, 2001). Enzymes such as active-site serine carbapenemases, plasmid-encoded class C cephalosporinases and acquired metallo-β-lactamases have also emerged (Miller et al., 2001; WHO, 2011). KPC, OXA-48, VIM and IMP-1 carbapenemases producers are currently the most widespread types of carbapenemase in *Enterobacteriaceae*. More recently, a new type of carbapenemase was reported - New Delhi metallo-β lactamase-1 (NDM-1) (Hughes, 2003). NDM-1 producers are becoming highly prevalent and some cases have been identified all over the world (Nordmann et al., 2011).

2.3.2. Target modification

Modification of target sites is a common mechanism of resistance and may occur for diverse classes of antibiotics which include tetracyclines, β-lactams and glycopeptides (Chopra et al., 1997). In the case of β-lactam antibiotics, their targets are the penicillin binding proteins (PBPs), which are important cell-wall structural enzymes present in almost all bacteria (Hoffman, 2001). Resistance to β-lactam antibiotics can be acquired by the acquisition and expression of the *mecA* gene which codes for an altered transpeptidase additional to PBP (PBP2a) (Chopra et al., 1997; Lambert, 2005; Shibata et al., 2003; Shimizu et al., 2001). PBP2a confers resistance to β-lactam antibiotics due to its low affinity for penicillins (Pinho et al., 2001). Alterations in PBPs are known to be responsible for specific resistances namely in MRSA and PRSP (Barker, 1999).

Other important examples of bacterial resistance mediated by modification of the targets of antibiotics are presented in TABLE 2.1.

TABLE 2.1 | Examples of antibiotic resistance mediated by target modification

Antibiotic class	Target	Resistance mechanism	Examples of bacteria
Quinolones	DNA gyrase or topoisomerase IV, (inhibition of DNA synthesis)	Chromosomal mutations in both target enzymes which promotes lower affinity for quinolones	Gram-positive bacteria, particularly *S. aureus* and *S. pneumoniae*
Glycopeptides (e.g. vancomycin, teicoplanin)	Peptidyl-d-alanyl-d-alanine (d-Ala-d-Ala) termini of peptidoglycan precursors (inhibition of cell wall synthesis)	Biosynthesis of peptidoglycan with altered glycopeptide recognition sites	VRSA, *S. aureus*, glycopeptide-intermediate *S. aureus*
		Acquisition of *vanA* and *vanB*, which encode for enzymes that produce a modified peptidoglycan precursor terminating in d-Ala-d-Lac	VRE, such as *E. faecium* and *E. faecalis*
Macrolides, lincosamides and streptogramin B	50S ribosomal subunit (block protein synthesis)	Post-transcriptional modification of the 23S rRNA component of the 50S ribosomal subunit involving methylation or demethylation of key adenine bases in the peptidyl transferase functional domain	Wide range of Gram-positive and Gram-negative bacteria
Oxazolidinones (e.g. linezolid)	50S ribosomal subunit (inhibition of formation of the initiation complex and interference with translocation of peptidyl-tRNA from A to P site)	Mutations in the 23S rRNA resulting in decreased affinity for binding; most mutations involve G to U substitutions in the peptidyl transferase region of 23S rRNA	Several microorganisms including enterococci
Aminoglycosides	16S rRNA in the smaller subunit of the bacterial ribosome, perturbing decoding of the mRNA	Mutations in the 16S rRNA gene	*Mycobacterium smegmatis, M. tuberculosis and M. abscessus*
Polymyxin B and Polymyxin E (e.g. colistin)	Lipopolysaccharide (LPS) component of the outer membrane	Modification of the phosphate esters linked to the diglucosamine components of the lipid A region by addition of 4-aminoarabinose groups to the phosphate esters	Gram-negative bacteria

Data obtained from (Barker, 1999; Hoffman, 2001; Lambert, 2005; Wright, 2003)

2.3.3. Alteration in the accessibility to the target by overexpression of efflux pumps

The overexpression of efflux pumps, which extrude antibiotics from the bacterial cell, is one of the most important mechanisms of microbial resistance to antimicrobials and can be observed in many clinically relevant pathogens (Gibbons and Udo, 2000; Stermitz et al., 2000a; Stermitz et al., 2000b). Although some are drug-specific, many efflux systems may transport a range of products with different structures and classes, contributing significantly to multidrug resistance (Kumar and Schweizer, 2005; Piddock, 2006). Indeed, the poly-specificity of efflux transporters can drive the acquisition of additional mechanisms of antibiotic resistance such as mutation of antibiotic targets or secretion of enzymes that degrade antibiotics (e.g. β-lactamases) (Pagès and Amaral, 2009). The overexpression of these pumps and synergy with other drug resistance mechanisms hampers effective antimicrobial treatment (Bhardwaj and Mohanty, 2012).

Antibiotic efflux transporters have been classified into five main families based primarily on amino acid sequence homology. These are the major facilitator superfamily (MFS), the resistance-nodulation-division (RND) family, the small multi-drug resistance (SMR) family, the ATP binding cassette (ABC) family and the multiple antibiotic and toxin extrusion (MATE) family (Lynch, 2006; Stavri et al., 2007). The efflux of drugs from Gram-positive bacteria is mainly mediated by a single cytoplasmic membrane-located transporter of the MFS, SMR or ABC families (Stavri et al., 2007; Tegos et al., 2008). Among Gram-negative bacteria, multidrug efflux pumps belonging to RND and SMR are common (Nikaido, 1998). The analysis of antibiotic efflux transporters has been already extensively reviewed (Lynch, 2006; Stavri et al., 2007). It is, however, interesting to highlight some important MDR efflux pumps, such as the protein NorA of *S. aureus* (significant for several fluoroquinolones, monocationic dyes and disinfectants), which is probably the most intensively studied pump in pathogenic Gram-positive bacteria and may be responsible for at least 10% of antibiotic resistance in MRSA (Kaatz et al., 2003; Sibanda and Okoh, 2007); TetK (mediates tetracycline efflux) and MsrA (for macrolides) in *S. aureus;* QacA, responsible for the export of several antimicrobial compounds and for acriflavine and EtBr resistance, the Bmr in *Bacillus subtilis*, the TetA(B) protein, among Gram-negative bacteria, also for tetracyclines and is also one of the most extensively studied members of the MFS (Borges-Walmsley et al., 2003; Gibbons et al., 2003a; Guay et al., 1994; Guz et al., 2000; Oluwatuyi et al., 2004; Sibanda and Okoh, 2007; Stavri et al., 2007). The ABC transporter LmrA from *Lactococcus lactis* has also been intensively studied (Infed et al., 2011). The AcrAB-TolC, which is the main efflux transporter in Enterobacteriaceae for diverse products (e.g. tetracyclines, fluoroquinolones, chloramphenicol), and the MexAB-OprM in *P. aeruginosa*, causing resistance to β-lactams, quinolones, tetracyclines and trimethoprim, are examples of the RND family (Lynch, 2006; Marquez, 2005; Sibanda, 2007; Stavri et al., 2007; Tegos et al., 2008).

2.3.4. Alteration in the accessibility to the target by reduced membrane permeability

The outer membrane (OM) of Gram-negative bacteria serves as a selective permeation barrier for many external hydrophobic solutes due to its high LPS, content and it forms specific contacts with integral outer membrane proteins (OMPs), such as porins (e.g., OmpF in *E. coli* and OprD in *P. aeruginosa*), which act as entry and exit points for antibiotics and other organic chemicals (Alekshun and Levy, 2007; Hemaiswarya et al., 2008; Nikaido, 2003; Nikaido and Vaara, 1985; Vaara, 1992). Changes in OMPs copy number, size and selectivity, promoted by the loss or modification of certain OMPs as a consequence of decreased expression, point mutations or insertion sequences in the encoding genes, or the production of modified OMPs with reduced permeability to antibiotics (Drawz and Bonomo, 2010; Hoffman, 2001), can alter the rate of diffusion and uptake of antibiotics (Pandra et al., 2010). Thus, porin mutations confer resistance to several antibiotics such as β-lactams, carbapenems, fluoroquinolones, tetracyclines, sulfonamides and chloramphenicol (Drawz and Bonomo, 2010; Hoffman, 2001).

2.3.5. Increased bacterial resistance by other mechanisms

2.3.5.1. By overexpression of the drug target

The overexpression of the drug target is also responsible for an increasing bacterial resistance. However, this may not be so simple. Palmer and Kishony (2014) interestingly found that target overexpression in *E. coli* has conflicting effects on drug resistance: it can increase, remain unchanged, decrease or even change non-monotonically. This highly depends on the properties of drug mechanism.

2.3.5.2. By stress-induced modifications

Some changes in the cell presumably have something to do with stress because they are affected by this stress locus (Levy, 2002). Stress derived from nutrient starvation, hypoxia, membrane damage, low pH, increased osmotic pressure, extreme temperature shifts, ribosome disruption or antimicrobial exposure, elicit a variety of specific and highly regulated adaptive responses that not only protect bacteria from the offending stress, but also manifest changes in the cell that impact innate antimicrobial susceptibility. This occurs indirectly, as a result of stress-induced growth cessation, since antimicrobials typically act on growing cells, or directly as a result of the stress-dependent recruitment of resistance determinants (e.g. antimicrobial efflux), changes to antimicrobial targets, generation of resistance mutations, promotion of resistant growth modes (biofilms) (Poole, 2012), etc.

2.3.5.3. Increased bacterial tolerance due to formation of biofilms

Chronic infections in which biofilms have been demonstrated to be involved are many and include periodontitis, cystic fibrosis pneumonia, and numerous infections associated with indwelling devices such as catheters, heart valves, and prostheses (Stewart, 2002). Also, biofilms constitute a major threat in the clinical environment by acting as reservoirs of multidrug resistant pathogenic bacteria.

A biofilm is a functional consortia of bacteria populations adherent to a surface and to each other and/or embedded within extracellular polymers matrices (glycocalyx), concentrated products of their own metabolism and ions and nutrients from the environment (Gilbert et al., 1997; Smith, 2005; Stepanović et al., 2007; Toté et al., 2008). This adaptation as adherent populations allow bacteria to survive within hostile environments and unfavorable conditions (including predation, antimicrobials presence and host immune responses) (Olson et al., 2002; Verstraeten et al., 2008).

A central tenet of biofilm formation is its dynamic nature. Most current models depict biofilm formation as a linear process that begins when free-floating bacterial cells attach to a surface (Shen et al., 2011). Then, it is believed to occur in a sequential process that includes initial reversible/irreversible adhesion to a solid surface and/or other microbes previously attached to the surface, cell–cell communication, formation of microcolonies on the surface, extracellular polymeric substances (EPS) production and, finally, differentiation of microcolonies into exopolymeric-encased, mature biofilms (FIGURE 2.6) (Costerton et al., 1999; Simões et al., 2010). EPS participate in the formation of microbial aggregates and are responsible for binding cells and other particulate materials together - cohesion - and to the surface – adhesion (Simões, 2005). Genetic analysis of biofilm formation has led to the proposal that extracellular signals and quorum-sensing (QS) regulatory systems are essential (Kjelleberg and Molin, 2002).

The rapid reversal of resistance upon dispersion from a biofilm suggests that this is an adaptive resistance mechanism rather than a genetic alteration (Stewart, 2002). Cells in the biofilm can return to a planktonic lifestyle by two possible ways. In order to colonize new areas or in response to environmental cues, such as starvation, a programmed set of events leads to hydrolysis of the extracellular polymeric matrix and conversion of a subpopulation of cells into motile planktonic cells, which can rapidly multiply and leave the sessile communities (Costerton et al., 1999). Also, biofilm dispersal can occur as a consequence of physical detachment or mechanical breakage of biofilms due to flow or shear stresses (Landini et al., 2010).

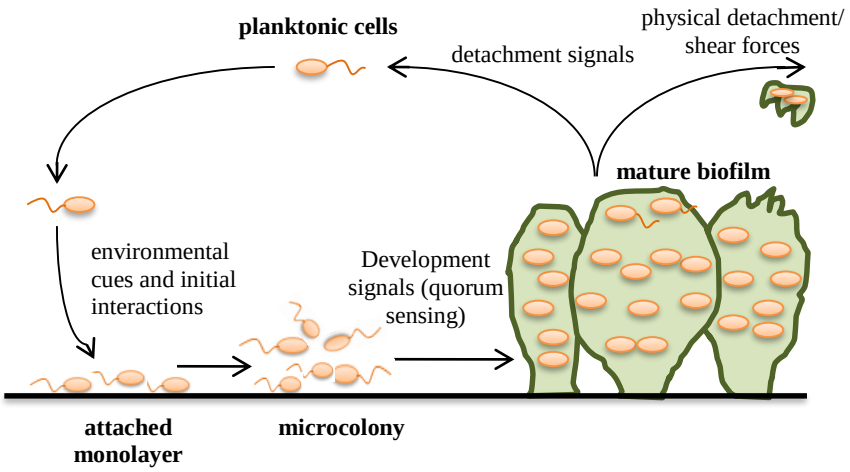


FIGURE 2.6 | Schematic representation of the steps involved in the formation of a biofilm. Planktonic cells can form cell-to-surface and cell-to-cell contacts resulting in the formation of microcolonies. Quorum-sensing leads to the formation of differentiated, thick and mature biofilm structures. Cells in the biofilm can return to a planktonic lifestyle. Figure adapted from O'Toole et al. (2000).

It is well known that bacteria in biofilms can be hundreds of times more resistant to antibiotics compared with planktonic cells. The microorganisms generate physiological changes when cells attach to a surface by expressing a biofilm phenotype that can confer resistance face to stress environmental conditions such as nutrient limitation, heat and cold shocks, changes in pH and to chemical agents. Biofilm resistance involves multiple mechanisms (Inoue et al., 2008). One obvious difference between planktonic cells and biofilms is the presence of a polymeric matrix enveloping the community that retards diffusion of antimicrobials into the biofilm (Brooun et al., 2000; Costerton et al., 1999; Fux et al., 2005).

Many studies have investigated the formation of biofilms as an explanation for microbial resistance (Mah and O'Toole, 2001; Smith, 2005; Stewart, 1996; Xu et al., 2000). FIGURE 2.7 represents some hypotheses explaining the higher resistance to antibiotics in biofilms. Antibiotics have been shown to penetrate biofilms readily in some cases and poorly in others, depending on the particular agent and biofilm (Costerton et al., 1999). However, given that, in many cases, biofilms consist of stacks of cells with aqueous channels flowing in between, only impenetrability seems unlikely (Smith, 2005). Because of the special structure of biofilms, there are gradients of nutrients and oxygen and, for this reason, cells can be in distinct growth states. Consequently, cells in different layers of the biofilm will be affected differently by different types of antimicrobials depending on their mechanism of action (Fernández et al., 2011). For example, penicillins, which target cell-wall synthesis, kill only growing bacteria (Stewart and William

Costerton, 2001). Other theories include a reduced susceptibility of biofilm microorganisms compared to their freely suspended counterparts (Stewart, 1996); and the existence of persister cells, a small population of cells with a highly protected phenotype (Brooun et al., 2000; Lai et al., 2009; Stewart, 2002).

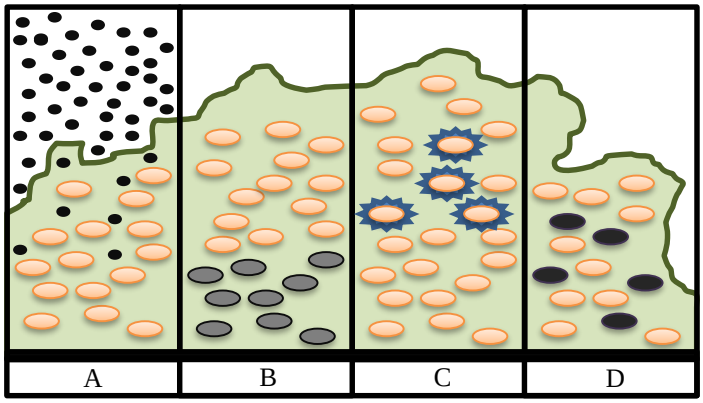


FIGURE 2.7 | Hypothesized explanations for the increased antibiotic resistance of biofilms. (A) the antibiotic (black points) penetrates slowly or incompletely the biofilm; (B) an adaptive stress response is expressed by some cells (marked cells); (C) a concentration gradient of a metabolic substrate or product leads to zones of slow non-growing bacteria (shaded cells); (D) a small fraction of cells differentiates into a highly protected persister state (dark cells).

2.4. HOW TO CIRCUMVENT BACTERIAL RESISTANCE TO ANTIBIOTICS?

New classes of antibiotics and more effective antimicrobial agents are needed. High-throughput methodologies combined with traditional molecular biology techniques have enabled the discovery of potential drug targets for new antibiotics and antibiotic potentiators. However, translating these targets from identification to actual drug compounds requires a significant amount of additional work and financial investment (Lu and Collins, 2009). A potential source of bioactive compounds is also the reinvestigation of metabolites previously assumed to be inactive, which represent ~60% of known metabolites (Berdy, 2012). For example, Naftifine®, an FDA-approved antifungal drug, was found to be a lead compound for potent Benzofuran-Derived Diapophytoene Desaturase (CrtN) inhibitors and to attenuate the virulence of a variety of clinical *S. aureus*, including MRSA, in mouse infection models (Chen et al., 2016). The total number of marketed drugs used in human therapy is estimated to be ~3500 compounds, representing less than 0.01% of all known chemical compounds (Berdy, 2012). Several additional approaches to antibiotic discovery have been pursued, including targeting virulence factors, antimicrobial

peptides, host-directed therapies, probiotics and phage therapy (Gill et al., 2015; Lewis, 2013). Also, the use of vaccines, monoclonal antibodies, immuno-regulatory cytokines and hematopoiesis-stimulating factors may have utility in the control of antibiotic resistant infections (Chopra et al., 1997).

Recently, multidrug therapy has gained a wider acceptance in the fight of multidrug resistant microbial strains. The use of drug combinations rather than single drugs provides better clinical outcomes, as the use of single agents is highly associated with occurrence of resistance (Kyaw et al., 2012). So, antimicrobial combinations are employed in order to prevent the emergence of resistant strains or to increase activity, in cases of mixed infections, or to reduce the toxicity of a substance without compromising the antimicrobial action. For example, it has become clear that a multidrug approach must be used to treat tuberculosis (e.g. isoniazid, rifampicin, pyrazinamide and ethambutol), to have a realistic chance of success (Lewis, 2013). Other effective synergistic pairing is trimethoprim and sulfamethoxazole, which have a great impact on folate metabolism (Brown and Wright, 2016).

Cases of synergy (where the combined effect of two drugs is greater than the sum of individual activities) between a broad range of antibiotics have been reported. In some cases, combining established antimicrobials has extended the useful life of an antibiotic. These combinatorial activities may be due to multi-target effects, or also to improved solubility, resorption rate or enhanced bioavailability (Wagner and Ulrich-Merzenich, 2009; Zimmermann et al., 2007). Other important potential strategy to help combat the resistance problem involves the discovery and development of new active agents capable of partly or completely suppressing bacterial resistance mechanisms (Malléa et al., 2003). This reversal of multidrug resistance represents a promising approach to mitigate the spread of resistance. Many strategies for avoiding, inhibiting, or bypassing resistance mechanisms in pathogens have been attempted (Davies and Davies, 2010). The development of RMAs, anti-resistance drugs or antibiotic adjuvants represents an attractive strategy to mitigate the spread of bacterial drug resistance since it could facilitate the recycling of well-established antibiotics that are often cheaper and less toxic than new candidate antimicrobials (Schelz et al., 2010; Sibanda, 2007).

Such non-conventional antibiotic treatments have shown promising results in preclinical studies but few of them have succeeded in demonstrating significant clinical outcomes (Chatterjee et al., 2016). Other possibilities of non-conventional antibiotic treatments include the use of compounds that inhibit quorum-sensing, target pathogen virulence, prevent biofilm formation or the adherence to the host tissues (Chatterjee et al., 2016), etc. FIGURE 2.8 exemplifies situations of synergy or potentiation between antibiotics and other bioactive compounds (Kalan and Wright, 2011; Wagner and Ulrich-Merzenich, 2009; Zimmermann et al., 2007).

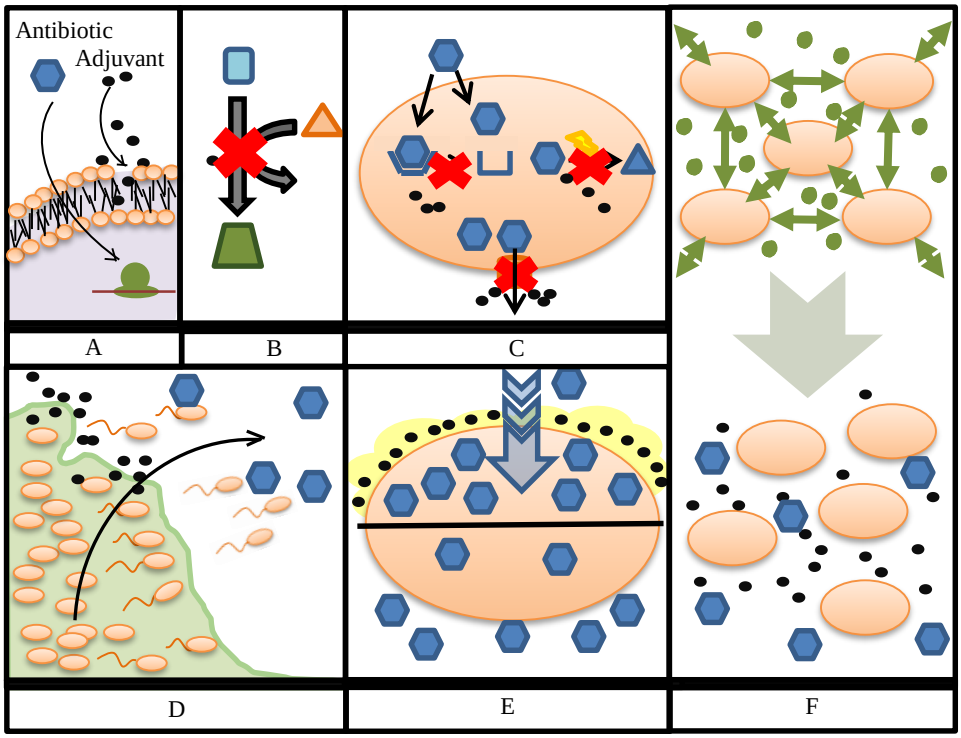


FIGURE 2.8 | Possible mechanisms of action of antibiotic adjuvants to improve therapy. (A) multi-target effect; (B) serial or orthogonal inhibition of vital physiological pathways; (C) inhibition of resistance enzymes, of compounds that block antibiotic efflux or that decrease the uptake into the cell; (D) dispersal of a biofilm to planktonically growing cells, resulting in increased susceptibility to antibiotics; (E) enhancement of the permeability of cell for antibiotics; (F) quorum-sensing inhibition, which may affect the behavior of the cells, such as pathogenicity, expression of resistance mechanisms, biofilm formation, etc.

An antibiotic potentiating compound may work by any of a variety of different mechanisms: it may or not affect the same molecular target or pathway (e.g., metabolic pathway, biosynthetic pathway) as the antibiotic(s) whose activity it potentiates; it may inhibit the metabolism of the antibiotic; it may be an inhibitor (e.g., a competitive or non-competitive inhibitor) of an enzyme that degrades the antibiotic; it may alter the mechanism of the antibiotic, e.g., may increase the metabolism to a more active form, shift the profile of metabolites; it may alter distribution, absorption, or excretion of the antibiotic in a way that effectively increases its activity in the body (Zechini and Versace, 2009), etc.

The inhibition of resistance mechanisms of bacteria to antibiotics has been an important topic and some current examples were made available. Bacterial enzymes that degrade or modify antibiotics are important targets for drug action since their inhibition protects the antibiotic from degradation (Chopra et al., 1997). The co-administration of β-lactamase inhibitors is an important

strategy for restoring the activity of β-lactam antibiotics enabling the antibiotic to more effectively interact with the PBPs (Drawz and Bonomo, 2010). Inhibitors of β-lactamases have long been known and their administration with antibiotics has been associated with considerable success, proving to be one of the most effective antibiotic combinations (Hemaiswarya et al., 2008; Miller et al., 2001). Penicillins, cephalosporins and carbapenems antibiotics with higher stability to hydrolysis were developed to circumvent the inactivating activity of these enzymes (Ulrich-Merzenich et al., 2010). An important example of such inhibitors is clavulanic acid, which binds with high affinity to many bacterial β-lactamases thus protecting antibiotics from destruction and is available commercially in combination with amoxicillin as Augmentin® and Ticarcillin (Timentin®) (Chopra et al., 1997; Miller et al., 2001; Sullivan et al., 2001). Interestingly, clavulanic acid is produced by *Streptomyces clavuligerus*, which also produces several β-lactam antibiotics. Other inhibitors of β-lactamases are sulbactam, marketed in combination with ampicillin (as Unasyn®), and tazobactam, marketed in combination with piperacillin (as Tazocin®) (Chopra et al., 1997; Drawz and Bonomo, 2010; Sullivan et al., 2001).

To inhibit cephalosporinase and other enzymatic activities, a possibility is to facilitate the diffusion of antibiotics through the bacterial envelope in order to increase their intracellular concentration. Some compounds, *e.g.*, polycationic cyclic lipopeptides and cationic antimicrobial peptides, which are amphiphilic in nature, targeting membranes thus increasing their permeability, have been assayed in combination with usual antibiotics to combat resistant clinical strains (Bolla et al., 2011). Many other compounds have also been reported to affect membrane permeability on a diverse range of microorganisms (Helander et al., 1998), mainly due to the perturbation of the lipid fraction of the cell membrane and, owing to their lipophilic character, increasing membrane permeability (Trombetta et al., 2005). Such permeabilizers, as they have been termed, can non-specifically enhance the permeability of bacterial cells to exogenous products, including antimicrobial agents and may therefore potentiate the antibacterial potential of antibiotics that interact with intracellular targets (Simões et al., 2009).

It is also of great interest to find co-therapeutic agents that inhibit PBP 2as. Some agents have already demonstrated the potential to either inhibit the modified targets or exhibit a synergy by blocking one or more of the other targets in the metabolic pathway, thus causing cell death (Hemaiswarya et al., 2008). A number of β-lactam antibiotics, including modified cephalosporins, carbapenems and trinem have been designed with enhanced activity against PBP2a (Hemaiswarya et al., 2008). Newer glycopeptide antibiotics have activity against vancomycin and teicoplanin-resistant Gram-positive bacteria, which express modified peptidoglycan structures refractory to the binding of the older glycopeptides (Chopra et al., 1997).

Since most bacteria can acquire MDR efflux pumps, the inhibition of these systems is a promising strategy for potentiating antibiotic effectiveness (Wagner and Ulrich-Merzenich, 2009). Recent studies suggest that efflux systems may be a first response to the antimicrobial making possible for the cell to survive and acquire other, more stable resistance mechanisms that will then provide a high-level resistance phenotype (Costa et al., 2013). EPIs would allow the enhancement of antibiotic uptake to overcome drug efflux (Kalan and Wright, 2011), and restore the antibacterial treatment by decreasing the intrinsic bacterial resistance to antibiotics, reversing the acquired resistance associated with efflux pumps overexpression, and reducing the frequency of the emergence of resistant mutant strains (Bhardwaj and Mohanty, 2012; Zechini and Versace, 2009). Also, an effective EPI would allow to decrease the antibiotic doses required for bacterial inhibition (Lechner et al., 2008; Mahamoud et al., 2007; Marquez, 2005; Smith et al., 2007b). Substantial efforts are needed to develop new, safe and effective bacterial EPIs that could be used in antimicrobial therapy (Lomovskaya et al., 2001; Marquez, 2005). Several ways of targeting efflux pumps are: alteration of pump gene expression, inhibition of membrane assembly of pump component, blocking OM exit duct, and collapsing the energy driven source (Bolla et al., 2011).

The process of discovery, testing and commercialization of drugs is rather slow (Bhardwaj and Mohanty, 2012). Despite that, a large number of MDR inhibitors have been discovered and patented (Gill et al., 2015) in last two decades. Structural and genetic studies have contributed to a better understanding of the transport mechanisms of the efflux pumps, and these include the identification of amino acid regions for rational design of drugs capable of evading efflux (Li and Nikaido, 2009). Globomycin from *Streptomyces hagronensis* blocks the functional assembly of some components of efflux pump, such as AcrA; carbonyl cyanide *m*-chlorophenylhydrazone and potassium cyanide affect the energy level of the bacterial membrane (Pagès and Amaral, 2009); Phe-Arg-β-naphthylamine resulted in a up to 2,000-fold reduction in the MICs of antibacterials known to be substrates of the *Campylobacter* CmeABC pump, and the frequency of emergence of erythromycin-resistant mutants in *C. jejuni* reduced more than 1,000 fold (Martinez and Lin, 2006).

A strategy already proposed to mitigate efflux-mediated resistance is the synthesis of analogs of macrolides, quinolones and tetracyclines that are not recognized by efflux pumps (Chopra et al., 1997). For example, the activities of tigecycline against various pathogens are at least partly attributable to being an inferior substrate for specific Tet transporters (Li and Nikaido, 2009). Research has also been conducted to engineer fluoroquinolones to avoid efflux via NorA pumps, such as garenoxacin (a dual topoisomerase IV and DNA gyrase inhibitor) and the lead stage compound DX-619 (an inhibitor of type II topoisomerase) (Gill et al., 2015). Nevertheless, very few bacterial EPIs had entered into a clinical trial phase (Li and Nikaido, 2009).

2.4.1. Current methods to assess *in vitro* synergy

Antibiotic combination therapy has become increasingly important in clinical area not only as an attempt to cover a broad spectrum of pathogens when the cause of the infection is unknown, but also to prevent and fight against drug-resistant infections (Odds, 2003; Sopirala et al., 2010). The accurate detection of clinically relevant antibiotic synergy based upon the results of *in vitro* testing has been a goal of researchers for some time (White et al., 1996). A number of methods used to detect *in vitro* synergy between antibiotics have been described (FIGURE 2.9).

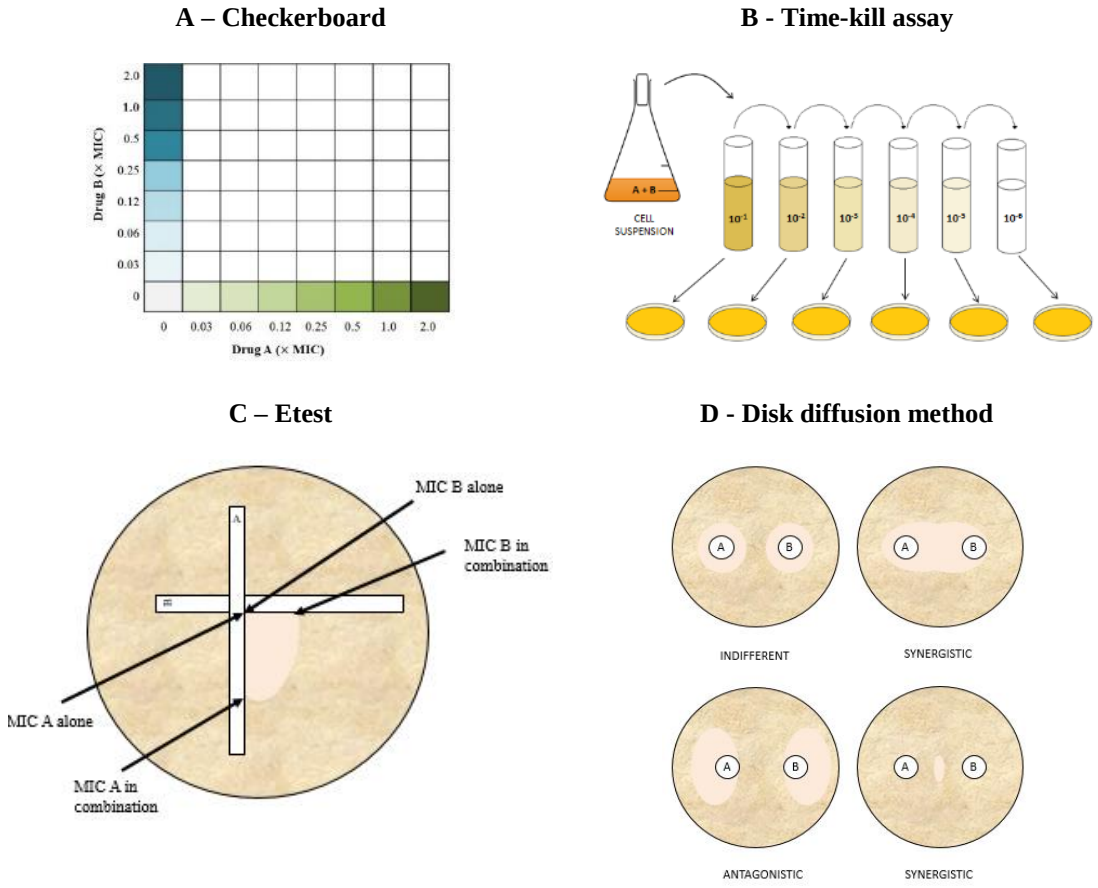


FIGURE 2.9 | Main methods to detect *in vitro* synergy between antibacterial compounds. (A) checkerboard microdilution method: the pattern of microtiter wells is formed by multiple two-fold dilutions of the two antibacterial compounds (drugs A and B), in concentrations that typically range from 0 to twice the anticipated MIC (or higher if antagonism is suspected) (Hemaiswarya et al., 2008; Kontoyiannis and Lewis, 2003); (B) time-kill assay to detect synergy between compounds; samples from the flask with the inoculum and the antimicrobials are taken along 24 h and colony counts of surviving bacteria over time are determined; (C) two Etest strips with antibiotics placed on the agar surface inoculated with the bacterium at a 90° angle with the intersection at the respective MICs; (D) possible results for the combination of two antimicrobials obtained with the double-disk diffusion test.

The microdilution checkerboard has been one of the most popular methods for the measurement of antibiotic synergy (Rand et al., 1996). In order to assess the effect of a given antimicrobial combination, each well of the checkerboard array contains a unique combination of the two drugs being tested (Kontoyiannis and Lewis, 2003). The time-kill assay is useful to study the dynamics of synergy between two antimicrobial agents by determining the number of viable bacteria remaining over time after exposure to each individual agent and the various combinations (Garcia and Isenberg, 2007).

Agar medium methods such as the Etest and the disk diffusion test have been applied, demonstrating good results in detecting synergy effects. Etest consists of a non-porous plastic strip containing a continuous gradient of antimicrobial concentrations covering 15 two-fold dilutions. For testing *in vitro* combinations, Etest strips of the two antimicrobials are placed on the agar at a 90° angle with the intersection at the respective MICs for the organism (Sueke et al., 2010). After incubation overnight, an elliptical zone of growth inhibition is formed and the MICs of the antimicrobial agents can be visualised (Garcia and Isenberg, 2007). Disk susceptibility testing methods remain the most commonly used techniques in clinical microbiology laboratories (Galani et al., 2008; Skov et al., 2003). Disk diffusion tests easily assess net antimicrobial activities of combinations for which commercially prepared disks are available (e.g. amoxicillin-clavulanate, trimethoprim-sulfamethoxazole). For other combinations, double-disk diffusion techniques have been revised to qualitatively determine drug interactions.

The conclusions of synergy/ indifference/ antagonism derived by each method may be unclear since there are several definitions to what constitutes synergy and numerous experimental techniques for synergy detection (Lambert et al., 2003). Typically, synergy indicates that the compounds in the mixture act via different mechanisms and/or targets (Lila and Raskin, 2005). In these cases, where both compounds have antimicrobial activity, it is only necessary to determine whether the combination is beneficial or not. However, some compounds, the so-called antibiotic adjuvants or RMAs, which possess weak or inexistent antibacterial activities by themselves, have been reported to potentiate antibacterial compounds against pathogens (Gill et al., 2015; Kalan and Wright, 2011). Further in this thesis, optimized methods to detect and characterize potentiation will be described. The adjuvant approach offers advantages such as the potential to decrease the therapeutic concentration of antibiotic that is needed, which can be beneficial in terms of emergence of resistance and toxicity issues, to extend the effective lifespan drugs, and to minimize barriers to be accepted into clinical setting for being already familiar compounds (Brown and Wright, 2016). However, the combination approach also presents challenges. It may not totally avoid resistance emergence in long term, as it is for any

antimicrobial therapy. Moreover, the potential for unexpected drug–drug interactions, side effects and toxicity issues that might arise from drug promiscuity must also be examined thoroughly.

2.5. SEARCHING FOR THERAPEUTIC ALTERNATIVES IN THE 'PREY' AND NOT IN THE 'PREDATOR'

Selection for antibiotic resistance in bacteria provides one of the most well-documented examples of an evolutionary response to natural selection (Walker and Levy, 2001). Similar resistance development has also been observed in viruses, fungi, plants and insects towards antiviral drugs, antifungals, herbicides and insecticides, respectively, creating increasing medical and economic problems (Maisnier-Patin and Andersson, 2004). Even before the beginning of the clinical use of antibiotics more than 60 years ago, resistant organisms had been isolated, which proves that this is not a recent problem (Levy, 2002). Indeed, antibiotic resistant genes have been characterized in coliforms from glacial water and ice in the Arctic estimated at 2000 years old (Barker, 1999).

Co-evolution between predator and prey has millions of years of experience to offer. Bacteria and fungi are the leading sources of therapeutic antimicrobials (Cowan, 1999). If one is indeed taking advantage of the natural weapons that microorganisms have been producing to fight with their 'enemies' for millions of years, perhaps one could expect that by co-evolution, resistance to these compounds would appear also naturally. The question then is, does the environmental resistome intersect with the resistome of clinical pathogens or are they distinct? Wright (2010) extensively reviewed connections that show a clearly link between resistance in the environment with the clinic: the CTX-M extended spectrum β-lactamase appears to have come from chromosomal genes of environmental genus *Kluyvera;* the *qnrA* gene associated with fluoroquinolone resistance has an environmental reservoir in the aquatic bacterium *Shewanella algae;* the gene cluster that confers resistance to vancomycin (a glycopeptide antibiotic) in *Enterococci* and *Staphylococci* have been identified in environmental *Bacilli*, in glycopeptide-producing bacteria, and in non-producing environmental strains (Wright, 2010). Additionally, culturable bacteria in soil were found to encode enzymes that degrade or inactivate antibiotics (Allen et al., 2010). Dantas et al. (2008) isolated hundreds of soil bacteria with intrinsic multidrug resistance phenotypes with the capacity to grow on antibiotics as a sole carbon source. In one soil species, over 400 actinomycetes cultured from forest, agricultural and urban soils were found to have highly varied resistance profiles and some of them even exhibited resistotypes that had not been seen before (D'Costa et al., 2006). Forsberg et al. (2012) describes MDR soil bacteria containing resistance cassettes against five classes of antibiotics (β-lactams, aminoglycosides,

amphenicols, sulfonamides, and tetracyclines) that have perfect nucleotide identity to genes from diverse human pathogens. Handelsman group identified and characterized several new antibiotic resistance genes from soil bacteria encoding for aminoglycoside acetyltransferases, tetracycline efflux (Riesenfeld et al., 2004), β-lactamases (Donato et al., 2010), fenicol efflux proteins (Lang et al., 2010), etc. Even in subsurface, bacterial antibiotic resistance was found to be common by Brown and Balkwill (2008): higher frequencies of resistance and multiple resistance bacteria to nalidixic acid, mupirocin or ampicillin were found.

The examples of resistance genes shared between environmental microbes and human pathogens raise questions regarding the clinical impact of environmental resistance. Microorganisms rapidly acquire resistance, which probably forces their competitors to produce inhibitors for such mechanisms. For example, plants have been extensively reported to be able to produce drug-resistance inhibitors in order to ensure the delivery of the antimicrobial compounds. These interesting compounds may have direct application on clinical setting. The chemical diversity between plants and microorganisms represents then a formidable possibility to identify MDR inhibitors from natural sources.

2.6. PLANTS DEFENSE SYSTEMS AS POTENTIAL SOURCES OF THERAPEUTIC STRATEGIES

In their ecosystem, plants are continuously exposed to a wide range of environmental stresses and hostile conditions. Stress factors affecting plant fitness include environmental (abiotic) factors, such as nutrient deficiency, hypoxia/anoxia, drought, salinity, adverse temperature fluctuations, high light intensity, and also those derived from anthropogenic activities, such as pesticides, pollutants and increased UV radiation (Iriti and Faoro, 2009; Suzuki et al., 2014). Besides, several living (biotic) factors are also stress-inducing factors, including bacteria, fungi, viruses, nematodes, insects and herbivore pests (Rausher, 2001).

Plants have faced most of their attackers for more than 350 million years. This allowed plants to co-evolved with their natural enemies in a reciprocal evolutionary interaction and to learn how to resist to their attacks. Although lacking mobile defender cells and a somatic adaptive immune system comparable to animals (Dangl and Jones, 2001), plants have the ability to recognize pathogen signals or elicitors and activate immune responses, such as the reinforcement of the cell wall, biosynthesis of lytic enzymes and production of secondary metabolites and pathogenesis related proteins (War et al., 2012).

Owing to their sessile lifestyle and this evolutionary arms race, plants have evolved a stunning broad array of structural, chemical defenses, formerly referred to as secondary metabolites,

designed to fight their invaders and protect themselves from damage. These compounds can be either constitutive, stored as inactive forms, known as phytoanticipins, or inducible in response to pathogen attack, known as phytoalexins (Fürstenberg-Hägg et al., 2013).

Phytoanticipins give the plant a characteristic odor (such as terpenoids), distinctive pigmentation (e.g., quinones and tannins) or their flavor (e.g., the terpenoid capsaicin from chili peppers) (Aiyegoro and Okoh, 2009; Simões et al., 2009; Zahin et al., 2010). Some phytoanticipins are found at the plant surface while others are present in vacuoles or organelles and released through a hydrolyzing enzyme after pathogen challenge (González-Lamothe et al., 2009).

Phytoalexins (including terpenoids, glycosteroids, and polyphenols) are small molecules (molecular weight < 500 g mol^{-1}) which are both synthesized and accumulated in plant after recognition of elicitors derived from the exposure to attackers (Hemaiswarya et al., 2008). There are four major pathways for synthesis of secondary metabolites: (1) shikimic acid pathway, (2) malonic acid pathway, (3) mevalonic acid pathway and (4) non-mevalonate pathway (Azmir et al., 2013). Polyphenolic compounds from plants show a huge structural diversity, including chlorogenic acids, hydrolyzable tannins, and flavonoids (flavonols, flavanones, flavan-3-ols, anthocyanidins, isoflavones, and flavones) (Marín et al., 2015). Other common classes of phytochemicals include alkaloids, lactones, diterpenes, triterpenes, glycosteroids and naphthoquinones (Cowan, 1999; Hemaiswarya et al., 2008; Lewis and Ausubel, 2006; Zahin et al., 2010).

Plant extracts have been utilized in the name of human health for centuries particularly in Asia to accelerate wound healing and to treat common infectious diseases. Such traditional medicines are still utilized in the routine treatment of various diseases. Examples include the use of bear-berry (*Arctostaphylos uva-ursi*) and cranberry juice (*Vaccinium macrocarpon*) to treat urinary tract infections, or essential oils of Tea Tree (*Melaleuca alternifolia*) as active ingredients in many topical formulations to treat cutaneous infections and also of *Hydrastis canadensis* and *Echinacea* species to "treat" tuberculosis infections (Gibbons, 2008; Nikaido, 1998; Schelz et al., 2010). Also, *Hydrastis canadensis* is used to combat inflammation and infection. Its antibacterial activity *in vitro* has been attributed to its alkaloids, the most abundant of which is berberine (Ettefagh et al., 2011).

Numerous clinical studies have proved the therapeutic value of molecules of plant origin on the human organism (Betoni et al., 2006; Gibbons, 2004; Schelz et al., 2010). Indeed, secondary metabolites are an important source of anticancer, antioxidant, antidiabetic, immunosuppressive, antifungal, anti-inflammatory, antimalarial, anti-oomycete, antibacterial, antifever, antidiabetic, insecticidal, nematicidal, and antiviral agents (Akram et al., 2014; da Silva et al., 2005; Dehghan

et al., 2016). In total, higher plant-derived products represent approximately 25% of drugs in current clinical use (Phillipson, 2007).

Due to their curative potential, plant extracts have been investigated for the development of novel drugs to control bacterial infections (Dickson et al., 2006; Jayaraman et al., 2010; Sibanda and Okoh, 2008). Of the more than 350000 species of higher plants currently recognized, only 5-10% have been investigated, and only ~15% phytochemically (Cragg and Newman, 2013), and considering that each plant species may contain 500-800 different secondary metabolites, the potential for the discovery of new therapeutic products in this largely untapped resource is considerable (Coutinho et al., 2010a; Miller, 2011; Schelz et al., 2010; Sibanda and Okoh, 2007).

Additionally, due to the current increase in awareness of the population of antibiotic resistance problems, the use of herbal medicinal products and supplements has increased tremendously over the past three decades with not less than 80% of people worldwide relying on them for some part of primary healthcare (Ekor, 2013). Indeed, in 2013, the global retail sale of herbal and botanical dietary supplements amounted to more than $6.032 billion in the United States, according to *HerbalGram* - the journal of the nonprofit American Botanical Council.

2.6.1. Synergistic interactions between plant phytochemicals

The therapeutic value of synergistic interactions has been known since antiquity, and many different cultural healing systems (such as Ayurveda and traditional Chinese herbal medicine) have relied on this principle and in the belief that combination therapy may enhance efficacy (van Vuuren and Viljoen, 2011). These synergistic interactions explain the efficacy of apparently low doses of active constituents in an herbal product (Aiyegoro and Okoh, 2009). This concept is based in the idea that an extract of a plant offers advantages over a single isolated ingredient. In fact, of the many medicines that have higher-plant origins, very few show strong antimicrobial activity. Phytochemical products usually produce MICs in the range of 100 to 1000 mg l^{-1} in *in vitro* susceptibility tests (Simões et al., 2009), much weaker than compounds produced by bacteria and fungi (MICs between 0.1 to 10 mg l^{-1}). Still, some products produced by plants can fight infections successfully (Sibanda and Okoh, 2007). In fact, the relative rarity of infectious diseases in wild plants is an indication of the effectiveness of their innate defense mechanisms (Hemaiswarya et al., 2008; Lewis and Ausubel, 2006). Hence, those phytochemicals are believed to work synergistically between each other, thus playing a role against infection in the plants defense system (Hemaiswarya et al., 2008; Sibanda, 2007; Tegos et al., 2002). Interaction with different targets by the different defensive components of the extract can promote synergistic effects and enhance the defensive system of plants. For example, in tomato, alkaloids, phenolics,

proteinase inhibitors and oxidative enzymes act synergistically, affecting the insect during ingestion, digestion and metabolism; in *Nicotiana attenuata*, trypsin proteinase inhibitors and nicotine expression contributed synergistically to the defensive response against *Spodoptera exigua* (Hub.) (Lepidoptera, Noctuidae) (War et al., 2012).

Some phytochemicals have been reported to inhibit important bacterial enzymes, thus having an interesting application in cotherapies with antibiotics. Myricetin, a flavonol found in many vegetables, herbs, berries and fruits, inhibited DNA B helicase in *E. coli*, which plays a central role during DNA replication initiation and elongation (Griep et al., 2007; Hemaiswarya et al., 2008). Moreover, myricetin inhibits a variety of DNA polymerases, RNA polymerases, reverse transcriptases and telomerases (Griep et al., 2007). This phytochemical also had a significant synergic activity against ESβL-producing *K. pneumoniae* in combination with cefoxitin, amoxicillin/clavulanate and ampicillin/sulbactam (Lin et al., 2005). Allicin is one of the most effective antimicrobial products isolated from garlic (*Allium sativum*) and was shown to potentiate the action of cefazolin and oxacillin against *Staphylococcus* spp., cefoperazone against *P. aeruginosa* (Cai et al., 2007), and vancomycin against VRE strains (Abascal and Yarnell, 2002). Allicin reportedly inhibits bacterial RNA synthesis (Hemaiswarya et al., 2008) and interacts with important thiol-containing microbial enzymes such as cysteine proteinases, acetate kinase, alcohol dehydrogenases, thioredoxin reductases and phosphotransacetyl-CoA synthetases (Ankri and Mirelman, 1999).

Nevertheless, many of the phytochemicals that are synthesized in response to pathogen invasion are not even antimicrobial. Most of the functions of such phytochemical products in plants are still unknown (Simões et al., 2009). They might have a regulatory function, indirectly increasing the level of resistance of the plant (Tegos et al., 2002), or can be produced if the interaction between constituents of the extract results in increased solubility and, thereby, enhance the bioavailability of active substances. A special synergy effect can then occur when antibiotics are combined with an agent that antagonizes bacterial resistance mechanisms (Wagner and Ulrich-Merzenich, 2009). The utility of plant products as antimicrobial potentiators and virulence attenuators has been intensively studied (González-Lamothe et al., 2009), much of it in the antifungal field. Some examples include marked synergy between azole antifungals and a group of small molecules called citridones, produced by *Penicillium* sp. (Kalan and Wright, 2011). The activity of rhein, the principal antimicrobial from rhubarb was potentiated 100- to 2000-fold (depending on the bacterial species) by disabling the MDR efflux pumps (Tegos et al., 2002). The following sections will focus on combinatorial activities of plant extracts and products with antibiotics, mainly due to a resistance-modifying activity.

2.6.2. *In vitro* antibiotic-potentiation by RMAs

The strategy of resistance-modifying activity implies that an antimicrobial product should be co-administered with an inhibitor that deactivates the bacterial resistance mechanism thus increasing the effectiveness of the antimicrobial product (Shahverdi et al., 2007). These products could be of significant benefit for the treatment of MDR infections. There are diverse phytochemical products which have already demonstrated the potential to act as RMAs and potentiated the antimicrobial effects of antibiotics. TABLE 2.2 shows several examples of such compounds described in bibliography. The chemical structures of these compounds can be found in Appendix A1.

TABLE 2.2 | Important examples of phytochemicals promoting antibiotic-potentiation due to a resistance-modifying activity

Phytochemical	Plant source	Antibiotic potentiated	Mechanisms of action	References
Carnosic acid; Carnosol	*Rosmarinus officinalis*	Tetracycline Erythromycin	MDR efflux pumps inhibition	(Gibbons et al., 2003a; Oluwatuyi et al., 2004; Stavri et al., 2007)
Reserpine	*Rauwolfia serpentina*	Fluoroquinolones Tetracycline	MDR efflux pumps inhibition	(Gibbons et al., 2003a; Gibbons and Udo, 2000; Markham et al., 1999; Marquez, 2005; Schmitz et al., 1998; Stavri et al., 2007)
Totarol Diterpene 416	*Chamaecyparis nootkatensis*	Norfloxacin Tetracycline Erythromycin Methicillin	NorA inhibition; Interference with PBP2a expression	(Gibbons, 2005; Nicolson et al., 1999; Simões et al., 2009; Smith et al., 2007a)
Berberine	*Berberis* spp.	Ampicillin Oxacillin	Intercalation into DNA; Increase membrane permeability	(Lewis and Ausubel, 2006; Simões et al., 2009; Stermitz et al., 2000b; Yu et al., 2005)
5*-methoxyhydno-carpin; pheophorbide *a*	*Berberis* spp.	Berberine Others (e.g. norfloxacin)	NorA inhibition	(Gibbons et al., 2003a; Guz et al., 2000; Stavri et al., 2007; Tegos et al., 2002)
Ferruginol 5-Epipisiferol	*Chamaecyparis lawsoniana*	Norfloxacin Erythromycin Oxacillin Tetracycline	EtBr efflux inhibition	(Sibanda and Okoh, 2007; Smith et al., 2007a)

Baicalein	*Scutellaria spp.*	Tetracycline β-lactams Gentamicin Ciprofloxacin	Inhibition of PBP2a; Reaction with the peptidoglycan; NorA inhibition	(Chan et al., 2011; Fujita et al., 2005; Wagner and Ulrich-Merzenich, 2009)
Catechin gallate; Epicatechin gallate; Epigallocatechin gallate	*Camellia sinensis*	β-lactams Norfloxacin Carbapenems Tetracycline	β-lactamases inhibition; PBP2a synthesis inhibition; Reaction with peptidoglycan; EtBr efflux inhibition; TetK inhibition	(Hu et al., 2002; Marquez, 2005; Roccaro et al., 2004; Shibata et al., 2005; Yam et al., 1998; Zhao et al., 2001)
Methyl-1α-acetoxy-7α-14α-dihydroxy-8,15-isopimaradien-18-oate; Methyl-1α,14α-diacetoxy-7α-hydroxy-8,15-isopimaradien-18-oate	*Lycopus europaeus*	Tetracycline Erythromycin	MDR efflux pumps inhibition	(Gibbons et al., 2003b)
Piperine	*Piper nigrum, Piper longum*	Ciprofloxacin	EtBr efflux inhibition	(Jin et al., 2011; Khan et al., 2006)
2,6-dimethyl-4-phenyl-pyridi-ne-3,5-dicar-boxylic acid diethyl ester	*Jatropha elliptica*	Ciprofloxacin Norfloxacin	NorA inhibition	(Marquez et al., 2005; Sibanda and Okoh, 2007)
Ethil gallate	*Caesalpinia spinosa*	β-lactams	Restriction of substrate diffusion for PBPs	(Shibata et al., 2005)
Cinnamaldehyde	*Cinnamomum zeylanicum*	Clindamycin	CdeA inhibition	(Shahverdi et al., 2007)
Tellimagrandin I; Rugosin B	*Rosa canina* L.	β-lactams	Inactivation of PBPs, particularly PBP2a	(Shiota et al., 2000; Shiota et al., 2004)
Corilagin	*Arctostaphylos uva-ursi*	β-lactams Cefmetazole	Inhibition of PBP2a activity or production	(Shimizu et al., 2001; Shiota et al., 2004)
Silybin	*Silybum marianum*	Ampicillin Oxacillin	MDR efflux pumps inhibition	(Kang et al., 2011; Stermitz et al., 2000b)
Polyacylated neohesperidoses	*Geranium caespitosum*	Berberine Ciprofloxacin Norfloxacin Rhein	MDR efflux pumps inhibition	(Stavri et al., 2007; Stermitz et al., 2003; Tegos et al., 2002)
Chrysosplenol-D Chrysoplenetin	*Artemisia annua*	Artemisinin Berberine Norfloxacin	MDR efflux pump inhibition	(Stavri et al., 2007)
Chalcone	*Dalea versicolor*	Berberine Erythromycin Tetracycline	NorA inhibition	(Belofsky et al., 2004; Stavri et al., 2007; Zdzisława, 2007)

4',5'-*O*-dicaffeoyl-quinic acid	*Artemisia absinthium*	Berberine Fluroquinolones	MFS family efflux systems inhibition	(Fiamegos et al., 2011)
Genistein; Orobol; Biochanin A	*Lupinus argenteus*	Berberine Norfloxacin	MDR efflux pump inhibition	(Morel et al., 2003)

Rosmarinus officinalis L. (commonly referred to as rosemary) extracts act as a soft analgesic and antimicrobial agent in traditional use (Lo et al., 2002; Pintore et al., 2002). Extracts of rosemary provide a major source of antimicrobials including carnosic acid and carnosol (Frankel et al., 1996; Oluwatuyi et al., 2004). These compounds at 10 mg l^{-1} were found to potentiate the activity of tetracycline (2- and 4-fold, respectively) against a TetK possessing *S. aureus* strain and carnosic acid showed a 8-fold reduction in MIC of erythromycin against *S. aureus* strains expressing MsrA (Sibanda and Okoh, 2007). Additionally, carnosic acid was shown to inhibit EtBr (a substrate for several MDR pumps) efflux in a NorA expressing *S. aureus* strain with an IC_{50} of 50 mM (1/4 of the MIC for the strain) (Oluwatuyi et al., 2004; Sibanda and Okoh, 2007). However, this activity is likely to be related to the inhibition of efflux pump(s) other than NorA.

The plant alkaloid reserpine produced by *Rauwolfia serpentina* was originally found to inhibit Bmr efflux pump (Guz et al., 2000; Stavri et al., 2007). Its EPI activity was also demonstrated against the NorA pump in *S. aureus* by reducing the MIC of norfloxacin at least 4-fold (Gibbons et al., 2003a; Markham et al., 1999). Reserpine reduced sparfloxacin, moxifloxacin and ciprofloxacin MICs up to 4-fold in several clinical isolates of *S. aureus* (Schmitz et al., 1998). Gibbons and Udo (2000) showed the same reduction for tetracycline in clinical isolates of MRSA with the TetK determinant. Reserpine also inhibited LmrA of *L. lactis* (Marquez, 2005). However, it was found that reserpine is toxic for humans at concentrations required for MDR pumps inhibition (Dickson et al., 2006; Markham et al., 1999). Moreover, bacterial resistance to this natural product was already observed (Hemaiswarya et al., 2008).

Berberine, a hydrophobic alkaloid produced by *Berberis* species (Hemaiswarya et al., 2008), is known to increase membrane permeability and to intercalate into DNA (Lewis and Ausubel, 2006). Yu et al. (2005) found an additive effect between berberine and ampicillin and a synergistic effect with oxacillin against MRSA. This product has the potential to restore the effectiveness of β-lactam antibiotics against MRSA (Stermitz et al., 2000b; Yu et al., 2005). However, berberine is rapidly extruded by multidrug efflux pumps (Stermitz et al., 2000a). It was observed that *Berberis* species also synthesize the 5'-methoxyhydnocarpin-D and pheophorbide a, which were identified as inhibitors of the NorA MDR efflux pump in *S. aureus* (Guz et al., 2000; Marquez, 2005; Sibanda and Okoh, 2007; Stermitz et al., 2000b; Tegos et al., 2002). These MDR inhibitors facilitate the penetration of berberine into *S. aureus* by completely inhibiting its efflux from the

cell (Gibbons et al., 2003a; Tegos et al., 2002; Wagner and Ulrich-Merzenich, 2009). This is an important example of synergy between components from the same plant. 5'-methoxyhydnocarpin-D also had a synergistic effect with several other products exported by NorA, including norfloxacin (Stavri et al., 2007). An extract of *Dalea versicolor*, chalcone, reportedly enhanced the activity of berberine, erythromycin and tetracycline against *S. aureus*, also apparently due to NorA inhibition (Belofsky et al., 2004; Stavri et al., 2007; Zdzisława, 2007). Chalcone increased the activity of berberine against *B. cereus* by 30-fold (Zdzisława, 2007). This is also an interesting case where products with strong direct antimicrobial activity and those with MDR pump inhibitory action were found in the same plant (Belofsky et al., 2004). 4',5'-*O*-dicaffeoylquinic acid from *Artemisia absinthium* also potentiated berberine and fluroquinolones against a wide panel of Gram-positive human pathogenic and was characterized by Fiamegos et al. (2011) as an efflux pump inhibitor with the potential to target efflux systems especially of the MFS family. The flavones chrysosplenol-D and chrysoplenetin isolated from *Artemisia annua* potentiated the activity of the antimalarial artemisinin against *Plasmodium falciparum* in the presence of berberine and norfloxacin (Stavri et al., 2007), due to NorA inhibition. The isoflavones genistein, orobol and biochanin A, isolated from *Lupinus argenteus*, potentiated the antibacterial activity of α-linolenic acid, also from the same plant, and enhanced the antibacterial activity of berberine and norfloxacin against *S. aureus* cells, indicating that they may inhibit MDR pumps (Morel et al., 2003).

Totarol, a diterpene isolated from immature cones of *Chamaecyparis nootkatensis*, reduced the MIC of tetracycline, norfloxacin and erythromycin for 4-, 4- and 8-fold, respectively, in MRSA strains (Smith et al., 2007b), suggesting that this product is a NorA EPI (Smith et al., 2007b). Totarol also potentiated by 8-fold activity of methicillin against MRSA (Nicolson et al., 1999). Despite the fact that the primary staphylococcal target for totarol is the respiratory chain, it was found that this synergic activity is due to the interference with PBP2a expression (Gibbons, 2005; Nicolson et al., 1999). Other studies suggest that this product may affect the production of macromolecules, including DNA and peptidoglycan, causing cell membrane disruption (Nicolson et al., 1999; Simões et al., 2009; Smith et al., 2007b). Other diterpenes, especially diterpene 416 (14-methylpodocarpa-8,11,13-trien-13-ol; 5), also potentiated methicillin, with a reduction on the MIC of more than 256-fold against MRSA, owing to a significant reduction in PBP2a expression (Nicolson et al., 1999). Two isopimarane diterpenes, methyl-1α-acetoxy-7α,14α-dihydroxy-8,15-isopi-maradien-18-oate and methyl-1α,14α-diacetoxy-7α-hydroxy-8,15-isopimaradi-en-18-oate, isolated from an extract of *Lycopus europaeus* by Gibbons et al. (2003b) demonstrated a 2-fold potentiation of tetracycline and erythromycin against two strains of *S. aureus* possessing the TetK, MsrA and NorA efflux-mechanisms.

Japanese green tea (*Camellia sinensis*) is consumed every day by billions of people worldwide for its antipyretic, antidotal, antidiarrheal and diuretic effects (Zhao et al., 2001). These benefits of green tea are not only due to the bacteriostatic and bactericidal activities of some constituents but also to the presence of several resistance-inhibitory products (Yam et al., 1998). Aqueous extracts of tea, especially catechin gallate, epicatechin gallate and epigallocatechin gallate, demonstrated the potential to reverse methicillin resistance in MRSA and penicillin resistance in β-lactamase-producing *S. aureus*, apparently by the prevention of PBP2a synthesis and the inhibition of β-lactamase secretion, respectively (Hu et al., 2002; Marquez et al., 2005; Roccaro et al., 2004; Yam et al., 1998). Moreover, in the presence of catechin gallate and epicatechin gallate the MIC of oxacillin was reduced from 256 and 512, respectively, to 1-4 mg l^{-1} against MRSA (Shibata et al., 2005). Epigallocatechin gallate had much lower activity than epicatechin gallate, although only differing by an extra hydroxyl group on the B-ring, causing a reduction in oxacillin MIC ranging between 4 and 64-fold (Smith et al., 2007a). Gibbons et al. (2004) showed that epicatechin gallate and epigallocatechin gallate caused a 4-fold potentiation of the activity of norfloxacin against a NorA over-expressing *S. aureus* strain. Another explanation for this synergy with β-lactams against MRSA is the possible interference of these products with the integrity of the cell wall through direct binding to peptidoglycan (Zhao et al., 2001). Additionally, epigallocatechin gallate was found to synergistically enhance the activity of carbapenems against MRSA (Hemaiswarya et al., 2008; Hu et al., 2002). It was also demonstrated that epicatechin gallate and epigallocatechin gallate are able to reverse tetracycline resistance in staphylococcal isolates expressing TetK and TetB efflux pumps, probably due to the inhibition of these pumps (Marquez, 2005; Roccaro et al., 2004).

Baicalein, isolated from extracts of the leaves of *Scutellaria baicalensis*, which is one of the most popular herbs in China for the treatment of bacterial and viral infections (Chan et al., 2011), had a synergistic effect with tetracycline against MRSA with and without TetK-genes and in TetK overexpressing *E. coli* (Fujita et al., 2005). This flavone potentiated the effects of β-lactam antibiotics against MRSA (Wagner and Ulrich-Merzenich, 2009). Chan et al. (2011) also demonstrated synergy between baicalein and ciprofloxacin against MRSA strains and with gentamicin against VRE, apparently by the inhibition of the NorA efflux pump. The synergistic actions of this compound on MRSA may therefore involve several mechanisms of action such bacterial efflux pumps inhibition (others different from TetK), PBPs inhibition or cell wall disintegration by interference with the peptidoglycan structure (Chan et al., 2011; Wagner and Ulrich-Merzenich, 2009). Tellimagrandin I and rugosin B, obtained from the extract of petals of *Rosa canina* L. (rose red), markedly reduced the MIC of β-lactams against MRSA (Shiota et al., 2000), possibly due to the inactivation of PBPs, especially of PBP2a (Shiota et al., 2004). Shimizu et al. (2001) found that corilagin from an extract of *Arctostaphylos uva-ursi* markedly reduced

the MIC of β-lactams, such as oxacillin and cefmetazole, by 100- to 2000-fold in MRSA, also possibly due to inhibition of PBP2a, its activity or its synthesis (Shimizu et al., 2001; Shiota et al., 2004).

Piperine, the major plant alkaloid in black pepper (*Piper nigrum*) and long pepper (*Piper longum*) reduced the MIC of ciprofloxacin (Khan et al., 2006) and mupirocin (Mirza et al., 2011) against *S. aureus*, including MRSA, and decreased EtBr efflux (Jin et al., 2011). Smith et al. (2007a) isolated active compounds from the cones of *Chamaecyparis lawsoniana* and observed that ferruginol and 5-epipisiferol were effective in increasing the efficacy of tetracycline, norfloxacin, erythromycin and oxacillin against resistant *S. aureus*. It was demonstrated that ferruginol inhibited in 40% the efflux of EtBr, using a NorA expressing *S. aureus* strain (Smith et al., 2007a). The penta-substituted pyridine 2,6-dimethyl-4-phenylpyridine-3,5-dicarboxylic acid diethyl ester, isolated from an ethanol extract of rhizome of *Jatropha elliptica* (Marquez et al., 2005), increased by 4-fold the activity of ciprofloxacin and norfloxacin against NorA expressing *S. aureus*, suggesting an efflux pump inhibition (Marquez et al., 2005; Sibanda and Okoh, 2007). Cinnamaldehyde from *Cinnamomum zeylanicum* bark essential oil may inhibit CdeA efflux pump in *Clostridium difficile* (Shahverdi et al., 2007), and reduced the MIC of ampicillin, tetracycline, penicillin, erythromycin and novobiocin in *E. coli* N00 666 (Palaniappan and Holley, 2010). Garvey et al. (2011) extracted *Levisticum officinale* and identified falcarindiol, oleic acid and linoleic acid in the fractions displaying the greatest synergy with five antibiotics; possibly by an efflux inhibition of AcrAB–TolC. Geraniol, found in the essential oil of *Helichrysum italicum*, significantly increased the efficacy of β-lactams, quinolones, and chloramphenicol in *E. coli*, *P. aeruginosa*, *Enterobacter aerogenes* and *A. baumannii* (Lorenzi et al., 2009). The flavonolignan silybin obtained from *Silybum marianum*, which is one of the oldest known traditional European medicine and has mainly been used for liver disorders (Kitajima and Yamaguchi, 2009), reduced by more than 4-fold the MIC of ampicillin or oxacillin against 20 clinical isolates of MRSA (Kang et al., 2011), possibly by a MDR pump inhibition (Park et al., 2012; Stermitz et al., 2000b). Two poliacylated neohesperidosides from *Geranium caespitosum* increased the activity of ciprofloxacin and norfloxacin, also apparently due to MDR pump inhibition (Stavri et al., 2007; Stermitz et al., 2003; Tegos et al., 2002).

2.6.3. *In vitro* antibiotic-potentiation by membrane permeabilizers

Another strategy to overcome resistance is to improve the delivery or enhance the accessibility of antibiotics to their sites of action. Many compounds have been reported to affect membrane permeability of a diverse range of microorganisms (Helander et al., 1998), mainly due to the perturbation of the lipid fraction of the cell membrane. Also, owing to their lipophilic character,

they can increase membrane permeability (Trombetta et al., 2005). Such permeabilizers can non-specifically enhance the permeability of bacterial cells to exogenous products, including antimicrobial agents, and may therefore potentiate the antibacterial activity of antibiotics that interact with intracellular targets (Simões et al., 2009).

Thymol and carvacrol, two main products of the essential oil of *Thymus vulgaris*, reportedly disintegrate the OM and thus increase membrane permeability and fluidity in Gram-negative bacteria, facilitating the penetration of antibiotics (Helander et al., 1998; Lambert et al., 2001; Schelz et al., 2010; Simões et al., 2009; Wagner and Ulrich-Merzenich, 2009). The combined application of both products resulted in a stronger antibacterial effect than if applied separately (Szczepaniak et al., 2011). Synergistic interactions have been reported between thymol and carvacrol with antibiotics against *Sphingomonas paucimobilis* and *Klebsiella oxytoca* (Zhang et al., 2011). These products increased the susceptibility of *Salmonella enterica* Typhimurium SGI-1 to ampicillin, tetracycline, penicillin, bacitracin, erythromycin and novobiocin and the resistance of *Streptococcus pyogenes* with the macrolide-resistant gene *ermB* to erythromycin (Palaniappan and Holley, 2010).

Ethyl gallate and other alkyl gallates purified from a dried pod of *Caesalpinia spinosa*, reduced the MIC of β-lactams against MRSA and methicillin-sensitive *S. aureus* (MSSA) strains (Shibata et al., 2005). The mechanism of action of these gallates is still unknown but, the possible perturbation on the membrane and the resulting restrictions on its components fluidity could difficult the diffusion of substrates for PBPs (Shibata et al., 2005).

Gallic acid from berry extracts has proven to be an efficient permeabilizer for several *Salmonella* strains (Nohynek et al., 2006). Moreover, synergic interactions were observed with the combination of gallic acid with tetracycline against *P. aeruginosa* strains (Jayaraman et al., 2010) and with streptomycin against *E. coli* and *P. aeruginosa* (Saavedra et al., 2010). The OM disintegrating activity of gallic acid was suggested to be based on the chelation of divalent cations from the OM. Additionally, the partial hydrophobicity of this product (Nohynek et al., 2006), decrease of negative surface charge, and occurrence of local rupture or pore formation in the cell membranes also allowed bacterial membrane destabilization (Borges et al., 2012).

A putatively synergic effect has been reported for the antibacterial constituents of *Humulus lupulus*, xanthohumol and lupulon, and some antibiotics such as polymyxin B sulfate, tobramycin and ciprofloxacin, against Gram-positive and Gram-negative bacteria (Wagner and Ulrich-Merzenich, 2009). The antimicrobial action of xanthohumol and lupulon was due to changes induced in the properties and permeability of the membrane (Natarajan et al., 2008).

2.6.4. Plant extracts with resistance-modifying activity *in vitro*

In addition to the synergistic effects observed for the previously described products, a large number of other *in vitro* studies have reported the capacity of plant extracts to potentiate the activity of antibiotics. Mechanistic studies into such synergistic effects is often complicated by the fact that plant extracts consist of complex mixtures of compounds, several of which can be involved in the final result (Wagner and Ulrich-Merzenich, 2009). The screening of these extracts is, however, expected to provide leads for the isolation of MDR inhibitors (Sibanda and Okoh, 2008). Some examples are described in this section.

Methanolic extracts of *Punica granatum* have shown synergistic activity by the broth dilution method and time-kill assays with chloramphenicol, gentamicin, ampicillin, tetracycline and oxacillin against strains of MRSA and MSSA (Braga et al., 2005). The extracts also demonstrated the potential to either inhibit the efflux pump NorA or to enhance the influx of the antibiotics (Braga et al., 2005). Aqueous crude extracts of *Catha edulis* at sub-MIC potentiated the action of tetracycline against *Streptococcus sanguis* TH-13 and *Streptococcus oralis* SH-2 (2- and 4-fold, respectively), and of penicillin-G against *Fusobacterium nucleatum* 9911 (4-fold) (Al-hebshi et al., 2006). Ethanol extracts of *Eugenia uniflora* L. and *Eugenia jambolanum* exhibited synergistic activity with gentamicin against *E. coli* (Coutinho et al., 2010b). Synergistic interactions were also observed between extracts of several Brazilian plants and eight antibiotics against *S. aureus*, indicating the presence of a range broad of inhibitors of protein synthesis, cell wall synthesis, nucleic acid synthesis and folic acid synthesis (Betoni et al., 2006). Methanolic extracts of 19 Jordanian plants had significant synergistic interactions against MRSA and MSSA strains in combination with chloramphenicol, gentamicin, erythromycin and penicillin G (Darwish et al., 2002). Ethanol extracts of the Chinese plants *Isatis tinctoria*, *Scutellaria baicalensis* and *Rheum palmatum* improved the activity of ciprofloxacin, penicillin, gentamicin and ceftriaxone against antibiotic resistant *S. aureus* strains (Yang et al., 2005). *Dalea spinosa* (smoke tree) extracts potentiated antibiotic activity against MRSA, due to MDR efflux pump inhibition (Belofsky et al., 2006; Simões et al., 2009). Coutinho et al. (2010a) demonstrated that an ethanol extract of *Momordica charantia* L. reduced the MIC for some aminoglycosides against MRSA. Extracts of *Securinega virosa* and *Mezoneuron benthamianum* exerted a potentiation activity against fluoroquinolone-, tetracycline- and erythromycin-resistant *S. aureus* strains (Stavri et al., 2007). Additionally, 4-fold potentiation of the activity of norfloxacin was observed with ethanol extracts of *M. benthamianum* and, in a less extension, with chloroform extracts of *S. virosa* (Dickson et al., 2006). Coutinho et al. (2009) reported that the ethanol extract of *Turnera ulmifolia* L. had a synergic effect with aminoglycosides. Acetone extracts of *Garcinia kola* seeds potentiated the

activity of tetracycline and chloramphenicol against *E. coli* and *K. pneumonia* and of amoxicillin and penicillin G against *S. aureus* (Sibanda and Okoh, 2008).

Adwan and Mhanna (2008) demonstrated a significant reduction in the MIC of several antimicrobial agents with water extracts of *Psidium guajava*, *Rosmarinus officinalis*, *Salvia fruticosa*, *Majorana syriaca*, *Ocimum basilucum*, *Syzygium aromaticum*, *Laurus nobilis* and *Rosa damascene* against MRSA and MSSA. Ethanolic extracts of some Indian medicinal plants, *Acorus calamus*, *Hemidesmus indicus*, *Holarrhena antidysenterica* and *Plumbago zeylanica*, demonstrated synergistic activities when combined with tetracycline, chloramphenicol, ciprofloxacin, cefuroxime and ceftidizime against several MRSA strains (Aqil et al., 2006) and with tetracycline and ciprofloxacin against ESβL-producing MDR enteric bacteria (Ahmad and Aqil, 2007). Synergistic interactions of crude extracts from *Camellia sinensis*, *Lawsonia inermis*, *Terminalia chebula* and *Terminalia belerica* have been reported with tetracycline against MRSA and MSSA strains and with ampicillin for *Camellia sinensis* extract (Aqil et al., 2005). Extracts of *Commiphora molmol*, *Centella asiatica*, *Daucus carota*, *Citrus aurantium* and *Glycyrrhiza glabra* had a good activity against *S. enterica* serovar Typhimurium overexpressing the AcrAB-TolC efflux protein (Stavri et al., 2007). Synergy was found between ethanolic extracts of *Rhus coriaria* (seed) and some antimicrobial drugs, including oxytetracycline HCl, penicillin G, cephalexin, sulfadimethoxine sodium and enrofloxacin against MDR *P. aeruginosa* strains, suggesting the presence of natural inhibitors, including EPIs (Adwan et al., 2010). The combinations of the methanol extract of the leaves of *Helichrysum pedunculatum* and 8 first-line antibiotics were investigated against several bacterial strains implicated in wound infections and 60% of the interactions were synergic (Aiyegoro et al., 2009b). The combination of methanol extracts of *Helichrysum longifolium* with penicillin G sodium, amoxicillin, chloramphenicol, oxytetracycline, erythromycin and ciprofloxacin against several bacterial isolates of referenced, clinical and environmental strains resulted in 61.7% of synergy for all interactions (Aiyegoro et al., 2009a). Touani et al. (2014) found that the methanol extracts of *Brassica oleacera* var. *butyris*, *Brassica oleacera* var. *Italica*, *capsicum frutescens* var. *facilulatum* and *Basilicum polystachyon* showed synergistic effects [fractional inhibitory concentration (FIC) ≤ 0.5] with an average of 75.3% of the tested antibiotics against MDR Gram-negative bacteria. Barreto et al. (2015) showed that the ethanol and hexane extracts of stem bark of *Anadenanthera colubrine* (Vell.) Brenan var. *cebil* enhanced the activity of neomycin and amikacin against *S. aureus* SA10 strain. Barreto et al. (2014) also showed a 10-fold reduction of the MIC of neomycin and amikacin when combined with the essential oil from *Lippia origanoides* H.B.K. against MRSA strain.

Many examples of synergistic activities in which essential oils have been found to reduce the minimum effective dose of antibiotics in the treatment of infections are described by Yap et al.

(2014). Aumeeruddy-Elalfi et al. (2015) reported the synergy of the essential oils of *Pimenta dioica*, *Psidia arguta* and *Piper betle* when combined with gentamicin against *E. coli* and *S. epidermis*. Fankam et al. (2015) investigated the antibacterial and antibiotic-resistance modifying activities of the methanol extracts from *Allanblackia gabonensis*, *Gladiolus quartinianus* and *Combretum molle* against 29 Gram-negative bacteria including MDR phenotypes. Percentages of antibiotic-modulating effects ranging from 67 to 100% were observed against MDR bacteria when combining the leaves extract from *C. molle* (½ and ¼ MIC) with chloramphenicol, kanamycin, streptomycin and tetracycline (Fankam et al., 2015). Tankeo et al. (2015) found synergistic effects against a panel of Gram-negative bacteria, including MDR phenotypes expressing active efflux pumps, obtained with *Beilschmedia acuta* bark extract and tetracycline as well as with *Polyscias fulva* leaves extract at MIC/2 and tetracycline and kanamycin. Also, in wild mushrooms extracts, especially from *Mycena rosea* and *Fistulina hepatica*, interesting synergistic activities between the extracts and commercial antibiotics (penicillin, ampicillin, amoxicillin/clavulanic acid, cefoxitin, ciprofloxacin, cotrimoxazol, levofloxacin) against *E. coli*, ESβL *E. coli* and MRSA (Alves et al., 2014).

2.6.5. Plant extracts promoting the dispersal of a biofilm to planktonically growing cells or quorum-sensing inhibition

Plant-based antimicrobial studies on planktonic microorganisms have been given extensive priority. The inhibition of biofilms, whether on independent or combined plant inhibitors, however, has been largely neglected (van Vuuren and Viljoen, 2011). Potential therapies for biofilm control include enzymes that dissolve the matrix polymers of the biofilm, chemical reactions that block biofilm matrix synthesis, and analogues of microbial signaling molecules that interfere with cell-to-cell communication, required for normal biofilm formation (Stewart and William Costerton, 2001).

Many bacteria are known to regulate diverse physiological processes through a mechanism called quorum-sensing which is accomplished through the production, secretion and subsequent detection of extracellular signal molecules called autoinducers (AIs). When these molecules reach a particular threshold concentration, they are taken up by other microbes and trigger adaptive changes appropriate to the community of organisms (Cvitkovitch et al., 2003; Fernández et al., 2011; Hammer and Bassler, 2003). QS has been found to regulate a number of physiological activities, including motility, conjugation, competence, sporulation, virulence and biofilm formation. Some of these quorum sensing molecules, such as the *Pseudomonas* quinolone signal (PQS) and the N-acyl homoserine lactones (AHL) from *P. aeruginosa*, possess antimicrobial activity at very high concentrations (Fernández et al., 2011). Consequently, QS pathways of

competing bacteria are potential targets for such nontoxic chemical defenses. Screening for natural products able to promote biofilm dispersal has led to the identification of inhibitors of AHL-based QS, such as bromoageliferin and oroidin (Landini et al., 2010). The aqueous extract of *Moringa oleifera* was found to inhibit violacein production, a QS-regulated behavior in *Chromobacterium violaceum* (Singh et al., 2009). In other study, the hexane, chloroform and methanol extracts of an Ayurveda spice, namely clove (*Syzygium aromaticum)*, shown anti-QS activity by inhibiting the response of *C. violaceum* CV026 to exogenously supplied *N*-hexanoyl-homoserine lactone, in turn preventing violacein production (Krishnan et al., 2012). Aqueous extracts of six plants, *Conocarpus erectus*, *Chamaesyce hypericifolia*, *Callistemon viminalis*, *Bucida buceras*, *Tetrazygia bicolor* and *Quercus virginiana*, caused the inhibition of QS genes and QS-controlled factors, with marginal effects on bacterial growth of *P. aeruginosa* (Adonizio et al., 2008). Also, common dietary fruit, herb and spice extracts showed a significant inhibition of QS (Vattem et al., 2007).

2.6.6. *In vivo* tests of phytochemicals

Extracts from many plant species have been previously tested against hundreds of bacterial strains *in vitro*, and some of them have demonstrated antimicrobial activity. All these findings have provided good indications for the potential of plants to be applied in combination with antibiotics to treat infectious diseases. Unfortunately, much of the data acquired are only preliminary, having been derived from *in vitro* antimicrobial assays, potentiation assays and efflux studies; and the mechanisms of action often remain unexplained (Stavri et al., 2007). Further research should therefore focus on the use of preclinical animal infection models, followed when appropriate, by clinical trials, enabling the definition of pharmacokinetic and pharmacodynamic targets and the measurement of many other parameters at the site of infection, such as antimicrobial efficacy, appropriate doses and safety data (Hemaiswarya et al., 2008; Renneberg, 1993). It is also required enormous investigation to elucidate side-effects and strategies of extraction and preservation of these products (Mendonça-Filho, 2006).

In this context, studies evaluating RMAs or phytochemicals-antibiotics therapies *in vivo* are few. To date, the efficacy of EPIs has been demonstrated in the therapy of malignancies *in vitro* and *in vivo*, as reviewed by Tegos et al. (2011). The only documented microbial EPI is currently MP-601,205, used for respiratory infections in patients with cystic fibrosis and ventilator-associated pneumonia (Tegos et al., 2011). The activity of levofloxacin plus an efflux pump inhibitor (a low molecular weight dipeptide amide) was evaluated in an animal model of a *P. aeruginosa* infection caused by a strain overexpressing the MexAB–OprM MDR efflux system and demonstrated efficacy (Renau et al., 1999). The combination of piperine with mupirocin in a

dermal infection model of mice showed better *in vivo* efficacy when compared with the commercially available formulation of 2% mupirocin (Mirza et al., 2011). The combination of Japanese green tea extract with levofloxacin against enterohemorrhagic *E. coli* infection in a gnotobiotic mouse model produced an increased survival rate and the prevention of tissue damage (Isogai et al., 2001). Isbrucker et al. (2006) developed safety studies of epigallocatechin gallate and demonstrated that this product caused minor dermal irritation in rats and guinea pigs, but not rabbits, and was a moderate dermal sensitizing agent in the guinea pig maximization test. Moreover, the dietary administration of epigallocatechin gallate to rats for 13 weeks was not toxic at doses up to 500 mg kg^{-1} day^{-1} (Isbrucker et al., 2006) and to 40 volunteers for 4 weeks at 800 mg day^{-1} only caused minor adverse effects (Doughari et al., 2009). Co-administration of EPIs with an antibiotic already progressed to human clinical trials (Guz et al., 2000; Lechner et al., 2008).

Other studies have focused on the *in vivo* antimicrobial activity of plant extracts or phytochemicals. For example, Chowdhury et al. (1991) demonstrated that allicin has promising *in vivo* antibacterial activity against *Shigella flexneri* when tested in the rabbit model of experimental shigellosis. Si et al. (2006) studied the antimicrobial activity of carvacrol, thymol and cinnamaldehyde against *Salmonella* in pig diets and reported that higher concentrations were needed to retain their antimicrobial activity when added to the diets. The effects of oolong tea polyphenols on dental caries in rats were tested and showing that total fissure caries lesions was significantly reduced by the addition of tea polyphenols to the diet or in the drinking water (Sakanaka et al., 1992). Tea catechins also inhibited the fluid accumulation induced by cholera toxin in sealed adult mice and reduced fluid accumulation by *Vibrio cholerae* O1 in intestinal loops of rabbits, suggesting that tea catechins may possess protective activity against *V. cholerae* O1 (Toda et al., 1992). *P. aeruginosa* lung infection of athymic rats was used to test subcutaneous administrations of ginseng, which seems to increase the resistance of the athymic rats to this bacteria (Song et al., 1997).

2.7. STUDYING PHYTOCHEMISTRY – IMPORTANT STEPS AND TECHNIQUES

To study phytochemistry, several factors must be considered for the preparation and extraction of the plant material, followed by the separation, purification and identification of the different constituents of plants. A brief description of important methods is made in this section.

2.7.1. Sample preparation and extraction

Sample preparation is the crucial first step in the analysis of plants because it is necessary to extract the desired chemical components (Huie, 2002). Proper actions must be taken to assure that potential active constituents are not lost, distorted or destroyed (by the presence of water, for example), etc. Preparation of plant material usually includes the following steps: pre-washing, drying (or freeze drying) and grinding to obtain a homogenous sample and increase the contact of sample surface with the solvent system (Sasidharan et al., 2011). Then, extraction plays a significant and crucial role on the final result and outcome (Azmir et al., 2013). The most common factors affecting extraction processes are matrix properties of the plant part, solvent, temperature, pressure and time (Huie, 2002; Wang and Weller, 2006). The selection of the solvent system largely depends on the chemical properties of the target compounds. Different solvent systems are available to extract the bioactive compound from natural products: polar solvents such as methanol, ethanol or ethyl-acetate are more suitable for the extraction of hydrophilic compounds, while for more lipophilic compounds, dichloromethane or a mixture of dichloromethane/ methanol in ratio of 1:1 can be used (Sasidharan et al., 2011). Extraction of plant materials can be done by various extraction procedures and the suitability of the methods of extraction according to characteristics of the target compounds must be considered. Methods such as ultrasound, sonication, pulsed electric field, enzyme digestion, extrusion, ohmic heating, soxhlet, heating under reflux can be applied. More modern methods such as surfactant-mediated extraction, supercritical-fluid extraction, pressurized-liquid extraction, microwave-assisted extraction and solid-phase extraction possess certain advantages. These are, e.g. the reduction in organic solvent consumption and in sample degradation, elimination of additional sample clean-up and concentration steps before chromatographic analysis, improvement in extraction efficiency, selectivity, and/ kinetics of extraction (Huie, 2002; Sasidharan et al., 2011).

2.7.2. Characterization of bioactive compounds

After extraction, further separation, identification, and characterization of bioactive compounds is required. Metabolomics, one of the ‘omic’ sciences in systems biology, is the discipline where endogenous and exogenous metabolites are assessed, identified and quantified within a biologic system (Zhang et al., 2012). Nowadays, ^{1}H NMR, Gas Chromatography–Mass Spectrometry (GC–MS) and Liquid Chromatography–Mass Spectrometry (LC–MS) are well-established powerful analytical methods for generating metabolomics profiles (Patel et al., 2010). These techniques have their advantages and disadvantages. For instance, GC–MS requires sample derivatization, which lengthens the sample preparation time (O'Gorman et al., 2013). In general, LC–MS and GC–MS are more time-consuming concerning the sample preparation. On the other

hand, GC–MS and LC–MS yield a higher sensitivity than NMR and therefore may detect metabolites that are present in a concentration below the detection limit of ^{1}H NMR (Scalbert et al., 2009). ^{1}H NMR is non-destructive, non-biased, and easily quantifiable, permits the identification of novel compounds and needs no chemical derivatization (Wishart, 2008). ^{1}H NMR may detect compounds that are too volatile for GC, while metabolites without proton (phosphoric acid) are not detected by ^{1}H NMR.

The technological developments in the field of NMR spectroscopy have enabled the identification and quantitative measurement of the many metabolites in a non-targeted and non-destructive manner (Smolinska et al., 2012). NMR-based metabolomics are finding use in plant science in discovery-oriented natural products chemistry (Kim et al., 2010a). Activity guided fractionation, a common approach in natural products research, fails when several metabolites act synergistically. A metabolomics approach has proved to be very efficient in detecting synergistic compounds. Through the statistical analysis of NMR spectra of complex mixtures of metabolites, unique spectral features can be identified and correlated to a phenotype or biological property of interest (Larive et al., 2015), FIGURE 2.10.

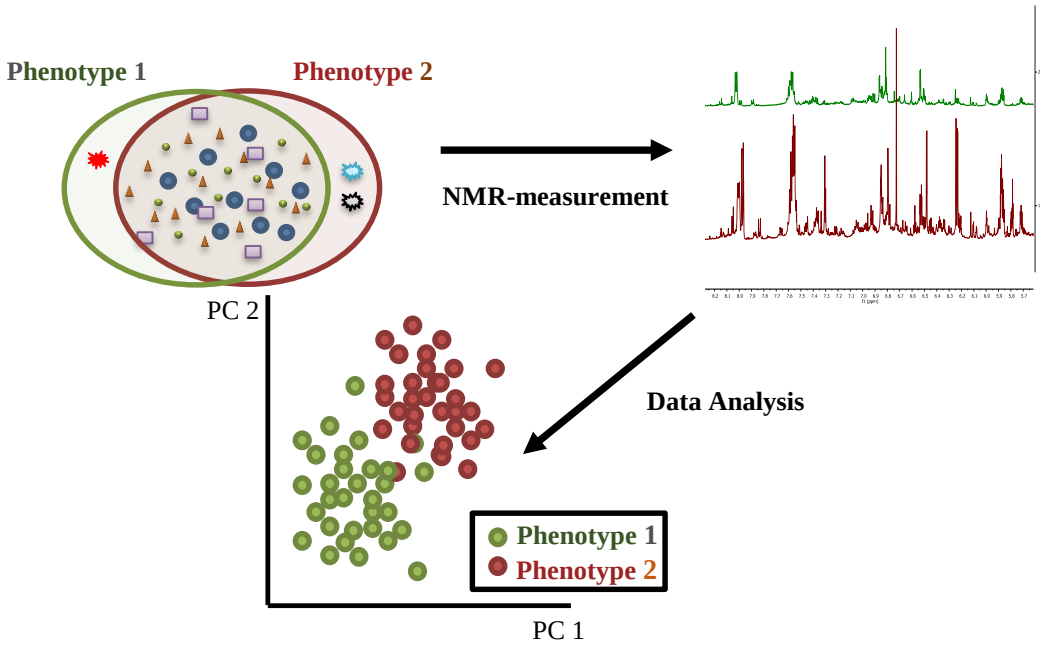


FIGURE 2.10 | Scheme of NMR-based metabolomics used to identify metabolites in complex mixtures and correlate them to a phenotype or biological property of interest. Multivariate data analysis (MVDA) methods aim to differentiate between classes in highly complex data sets, despite within class variability.

Also, multidimensional NMR experiments spectra [such as 2D-NMR technique: *J*-resolved, ^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H-^{13}C heteronuclear multiple bond correlation (HMBC), and ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC)] can aid in the process of assigning resonances, despite this strategy can be time-consuming (Kim et al., 2010a).

When the identity of the metabolites in a sample is known (or suspected), resonance assignments can be facilitated using libraries or databases. Public and commercial databases, such as HMDB, LipidMaps and Metlin (Bartel et al., 2013) now contain experimental 1D ^{1}H, ^{13}C and 2D ^{1}H-^{13}C spectra and extracted spectral parameters for over a thousand compounds and theoretical data for thousands more (Ellinger et al., 2013).

2.7.3. Multivariate Data Analysis (MVDA) in Metabolomics

Usually, scientific phenomena cannot be interpreted by a single variable but by multi ones. To handle the obtained multivariates, specific methods are required for data reduction, multicomponent statistics and prediction (Berrueta et al., 2007). A characteristic of metabolomics is the large amount of data generated and an important part of any metabolomics study is the analysis of these data using multivariate statistics (Brennan, 2013).

One approach to find meaning in metabolomics datasets involves MVDA methods that seek to capture not only changes of single metabolites between different groups, but also to utilize the dependency structures between the individual molecules. MVDA can be performed by unsupervised or supervised methods.

The unsupervised methods seek discriminating factors between the independent variables with the aim to obtain a graphical representation as the result of maximization of variances. For example, principal component analysis (PCA), is an unsupervised linear mixture mode. PCA is arguably the most widely used multivariate analysis method for metabolic fingerprinting and in chemometrics in general. Principal component analysis is often used as a starting point for data analysis, especially in a hypothesis free, exploratory experimental setup and attempts to identify inherent grouping of samples as a result of the similarity of the metabolic composition by a smaller number of mutually decorrelated principal components (PCs) (Bartel et al., 2013; Brennan, 2013). So, principal component regression analyzes X in order to obtain components which can explain X in the best way.

Supervised methods find the best fitting relationship between independent and dependent variables. Examples of supervised techniques is partial least-squares (PLS) and orthogonal projection to latent structures (OPLS) modeling (Brennan, 2013; Worley and Powers, 2013). PLS is a model for relating two data matrices of X and Y by a multivariate linear model. PLS regression

finds components of X which can predict Y in the best way. OPLS method is the improved form of PLS was first presented in 2002 and removes X changes that have no correlation with Y. Here the S-plot is proposed as a tool for visualization and interpretation of OPLS helping to identify statistically significant metabolites, based both on contributions to the model and their reliability (Sugimoto et al., 2012).

Chapter

3

MATERIALS AND METHODS

In this section, a description of all the biological and chemical materials used in this study is made, along with important observations necessary for their preparation. A protocol and a set of parameters adjusted for the identification of RMAs and other types of antibiotic adjuvants are defined. All the methods used in this thesis are described, highlighting crucial and important steps, appropriate conditions and a number of requirements that must be considered and fulfilled. A detailed chronological and methodological description of the project is given.

3.1. CRUCIAL STEPS AND PREPARATION OF MATERIAL BEFORE TESTING

3.1.1. General considerations

Material from any source is appropriate for these protocols, but initial pretreatment and preparation may vary accordingly. The protocol as written is focused on plant extracts, plant-isolated compounds or phytochemicals combined with commercial antibiotics. Any other natural extracts (from fungi, bacteria, algae, marine samples, etc.) and single isolated phytochemicals or synthetized compounds could be tested in combination with antibiotics as well. Also combinations with other antibacterial compounds that not commercial antibiotics can be performed. These protocols are also focused on *S. aureus* and MRSA clinical isolates but other aerobic bacteria can be tested as well. However, this requires optimization procedures and selection of appropriate conditions.

When assessing the susceptibility of bacteria to antimicrobial products it is important to promote the uniform application of terminology to the methods used (Simões et al., 2009). According to the European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), the dilution methods (agar dilution or broth microdilution) are the designated reference methods for antimicrobial susceptibility testing (EUCAST, 2000). As with any test of antibiotic activity over time, it is of utmost importance specifications regarding inoculum size, growth phase and medium composition (Eliopoulos and Eliopoulos, 1988). So, to produce accurate and reproducible results for the test of antibiotics and antibiotic-adjuvant combinations, a number of requirements must be considered and fulfilled. If implemented rigorously according to the procedures described herein, these modifications allow the generation of reliable data that will be comparable between different laboratories.

3.1.2. Choice of bacteria growth medium

The use of all methods of this protocol is limited to aerobic bacteria that grow well within 24 h in the CLSI (2015b) and EUCAST (2000; 2003) recommended test Mueller–Hinton (MH) growth medium. Preparation of growth medium can be found later in *Section 3.1.8.* For other organisms, specific recommendations for medium composition and testing conditions can be found in the CLSI (2015b) guidelines.

3.1.3. Preparation of bacterial inoculum

Bacteria must be isolated to a pure culture. The standardization of the bacterial cell numbers used is of critical importance for obtaining accurate and reproducible results. Both inoculum size and growth phase may lead to false susceptible results. A fresh pure culture should be used for the preparation of the inoculum.

Different methods for the preparation of the bacterial suspension can be used: direct colony suspension into liquid; growth method using either fresh or overnight cultures. In this thesis, preparation of the inoculum was carried out using the growth method using overnight cultures. The density of the cell suspension was assessed spectrophotometrically at 600 nm. Alternatively, a turbidity standard can be used as a visual yardstick. The McFarland 0.5 turbidity standard is equal to 1–2 × 10^8 CFU ml^{-1} (Wiegand et al., 2008). CLSI (2015b) has recommended a final inoculum size for susceptibility testing of aerobic bacteria in broth of 5 × 10^5 CFU ml^{-1}. The final inoculum size was always confirmed by a colony count for interpretation of killing endpoints on the following day of each experiment. Preparation of *saline tubes* (0.9% wt vol^{-1} NaCl) for performing dilutions in preparation for colony counts will be further explained in *Section 3.1.8*.

3.1.4. *Staphylococcus aureus* strains

In total, fifteen *S. aureus* strains were tested in this thesis. Their characteristics are shown in TABLE 3.1. As previously mentioned in *Section 2.2.1*, ST239 type MRSA strains are probably the most predominant clone of MRSA causing hospital-acquired infections (Abimanyu et al., 2012). The CA-MRSA strain USA300 is the most predominant MRSA strain type causing infections outside the health care environment in the United States (David et al., 2013). This strain is PVL-positive, meaning that has been epidemiologically associated with severe skin infections and pneumonia (Chambers and DeLeo, 2010).

Prior to use, each strain at -80ºC was transferred onto MH (Merck, Darmstadt, Germany) agar plate, grown overnight, and inoculated into MH broth at 37ºC and under agitation (150 rpm). There are several sets of controls that should be carried out. American Type Culture Collection (ATCC) or other national collections should be used to perform a better quality control. Thus, to be able to compare individual results and to validate the method, a quality control organism was always inserted for MIC characterization (*S. aureus* CECT 976 was used in this project).

TABLE 3.1 | Description of the fifteen *Staphylococcus aureus* strains used in this study according to their origin and genetic profiles

S. aureus		**Origin**	**Observations**
1	CECT 976	Laboratory strain	Spanish Type Culture Collection; equivalent to ATCC 13565; no antibiotic resistance; enterotoxin A gene
2	SA1199B	Laboratory strain	Derived from a MSSA bloodstream isolate from a patient with endocarditis; NorA efflux pump overexpresser
3	XU212	Laboratory strain	TetK efflux pump overexpresser; MRSA
4	RN4220	Laboratory strain	MsrA efflux pump overexpresser (plasmid pU5054)
5	MJMC001	CHTMAD, Portugal	MRSA, isolated from a diabetic foot
6	MJMC002	CHTMAD, Portugal	MRSA, isolated from a diabetic foot
7	MJMC003	CHTMAD, Portugal	MSSA, isolated from a diabetic foot
8	MJMC004	CHTMAD, Portugal	MRSA, isolated from a diabetic foot
9	MJMC009	CHTMAD, Portugal	MSSA, isolated from a diabetic foot
10	MJMC010	CHTMAD, Portugal	MSSA, isolated from a diabetic foot
11	M116	Indonesia	ST239-MRSA
12	RWW337	Malaysia	ST239-MRSA
13	RWW50	United States	MLST ST8
14	M82	Indonesia	MSSA CC20 strain, Bovine lineage
15	RN6390	Laboratory strain	ALC132; no antibiotic resistance

CHTMAD: Centro Hospitalar de Trás-os-Montes e Alto Douro (Vila Real, Portugal)

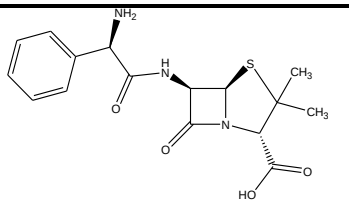
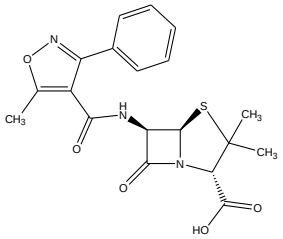
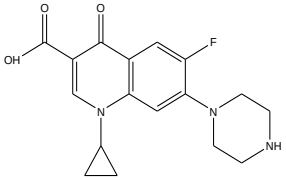
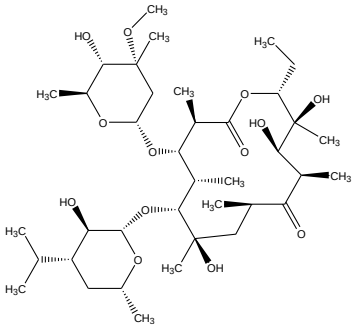
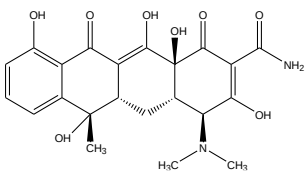
3.1.5. Preparation of antibiotics

Five antibiotics were used in this thesis: ampicillin, oxacillin, ciprofloxacin, erythromycin and tetracycline. Description and characterization of these antibiotics regarding their mode of action and spectrum of activity can be found in TABLE 3.2.

The antibiotics were obtained from Sigma-Aldrich (Sintra, Portugal). Powders were accurately weighed and dissolved in the appropriate solvents, following the manufacturer's recommendations and CLSI (2015b) and EUCAST (2003) requirements, to yield the desired concentration, using sterile glassware. Stock solutions for all antimicrobials were always prepared as well as several range of intermediate concentrations of antibiotic, such as 0.1, 1, 10, 100 and 1000 mg l^{-1}. Aseptic precautions were followed when further dilutions were warranted. Sterilization was guaranteed by a membrane filtration (care should be taken to avoid the use of fiber pads owing to their absorbent nature) and samples before and after sterilization were

compared by assay to ensure that adsorption to the membrane had not occurred. Stock solutions were frozen as soon as possible after preparation. The stock was aliquoted in 1 ml volumes and frozen at -20ºC for periods up to six months. Stock solutions were used promptly on defrosting and not re-frozen. Standard strains of stock cultures were used to evaluate the antibiotic stock solutions. MICs were compared with CLSI (2015b) guidelines for a collection strain (for control), in order to be sure that the antibiotic was well prepared.

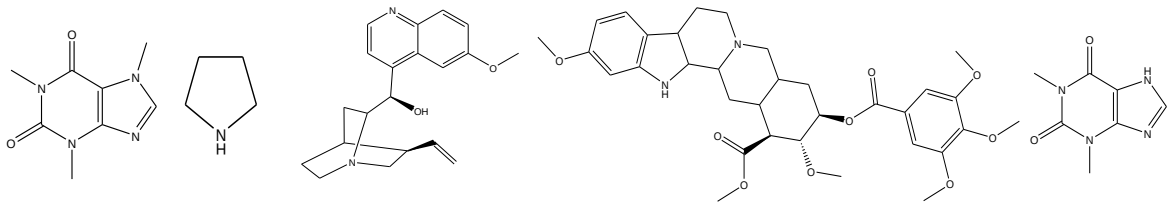
TABLE 3.2 | General characterization of the five antibiotics tested along this study. Information of mechanisms of action and spectrum of activity are described and chemical structures represented

Antibiotic	**Class**	**Mechanism of action**	**Spectrum**	**Chemical structure**
Ampicillin	β-lactam	Inhibition of cell wall synthesis through binding to PBPs	Gram-positive and some Gram-negative bacteria	
Oxacillin	β-lactam	Inhibition of cell wall synthesis mediated binding to PBPs	Gram-positive and some Gram-negative bacteria	
Ciprofloxacin	Quinolone	Inhibition of topoisomerase II (DNA gyrase) and topoisomerase IV	Gram-positive cocci (in urinary tract infections), some Gram-negative bacteria	
Erythromycin	Macrolide	Reversibly binding to the 50 S ribosomal subunit	Gram-positive cocci (mainly staphylococci and streptococci) and bacilli, and to lesser-extent gram-negative cocci; Mycoplasma, *Legionella*	
Tetracycline	Polyketide	Reversibly binding to the bacterial 30S ribosomal subunit	Several bacterial infections such as Rocky Mountain spotted fever, typhus fever, tick fevers, Q fever, rickettsialpox	

3.1.6. Preparation of phytochemicals

The phytochemicals selected and tested in this thesis are described in this section and their chemical structures are represented.

- **Alkaloids:** caffeine, pyrrolidine, quinine, reserpine, theophylline – purchased from Sigma-Aldrich (Sintra, Portugal) (FIGURE 3.1);



Caffeine Pyrrolidine Quinine Reserpine Theophylline

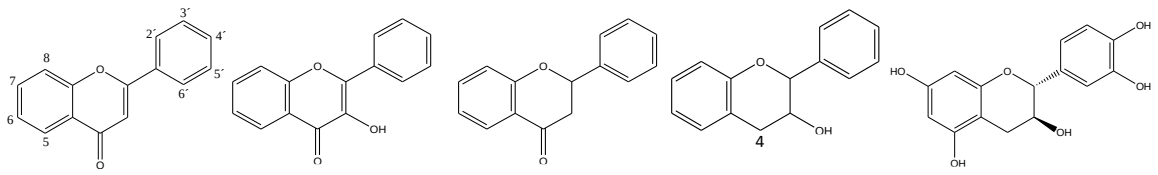
FIGURE 3.1 | Chemical structures of the five alkaloids tested in this study.

- **Flavonoids:** apigenin, (+)-catechin, chrysin, hesperidin, luteolin, morin, quercetin, rutin – purchased from Sigma-Aldrich (Sintra, Portugal) (TABLE 3.3);

TABLE 3.3 | A summary of the chemical structures of the eight flavonoids tested in this study

	Substituents at carbon position:								
Flavonoids:	**3**	**5**	**6**	**7**	**8**	**2′**	**3′**	**4′**	**5′**
Apigenin[1]		OH	-	OH	-	-	-	OH	-
Chrysin[1]		OH	-	OH	-	-	-	-	-
Luteolin[1]		OH	-	OH	-	-	OH	OH	-
Morin[2]	OH	OH	-	OH	-	OH	-	OH	-
Quercetin[2]	OH	OH	-	OH	-	-	OH	OH	-
Rutin[2]	O-rut	OH	-	OH	-	-	-	OH	OH
Hesperidin[3]		OH	-	O-rut	-	-	OH	OCH_3	-
(+)-catechin[4*]	OH	OH	-	OH	-	-	-	OH	OH

[1]Flavone [2]Flavonol [3]Flavanone [4]Flavan-3-ol *(+)-catechin



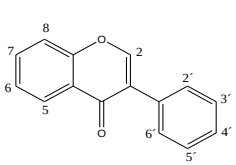
- **Isoflavonoids:** biochanin A, calycosin, calycosin-7-*O*-β-D-glucoside, corylifol A, daidzein, daidzin, genistein, genistin, glycitein, glycitin, formonenetin, 3'-hydroxypuerarin, iridin, irigenin, irisflorentin, 3'-methoxypuerarin, neobavaisoflavone, ononin, orobol, puerarin, tectoridin and tectorigenin - purchased from Biopurify (Chengdu, China) (TABLE 3.4).

TABLE 3.4 | A summary of the chemical structures of the twenty-two isoflavonoids tested in this study

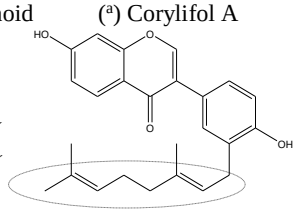
	Substituents at carbon position:*								
Isoflavonoids:	**3**	**5**	**6**	**7**	**8**	**2′**	**3′**	**4′**	**5′**
Daidzein		-	-	OH	-	-	-	OH	-
Daidzin		-	-	O-glc	-	-	-	OH	-
Corylifol A[(a)]		-	-	OH	-	-	-	OH	2Xprenyl
Neobavaisoflavone[(b)]		-	-	OH	-	-	prenyl	OH	-
Genistein		OH	-	OH	-	-	-	OH	-
Genistin		OH	-	O-glc	-	-	-	OH	-
Orobol		OH	-	OH	-	-	OH	OH	-
Calycosin		-	-	OH	-	-	OH	OCH_3	-
Calycosin-7-*O*-β-D-glucoside		-	-	O-glc	-	-	OH	OCH_3	-
Formononetin		-	-	OH	-	-	-	OCH_3	-
Ononin		-	-	O-glc	-	-	-	OCH_3	-
Biochanin A		OH	-	OH	-	-	-	OCH_3	-
Tectorigenin		OH	OCH_3	OH	-	-	-	OH	-
Tectoridin		OH	OCH_3	O-glc	-	-	-	OH	-
Glycitein		-	OCH3	OH	-	-	-	OH	-
Glycitin		-	OCH_3	O-glc	-	-	-	OH	-
Irigenin		OH	OCH_3	OH	-	-	OH	OCH_3	OCH_3
Iridin		OH	OCH_3	O-glc	-	-	OH	OCH_3	OCH_3
Puerarin		-	-	OH	glc	-	-	OH	-
3'-Hydroxypuerarin		-	-	OH	glc	-	OH	OH	-
3'-Methoxypuerarin		-	-	OH	glc	-	OCH_3	OH	-
Irisflorentin[(c)]		OCH_3	R_3	R_3	-	OCH_3	OCH_3	OCH_3	-

(glc – glucose)

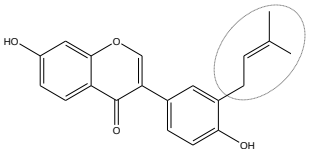
*Structure of isoflavonoid



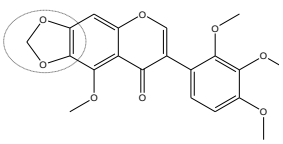
([a]) Corylifol A



([b]) Neobavaisoflavone



([c]) Irisflorentin



- **Triterpenoids**: betulinic acid, lupeol, betulin, hederagenin, ursolic acid and oleanolic acid - purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) (FIGURE 3.2).

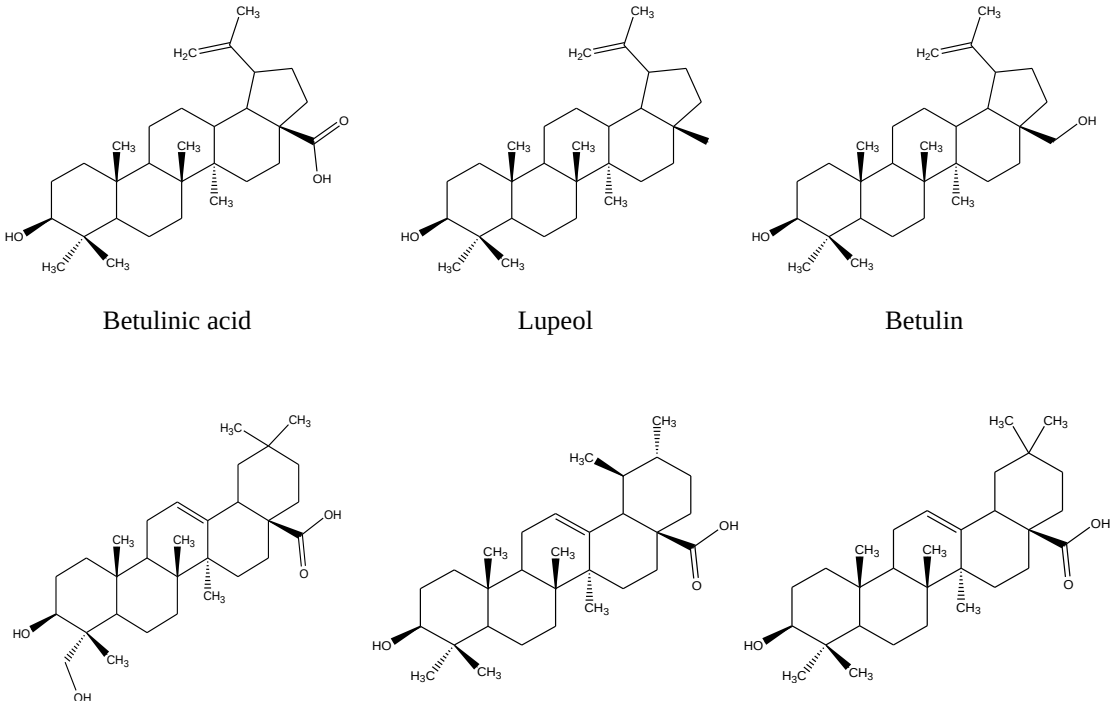


FIGURE 3.2 | Chemical structures of the six triterpenoids tested in this study.

For susceptibility testing, all phytochemicals should ideally be prepared in the same solvent. For plant-derived compounds dimethyl sulfoxide (DMSO) is recommend since water solubility is usually low. Additionally, DMSO is not toxic for most bacteria under the concentration of approximately 5% v v^{-1}. Therefore, all the phytochemicals tested in this study were dissolved in DMSO (Sigma-Aldrich, Sintra, Portugal). The phytochemicals were prepared at a certain initial concentration considering that only a volume ideally less than 5% v v^{-1} should be applied either on, depending on the method, broth or on agar medium (in *Section 3.1.8* it is described how to add the phytochemicals in MH agar), so to ensure that the solvent is not promoting growth inhibition and thus interfering with the results. When testing one phytochemical for its antibacterial activity, at least one antibiotic was included in the same assay to ensure that the bacterial strains present the normal susceptibility profiles and are in the expected conditions.

3.1.7. Collection and plant samples preparation

Twenty-nine Portuguese medicinal, invasive and fruit plant species were mostly collected in the region of Trás-os-Montes and Beira Transmontana (Portugal) and characterized by the Botanical Garden from Vila Real (Portugal). All plants tested are described in TABLE 3.5. Information about the ethnopharmacological uses of these plants can be found in Appendix A2.

TABLE 3.5 | Description of the twenty-nine species tested in this study

Plant name		Common name	Family
1.	*Acacia dealbata*	*Mimosa*	Fabaceae
2.	*Cytisus striatus*	*Giesta*	Fabaceae
3.	*Genista tridentate*	*Carqueja*	Fabaceae
4.	*Prunus domestica*	Plum	Rosaceae
5.	*Prunus avium*	Wild cherry	Rosaceae
6.	*Prunus persica*	Peach tree	Rosaceae
7.	*Pyrus communis*	Pear tree	Rosaceae
8.	*Agrimonia eupatoria*	Church Steeples	Rosaceae
9.	*Eriobotrya japonica*	Loquat	Rosaceae
10.	*Crataegus monogyna*	Hawthorn	Rosaceae
11.	*Rubus idaeus*	Wild Raspberry	Rosaceae
12.	*Malus communis*	Apple tree	Rosaceae
13.	*Eupatorium cannabinum*	Hemp agrimony	Asteraceae
14.	*Centaurea nigra*	Black knapweed	Asteraceae
15.	*Physalis angulata*	Cutleaf Ground-cherry	Solanaceae
16.	*Cyphomandra betacea*	Tree tomato	Solanaceae
17.	*Nerium oleander*	Oleander	Apocynaceae
18.	*Trachelospermum jasminoides*	Star jasmine	Apocynaceae
19.	*Eucalyptus globulus*	Blue Gum Tree	Myrtaceae
20.	*Calluna vulgaris*	Calluna	Ericaceae
21.	*Ficus carica*	Fig tree	Moraceae
22.	*Castanea sativa*	Sweet chestnut	Fagaceae
23.	*Juglans regia*	Walnut	Juglanduceae
24.	*Diospyros kaki*	Japanese Persimmon	Ebenaceae
25.	*Vitis vinifera*	Grape Vine	Vitaceae
26.	*Fraxinus excelsior*	European ash	Oleaceae
27.	*Actinidia chinensis*	Chinese gooseberry	Actinidiaceae
28.	*Buxus sempervirens*	Common box	Buxaceae
29.	*Pteridium aquilinum*	Bracken	Dennstaedtiaceae

The leaves of all plants were used for the extraction, excepting for *C. striatus*, for which flowers and twigs were also separately extracted and tested.

The leaves of all plants as well as the flowers and twigs of *Cytisus striatus* were harvested, separated and immediately frozen in liquid N_2 in order to avoid unwanted enzymatic reactions and stored at -20°C until analysis.

3.1.8. Reagent setup

- *Physiological saline solution [0.9% (wt/vol) NaCl] sterilized by autoclaving dispensed in 15 ml polystyrene capped tubes:* saline volumes for performing dilutions in preparation for colony counts were dispensed aseptically after autoclaving to minimize volumes inconsistencies that may occur during autoclaving of small volumes. The volume of saline needed for serial dilutions is 9 ml. cap tubes tightly for storage to prevent evaporation. These tubes were stored at 4ºC.

- *Mueller-Hinton (MH) broth medium preparation:* MH broth was prepared according to the manufacturer's instructions, autoclaved and cooled to 2–8ºC before the addition of the cation solutions. Additional cation supplementation (Ca^{2+} and Mg^{2+} ions) may be made as indicated by CLSI (2015b) when testing several antibiotics and strains. The final concentration should be 20–25 mg Ca^{2+} and 10–12.5 mg Mg^{2+} per liter (Wiegand et al., 2008), which reflects the divalent cation concentration in blood. The pH of each batch of MH broth was checked with a pH meter when the medium is prepared (between 7.2 and 7.4).

- *Preparation of MH agar:* MH was added to a 1 l flask according to the manufacturer's instruction with 12-15 g of agar and add 1 l of distilled water. The solution was autoclaved and cooled down until it could be poured into 90 mm Petri dishes to give a uniform depth of approximately 4 mm as previously refereed. The agar medium was allowed to set and to cool to room temperature and stored at 4ºC.

- *Preparation of MH agar with the phytochemical/plant extract to test:* MH was added to a 1 l flask according to the manufacturer's instruction with 12-15 g of agar and add 1 l of distilled water. Depending on the amount of plates necessary, other containers with other volumes can be used. One flask was prepared for each candidate as adjuvant to test. After autoclaving the MH broths and when the solution was cool enough, the adjuvant solutions were added as described in *Section 3.1.6*, yielding the final concentration desired (no more than 5% v v^{-1} of medium). Dried plant extracts prepared in DMSO were used in agar medium in the same way. The cooled supplemented medium was poured into 90 mm Petri dishes to give a uniform depth of approximately 4 mm. The agar medium was allowed to set and cool down to room temperature. These plates were usually prepared and used immediately to avoid drug deterioration on the medium. Unless, the plates were stored at 4ºC for a maximum period of 5 days.

3.2. DESCRIPTION OF METHODOLOGY STEP-BY-STEP

3.2.1. Task 1. Evaluation of the best method to assess antibiotic potentiation by phytochemicals against *Staphylococcus aureus*

Small description. In this section, a protocol and a set of parameters adjusted for the identification of RMAs and other types of antibiotic adjuvants are described. Checkerboard, time-kill assay, Etest and the disk diffusion methods were optimized and adjusted to provide such an analysis. Five alkaloids and five flavonoids were tested in combination with five antibiotics against *S. aureus* strains, including MRSA. A comparison of the results of potentiation given by these methods was evaluated to choose the best one.

Bacterial strains. Three clinical MRSA (MJMC001, MJMC002, MJMC004) and three clinical MSSA (MJMC003, MJMC009, MJMC010), *S. aureus* SA1199B, RN4220, XU212 and CECT 976. Information of bacterial strains can be found in *Section 3.1.4*.

Antibiotics. Ampicillin, ciprofloxacin, erythromycin, oxacillin and tetracycline. Preparation and specificities of the antibiotics are described in *Section 3.1.5*. Etest strips of antibiotics (AB Biodisk, Marcy L´Étoile, France) were obtained from Izasa (Oeiras, Portugal).

Phytochemicals. Alkaloids: caffeine, pyrrolidine, quinine, reserpine and theophylline; flavonoids: (+)-catechin, hesperidin, morin, quercetin and rutin. Preparation and specificities of the phytochemicals are described in *Section 3.1.6.* FIGURE 3.1 and TABLE 3.3 show the chemical structures of the alkaloids and flavonoids tested, respectively.

3.2.1.1. Experimental design

The concept of restoring the utility of already known antimicrobials by employing RMAs seems to be a remarkable alternative, despite of some concerns of possible toxicity that might arise from drug promiscuity. The combination of bioactive compounds is expected to exert a synergistic outcome or to reduce possible adverse side effects. Also, the development of active compounds in conjugation with existing antibiotics could probably avoid the emergence of resistant variants that might otherwise arise during treatment (Gibbons et al., 2003a; Olajuyigbe and Afolayan, 2012). There are several evidences of non-antimicrobial molecules that enhanced the activity of antimicrobial agents (Gill et al., 2015; Kalan and Wright, 2011), usually for improving solubility, absorption, safety, stability, or bioavailability of the active principle or due to a resistance-modifying effect.

A number of conceptual and methodological gaps are barring the identification of RMAs for clinical implementation. The methods reported in bibliography are more suitable for multi-antibacterial drugs therapy and not for the application of one antibacterial compound coupled with RMAs or antibiotic adjuvant. Also, most plant-related studies are still based on simple antibacterial tests against a range of bacteria, and the outcome is interpreted according to the simple classification of being antibacterial active or not. This classification as "antibacterial" is done even when plant extracts are tested at concentrations that exceed a lot the acceptable limits of clinical standards. It is important to highlight that researchers should continue investigating the synergistic capacity of plant extracts or other natural products, independently of the antimicrobial activity they might have. Thus, a more integrated approach is necessary to fully and deeply evaluate the defense features of each plant. A strategy to evaluate all interesting antibacterial activities shown by a plant extract or phytochemical is proposed as schematized in FIGURE 3.3.

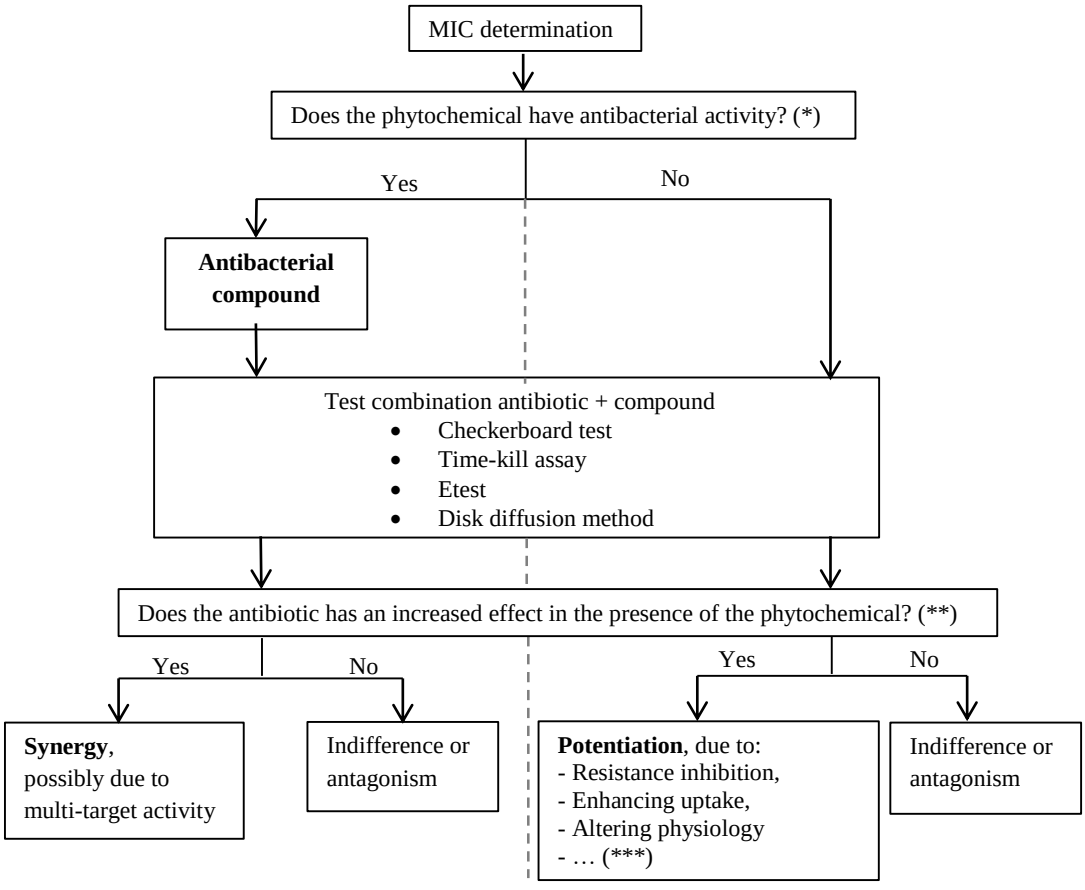


FIGURE 3.3 | Characterization of phytochemicals as antibacterials or antibiotic-adjuvants. (*) at doses that can be tolerated according to sound medical practice; (**) the definition of synergy or potentiation should be given according to the classification systems for each method that will be further explained in *Section 3.2.1.7*; (***) additional methods are required to assess the mode of action of the adjuvant and its interaction with the antibiotic.

The analysis explained in the left part of FIGURE 3.3 is generally made for compounds or extracts that have shown interesting antibacterial activity. The definition of being 'antibacterial' can be controversial. An antibacterial drug can be defined as anything that destroys bacteria or suppresses their growth or their ability to reproduce. However, the minimal concentration required for this activity is important if one consider a clinical application. It is known that antibacterial compounds from bacteria and fungi sources are orders of magnitude stronger (0.01-10 mg l^{-1}) than plant-derived antibacterials (100-1000 mg l^{-1}) (Tegos et al., 2002). Plant-derived compounds have not been considered for clinical application, since they can be toxic and effective at concentrations higher than the dose-related toxicity. For more toxic drugs, clinical use is based on weighing the favorable and unfavorable effects at a particular dose. Antibacterial compounds can play synergistically between each other throughout a multi-target therapeutic effect. Here, the components can have either separate targets or separate sites on the same target to create a combination effect and increase the pharmacological action (Zimmermann et al., 2007).

In the case that the compound/extract of interest has no antibacterial activity, the strategy is explained in the right part of the scheme (FIGURE 3.3). For detecting RMAs or antibiotic coadjuvants, it is expected to observe a high inactivation of resistant bacteria when the combination of antibiotic/plant compound is applied and only minor or no antibacterial activity of the phytochemical at doses that can be tolerated according to sound medical practice.

For all the methods, determination of the ability of a candidate compound to potentiate the activity of an antibiotic agent includes comparison of cell growth or survival in test cells and control cells, wherein test cells are incubated in the presence of the antibiotic agent and candidate compound, while control cells are incubated only in the presence of the antibiotic agent under the initial conditions of bacterial inoculum, the same concentration of the antibiotic and the time of exposure. In such methods, a candidate compound is identified as a potentiating compound of the antibiotic agent if the growth or survival of cells in the presence of the antibiotic agent and the candidate compound is less than the growth of the cells in the presence of the antibiotic agent alone.

Growth or survival can be assessed using cells growing in liquid or solid media. Any method known in the art can be used to determine inhibition of growth, proliferation and/or survival. Examples include measuring optical density in liquid culture, measuring colony formation, or measuring bacterial viability. Reproducibility of the results obtained may be tested by repeating the experiment and, in the case of a high-throughput assay, by incubating cells in more than one well of the assay plate e.g., in triplicate, with the same concentration of the same candidate compound. Additionally, since candidate compounds may be effective at varying concentrations depending on their mechanisms of action, varying concentrations of the candidate compound may

be added to different wells containing cells. In certain embodiments, the methods should involve the use of one or more negative or positive control compounds. A positive control compound may be any molecule, agent, moiety or drug that is known to potentiate the activity of the antibiotic under investigation. A negative control compound may be any molecule, agent, moiety or drug that is known to have no significant potentiating effect on the activity of the antibiotic agent. The solvent used for preparing the compounds could be used. Further comparison of the potentiating effect of the candidate compound to the potentiating effect (or lack thereof) of the positive (or negative) control compound is required (Cottarel et al., 2010).

The term "synergy" might not be a valid way to characterize positive interactions intermediated by these agents with no antimicrobial activity. Synergy by definition means that two compounds are potentiating each other. In this case it would be better to only consider this as a potentiation. Several explanations of possible interactions between antibiotics and adjuvants were previously made in FIGURE 2.8 in *Section 2.4*. Methods used so far to evaluate combinations between one antibiotic and an antibiotic-adjuvant are based on the same outcome used to classify synergy between two antibacterial compounds. This section aims to describe optimization of methods for detection of antibiotic adjuvants. As previously mentioned, the protocols described in this section are adjusted to a step-by-step format and for testing the susceptibility to natural extracts and phytochemicals and follow the guidelines of the two established organizations and committees, CLSI (2015a; 2015b) and EUCAST (2000; 2003), in order to assure intra- and inter-laboratory reproducibility.

3.2.1.2. Antibacterial susceptibility testing

The MIC of each agent was determined by microdilution techniques according to CLSI (2015a) guidelines. Bacteria (~10^6 CFU ml^{-1}) were inoculated into MH broth and dispensed at 200 µl $well^{-1}$ in polystyrene sterile 96-well microtiter plates, along with 2-fold dilutions of the compounds to test. Two-fold dilution series with around 10–12 dilutions down from 512 mg l^{-1} are conventionally used for most antibacterial agents for MIC determination. However, the concentration range will depend on the bacterial isolates to be tested and the antibacterial agent itself. MIC was defined as the lowest concentration of the antimicrobial compound that inhibited bacterial growth after 24 h of incubation at 37ºC. The bacterial growth was determined at 600 nm using a microplate reader (Spectramax M2e, Molecular Devices, Inc., Sunnyvale, CA, USA). At least three independent experiments were always performed for each compound. A drug-free control was included to evaluate the growth behavior of cells in normal condition. The solvents used for the preparation of the compounds were tested for their antimicrobial activity (as negative controls). The highest concentration of DMSO remaining after dilution (5% v v^{-1}) caused no

inhibition of bacterial growth. Bacteria-free controls constituted by the drugs in fresh medium were always prepared, as well as sterility controls containing only the medium.

3.2.1.3. Checkerboard microdilution assay

The checkerboard assay was performed in 96-well microtiter plates according to CLSI (2015a) guidelines with some modifications. First, final concentrations of user-defined panel configuration should be planed. If the phytochemical to be tested with the antibiotic has detectable MIC, concentrations ranging from, for example, 1/64 MIC to 4 × MIC should be included in order to observe the occurrence and magnitude of synergy or antagonism. For combinatorial testing, one compound is being diluted along the *x* axis of the checkerboard array while the other is diluted along the *y* axis, so each well has a different combinations of the compounds. An intermediate solution of the compounds to test should be prepared from the stock solution so that a certain volume (< 2.5% v v^{-1} of the well) of this solution is added to the wells of the microtiter plate in order to generate a final highest desired concentration. The other concentrations will be further produced in the microtiter plate by serial dilution. The volume added of both compounds should be ≤ 5% v v^{-1} for a final volume of the well of 200 µl. Nevertheless, it is necessary to be sure that the solvent is not promoting growth inhibition and interfering with the results.

In the case that the phytochemical to be tested in combination with an antibiotic has no detectable MIC, an initial range of concentrations down from 256 or 512 mg l^{-1} of the phytochemical was applied. In order to optimize the method, depending on the number of adjuvants or antibiotics to test, the space of the checkerboard array can be adjusted and, for an initial screening, each adjuvant can be applied only at two or three concentrations. After initial screening, best combinations can be assessed for optimal concentrations. It is expected to find the minimal and optimal concentration of the adjuvant causing the highest MIC fold reduction of the antibiotic.

After an overnight incubation into MH broth at 37ºC, bacterial cultures were adjusted in fresh broth to approximately 10^6 CFU ml^{-1}. Bacterial suspensions were added to each well along with the antibiotics and phytochemicals (in a total volume of 200 µl) according to the panel configuration. Negative growth controls were performed in a separate microtiter plate by mixing sterile medium with the drugs. Readings were determined in a spectrometer at 600 nm.

3.2.1.4. Etest

This method was performed according to the manufacturer's instructions. Each phytochemical was added to MH agar (after autoclaved and cooled) yielding the final concentration desired,

which was chosen according to the checkerboard results. Then, the medium was poured into 90 mm Petri dishes to give a uniform depth of approximately 4 mm (~20 mL). The bacterial suspensions were adjusted to 0.5 McFarland standards and seeded over hardened MH agar Petri dishes using a sterilized cotton swab and allowed to set (for 10 to 15 min). Antibiotic Etest strips were applied in duplicate on the MH agar plates. As control, the antibiotic strips were applied on simple MH agar plates (without the phytochemical). Plates were incubated for 24 h at 37ºC. MICs were read on plates with the combination of the two agents and on antibiotic control plates.

3.2.1.5. Disk diffusion method

This method was a modification from the Kirby-Bauer one and it was already applied in other studies (Saavedra et al., 2010). The bacterial inoculum and the MH agar plates were prepared in the same way of that described for Etest. Sterile blank disks (6 mm diameter; Oxoid, Madrid, Spain) were placed on the agar plate seeded with the respective bacteria. A volume of 15 µl of each antibiotic, prepared according to CLSI (2015b) guidelines (ampicillin – 10 µg $disk^{-1}$; ciprofloxacin – 5 µg $disk^{-1}$; erythromycin – 15 µg $disk^{-1}$; tetracycline – 30 µg $disk^{-1}$; oxacillin – 1 µg $disk^{-1}$), was added to blank disks. Disks of antibiotics on simple MH agar plates (without the phytochemicals) were used as positive controls (FIGURE 3.4). Disks impregnated with DMSO were used as negative controls. The plates were incubated at 37°C for 24 h. After incubation, each inhibition zone diameter (IZD) was recorded and analysed according to CLSI (2015b) guidelines.

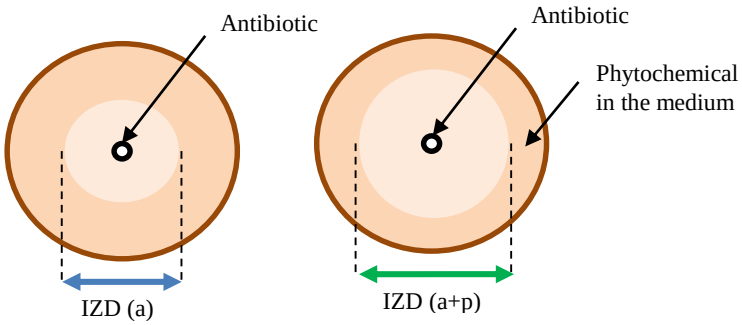


FIGURE 3.4 | Disk diffusion method for detection of potentiation of antibiotic activity. The phytochemical is inserted in MH agar (≤ 5% v v^{-1}). Inhibition zone diameters (IZDs) promoted by antibiotic disks are measured in plates with [IZD (a+p)] and without [IZD (a)] the phytochemical and compared.

3.2.1.6. Time-kill assay

Time-kill studies were performed according to Roccaro et al. (2004) with some modifications. Overnight cultures were diluted with MH broth to a starting inoculum of 10^6 CFU ml^{-1}. Each

antibiotic was tested at MIC, ½ × MIC and ¼ × MIC. The concentration of the phytochemicals was chosen according to the checkerboard results. The combinations were added to sterilized 50 ml polystyrene capped tubes and inoculated with each isolate in a total volume of 10 ml. The antimicrobial combinations did not exceed 5% v v^{-1} of the final volume. Controls were performed with each compound separately. The tubes were incubated at 37ºC and 150 rpm for 24 h. CFU counts in MH agar were performed after 0, 4, 8 and 24 h of the beginning of the incubation.

3.2.1.7. Classification of the interaction between two antibacterial compounds or between one antibacterial compound and an adjuvant

In the combination between two antibacterial drugs, there are four possible effects:

- indifference (when the combination of antibacterial products promotes equal effects to these of the most active product);
- additive effect (when the combination of antibacterial products is equal to that of the sum of the effects of the individual products);
- synergy (when the combination of antibacterials exceeds the sum of the effects of the individual products);
- antagonism (when the combination of antibacterial products promotes a reduced effect compared to the effect of the most efficient individual product) (Simões et al., 2009).

Additive and synergistic effects may be experimentally characterized and quantified. Negative interactions (interferences) occur when certain components of the mixture inhibit full biological activity of pharmacologically-active compounds by reducing their stability or bioavailability or by enhancing their metabolism (Lila and Raskin, 2005).

Generally, for checkerboard, if the two combined compounds are antibacterial, in order to assess the effect of any given antimicrobial combination, the fractional inhibitory concentration (FIC) index is usually determined. FIC index FICI = FIC(A) + FIC(B), where FIC(A) is the ratio between the MIC of drug A in combination and the MIC of drug A alone and FIC(B) is the ratio of the MIC of drug B in combination and the MIC of drug B alone (Sopirala et al., 2010). Synergy has generally been defined as requiring a 4-fold reduction in the MIC of both antimicrobial in combination, compared with each used alone, *i.e.* a FICI of 0.5 (Odds, 2003). For detecting antagonism, the > 4 rule have been widely accepted (Mackay et al., 2000; Rand et al., 1996). The definition of indifference or additivity has been debated for a long time and cannot always be distinguished.

For Etest, if the MIC of antibiotic is reduced by > 3-fold, the result is considered synergic; an increase ≥ 3-fold of the MIC is usually classified as antagonistic (Sopirala et al., 2010). Otherwise, FICI can also be determined as previously explained.

For time-kill assays, a ≥ 2-$\log_{10}$ decrease and a ≥ 2-$\log_{10}$ increase in CFU ml^{-1} between the combination and its most active constituent at the designated sample time, is considered synergy and antagonism, respectively (Eliopoulos and Eliopoulos, 1988). Additivity/indifference is usually defined as any scenario not meeting the criteria for either synergy or antagonism.

This classification derived by each method may be confusing since there are several definitions to what constitutes synergy and numerous experimental techniques for synergy detection (Lambert et al., 2003). For example, it is not surprising that with two different methods such as checkerboard and time-kill assay, results might vary as these techniques measure two different endpoints: inhibition of grow and killing (Bonapace et al., 2002). As the test results vary widely under different test conditions, the procedures have to be standardized for intra- and inter-laboratory reproducibility.

A new approach was established for interactions between compounds that have no antimicrobial activity but that can potentiate antimicrobials. Positive interactions that intensify the potency of a bioactive product are generally called potentiation. This approach is based on some synergistic definitions applied for combinations involving two antimicrobials using checkerboard (Mackay et al., 2000; Rand et al., 1996), Etest (Cantón et al., 2005; Lewis et al., 2002; Sopirala et al., 2010), and time-kill assay (Eliopoulos and Eliopoulos, 1988; NCCLS/ CLSI, 1999). In order to propose a classification scheme for the interactions obtained with disk-diffusion method, simple linear regressions were performed in order to define linear functions correlating the data from checkerboard and Etest (MIC fold reductions) to the IZDs obtained with the disk-diffusion method (FIGURE 3.5). The Etest and checkerboard variables were linearized by logarithmic conversions. The population (n) used for this correlation included the combinations between the antibiotics with the phytochemicals tested, five alkaloids and five flavonoids (380 combinations in total). The strength of the linear association between pairs of variables was determined by coefficients of determination (*R*-square). According to Nicodemo et al. (2004), a strong correlation was found between Etest and disk-diffusion method (*R*-square = 73.5%; $P < 0.05$; n = 240) and between checkerboard and disk-diffusion method (*R*-square = 71.4%, $P < 0.05$; n = 380).

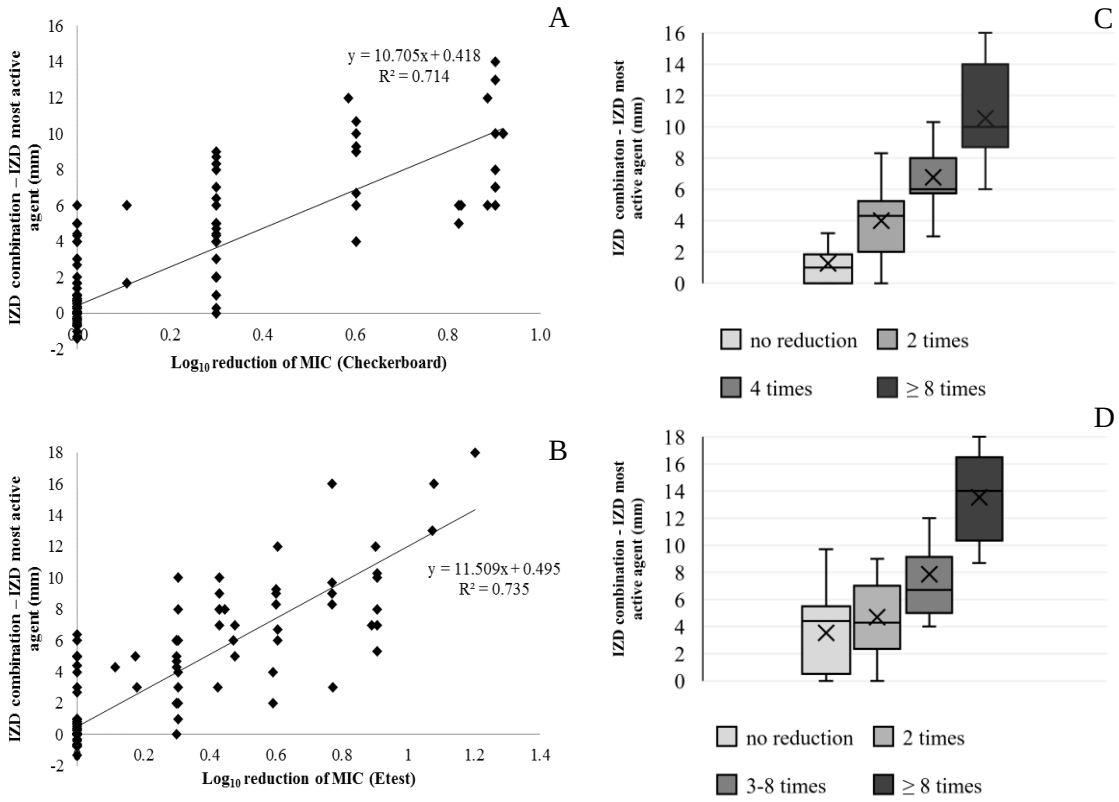


FIGURE 3.5 | Correlation between the disk-diffusion method results (IZD combination – IZD most active agent) and the MIC reductions given by checkerboard (A, C) and by Etest (B, D). Representations B and D allow to visualize a box between the lower quartile (e.g. the median of the first half of the data corresponding to the inferior limit of box) and the upper quartile (e.g. the median of the second half of the data corresponding to the superior limit of the box), the median of the data (represented by the line inside the box) and the data average (represented by a cross). Error lines show the lowest and highest values of the data. Data comprises 380 combinations between five antibiotics (ciprofloxacin, tetracycline, erythromycin, oxacillin and ampicillin) and the phytochemicals (five alkaloids – caffeine, pyrrolidine, quinine, reserpine, theophylline, - and five flavonoids – (+)-catechin, hesperidin, morin, quercetin, rutin). For Etest, the β-lactam antibiotics were not included.

This analysis is valid for the antibiotics used in this study, where only ciprofloxacin, erythromycin and tetracycline were potentiated by the phytochemicals (4-8 times reduction). However, by using other antibiotics, results can differ.

According to these correlations, TABLE 3.6 shows the scheme proposed for disk diffusion method to classify an interaction between the antibiotic and one adjuvant as negative, indifferent, additive or potentiation. For checkerboard, when the MIC of the phytochemical alone is not

detected in the range of tested concentrations, the double of the highest concentration tested for MIC determination can be used for FICI determination and if FICI value was ≤ 0.5 this interaction was determined to be potentiation (Sopirala et al., 2010).

TABLE 3.6 | Characterization of the effect promoted by a phytochemical in the activity of one antibacterial agent. Indifferent effect is considered between the limits proposed for additive and negative interactions

Interaction	Checkerboard	Etest	Disk-diffusion	Time-kill assay
Potentiation	MIC_a reduction > 4-fold dilutions	MIC_a reduction > 3-fold dilutions	$(IZD_{a+p} - IZD_a) \geq$ 6 mm	≥ 2 $\log_{10}$ decrease in CFU of the antibiotic
Additive	MIC_a reduction ≥ 2-fold and < 4-fold dilutions	MIC_a reduction ≥ 2-fold and < 3-fold dilutions	$4 \leq (IZD_{a+p} - IZD_a) < 6$ mm	$\log_{10}$ decrease ≥ 1 and < 2 in CFU of the antibiotic
Negative	MIC_a increase > 4-fold dilutions	MIC_a increase ≥ 3-fold dilutions	$(IZD_a - IZD_{a+p}) \geq$ 6 mm	$\geq$ 2-$\log_{10}$ increase in CFU of the antibiotic

The classification of each combination was compared between methods. Agreement between methods was defined as all methods having the same interpretative category, minor disagreement as only one method displaying disagreement results and major disagreement was defined when two or more methods displayed disagreement results.

3.2.1.8. Statistical analysis

For statistical analysis, the *in vitro* results were analysed by Student's *t* test using the statistical program SPSS (Statistical Package for the Social Sciences) version 19.0 (IBM Corp., Armonk, NY, USA). Statistical calculations were based on a confidence level ≥ 95% ($P < 0.05$) which was considered statistically significant. Relatively to the simple linear regressions performed correlating the checkerboard and Etest data to the disk-diffusion results, the validation of these linear models was carried out by F-test. All *P* values reported were two-tailed and values lower than 0.05 were considered significant.

3.2.2. Task 2. Combinatorial approaches with selected phytochemicals to increase antibiotic efficacy against *Staphylococcus aureus* biofilms

Small description.	Selected phytochemicals shown to potentiate antibiotics against planktonic *S. aureus* cells were tested on the prevention and control of *S. aureus* biofilms. The phytochemicals were also studied for their ability to avoid antibiotic adaptation and to inhibit NorA efflux pumps (by measuring EtBr accumulation) in SA1199B. Additionally, cytotoxicity of phytochemicals on lung fibroblast cell lines was assessed.
Bacterial strains.	*S. aureus* SA1199B, RN4220, XU212 and CECT 976. Information of bacterial strains can be found in *Section 3.1.4.*
Antibiotics.	Ciprofloxacin, erythromycin and tetracycline. Preparation and specificities of the antibiotics are described in *Section 3.1.5.*
Phytochemicals.	Morin, pyrrolidine, quercetin, quinine and reserpine. Preparation and specificities of the phytochemicals are described in *Section 3.1.6.* FIGURE 3.1 and TABLE 3.3 show the chemical structures of the alkaloids and flavonoids tested, respectively.

3.2.2.1. Biofilm formation, prevention and control

The MIC of antibiotics and phytochemicals were determined against planktonic cells according to CLSI (2015a) guidelines in the previous *Section 3.2.1.2.* Antibiotics were applied at MIC, 10 × MIC and 50 × MIC against biofilms. The phytochemicals were found to have no antimicrobial activity for the concentrations tested (up to 1500 mg l^{-1}). Consequently, the concentrations applied correspond to the optimal concentrations previously found to potentiate the antibiotics against the tested bacteria in planktonic state: 100 mg l^{-1} for reserpine and quinine and 500 mg l^{-1} for pyrrolidine, quercetin and morin.

Antibiotics and phytochemicals were tested individually and in combination in order to assess their ability to control biofilms and prevent their formation. Two exposure times were tested for the control of established biofilms (1 and 24 h). Overnight cultures adjusted to a cell density of 10^6 cells ml^{-1} were added to sterile 96-well polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) to form biofilms during 24 h at 37ºC and 150 rpm. Afterwards, the medium was removed and the biofilms were exposed to the antibiotics, phytochemicals and their

combinations (at 5% v v^{-1} of the well for a final volume of 200 µl), for 1 h at 37ºC and 150 rpm. The same protocol was performed for an exposure time of 24 h.

In order to assess the combinations towards the prevention of biofilm formation, overnight cell suspensions were added to microtiter plates along with antibiotics, phytochemicals and their combinations (at 5 % v v^{-1} of the well). The plates were incubated for 24 h at 37ºC and 150 rpm. After incubation, biofilms were scraped and diluted in saline solution (0.85% NaCl). The numbers of CFU per unit of adhesion area (CFU cm^{-2}) were assessed in MH agar for both biofilm control and prevention experiments.

A phytochemical-antibiotic synergy assay was performed according to Monzón et al. (2001). The combination was considered synergic when the log_{10} reduction CFU cm^{-2} caused by the combination was significantly higher ($P < 0.05$) than the sum of reductions of individual treatments, and antagonistic when the log_{10} reduction in CFU cm^{-2} of the combination was significantly lower ($P < 0.05$) than that obtained with the more active compound.

3.2.2.2. Antibiotic adaptation assay

The most promising phytochemical-antibiotic synergy results on biofilms were obtained with *S. aureus* SA1199B. Therefore, this strain was selected for an antibiotic adaptation assay aiming to understand if the phytochemicals were effective in reversing bacterial resistance or avoiding the development of resistance due to prolonged exposure. The antibiotic adaptation assay was performed with *S. aureus* SA1199B growing with increasing sub-inhibitory concentrations of ciprofloxacin for 15 days, according to FIGURE 3.6.

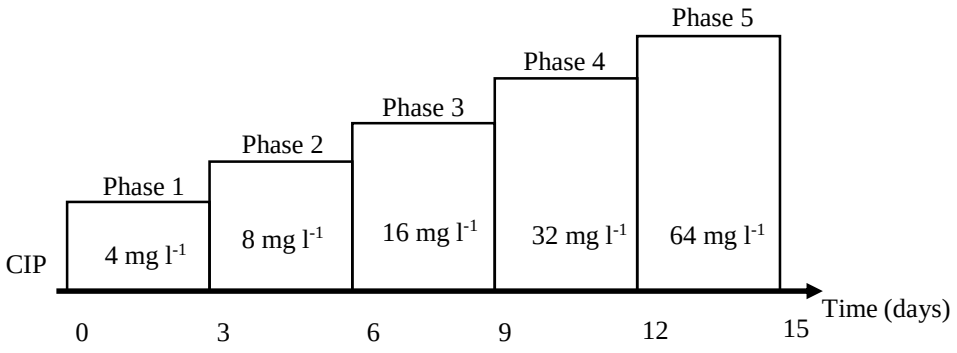


FIGURE 3.6 | Administration of ciprofloxacin (CIP) with phytochemicals during 15 days to *Staphylococcus aureus* SA1199B to assess bacterial adaptation to this antibiotic. Bacterial cultures incubated with sub-inhibitory doses of ciprofloxacin increasing every 3 days (from 1/32 × MIC to 1/2 × MIC) for a total of 15 days. Bacterial susceptibility to ciprofloxacin was determined in the end of each cycle by disk diffusion method (CLSI, 2015b).

S. aureus SA1199B was grown overnight in MH broth at 37 °C, adjusted to a cell density of 10^6 CFU ml^{-1} and incubated with ciprofloxacin at 1/32 × MIC (4 mg l^{-1}, phase 1) for 24 h (37ºC and 150 rpm agitation) in the presence of each phytochemical. After 24 hours, the bacterium was refreshed: centrifugation was performed at 3999 *g* for 10 min, the supernatant discarded, the pellet was washed twice and resuspended in MH broth with the same concentrations of antibiotic and phytochemical. This procedure was repeated after 48 hours. In the 3rd day, after washing the cultures, the concentration of the antibiotic was increased to 1/16 × MIC (8 mg l^{-1}, phase 2), and consecutively, for a total of 15 days, until a final concentration of 1/2 × MIC. The concentration of each phytochemical was maintained along the assay. Controls were performed in the absence of phytochemicals or ciprofloxacin and with DMSO (5% v v^{-1}). Bacterial samples were taken every 3 days, washed as previously described and susceptibility profiles of bacterial populations to ciprofloxacin were evaluated by the disk diffusion method according to CLSI (2015b) guidelines. The final population grown in the presence of only ciprofloxacin [named SA1199B(r)] was recovered after the 15 days, washed and stocks were prepared for further experiments to assess efflux pump inhibition with EtBr. Triplicates of each combination were performed.

3.2.2.3. Disk diffusion method

Bacterial cultures obtained from adaptation experiment were prepared as described in *Section 3.2.1.5* and seeded over hardened drug-free MH agar Petri dishes using a sterilized cotton swab. A volume of 10 µl of ciprofloxacin prepared according to CLSI (2015b) guidelines (ciprofloxacin – 5 µg $disk^{-1}$) was added to the blank disks. Disks with 10 µl of DMSO were used as negative control. After incubation each IZD was recorded and antibiotic susceptibility was analysed according to CLSI (2015b) guidelines.

3.2.2.4. Ethidium bromide accumulation assay by fluorometry

Ethidium bromide is a substrate for many efflux systems in various MDR microorganisms, including *S. aureus* (Jin et al., 2011), and has been shown to be a particularly suitable probe for these studies, since it emits weak fluorescence in aqueous solution (external to the cell) and becomes strongly fluorescent when it binds to cellular components (Paixão et al., 2009). EtBr accumulation assay was performed in order to assess the putative action of phytochemicals as NorA inhibitors. First, the MIC of EtBr (prepared in a stock of 10 g l^{-1}) was determined according to CLSI (2015a) guidelines as described in *Section 3.2.1.2*. The detection of EtBr accumulation in *S. aureus* strains was performed using a fluorometric method according to Rodrigues et al. (2013) and Jin et al. (2011). Briefly, bacteria were grown in MH broth at 37°C until mid-log phase

(OD_{600} of 0.6 to 0.7). Bacterial suspensions were centrifuged at 3999 *g* for 10 min, the supernatant was discarded, the pellet was washed in phosphate buffered saline (PBS; pH 7.4), and bacterial suspension were adjusted to 10^8 CFU ml^{-1} with PBS. Aliquots of 100 µl of bacterial suspensions were transferred to wells of a 96-well plate containing serial dilutions of EtBr at concentrations ranging from 80 to 0.06 mg l^{-1}. In order to assess the effect of phytochemicals on EtBr accumulation, EtBr at ½ MIC (to not compromise the bacterial viability) was applied in the absence or presence of each phytochemical: reserpine and quinine at 100 mg l^{-1}; pyrrolidine, quercetin and morin at 500 mg l^{-1}. The negative control was performed with DMSO (5% v v^{-1}). Also, controls for each phytochemical with EtBr in PBS were performed in order to detect possible fluorescence emitted by the products themselves. Fluorescence was acquired every 1 min for 60 min at 37°C in a microplate reader (Spectramax M2e) using 530 nm and 590 nm as excitation and detection wavelengths, respectively.

3.2.2.5. Cytotoxicity tests

The cytotoxicity of some phytochemicals was evaluated according to ISO/EN 10993 (part 5) guidelines with some modifications (ISO, 1992). L929 cells (ATCC CCL 1), derived from an immortalized mouse lung fibroblast cell line and routinely used in *in vitro* cytotoxicity assessments, were used. Cells were grown in 175 cm^2 culture flasks using Dulbecco's modified eagle medium (DMEM, Sigma-Aldrich, Sintra, Portugal) supplemented with 1% of penicillin/streptomycin solution (Sigma-Aldrich, Portugal) and 10% of foetal bovine serum (FBS, Biochrom, São Mamede de Infesta, Portugal). The flasks were incubated at 37°C for 72 h in a 95% air 5% CO_2 atmosphere with 100% humidity. Twenty-four hours before the cytotoxicity tests starts, cells were trypsinised, seeded in 96-well microtiter plates at a concentration of 1×10^4 cells per well and left to adhere at 37°C in a 95% air 5% CO_2 atmosphere with 100% humidity. Then, the medium in each well was replaced by 200 µl of fresh DMEM with the phytochemicals at several concentrations (chosen according to the antimicrobial assays). The phytochemicals did not exceed 2% v v^{-1} of the well final volume. The positive control was performed by adding fresh medium without any phytochemical and the negative control by adding DMSO at 2% v v^{-1}. Each condition was performed in 5 wells and in triplicate. The plates were incubated for 72 h, at 37°C, in a 95% air 5% CO_2 atmosphere with 100% humidity. Then, the cell viability was assessed by using the Cell Titer 96® One solution Cell Proliferation Assay Kit (Promega, Wisconsin, USA). This assay involves the bioreduction of the substrate, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyl-methoxy-phenyl)-2(4-sulfophenyl)-2H tetrazolium) (MTS) into a brown formazan product by NADPH or NADP produced by dehydrogenase enzymes in metabolically active cells (Baran et al., 2004). After incubation, the medium with phytochemicals was removed and

replaced by 200 µl of a mixture of DMEM without FBS and MTS (1:5 ratio) and incubated at the same conditions for 3 h. The OD_{490} of each well was measured using a plate reader (Molecular Devices, USA). From the dose-response curves obtained, the half maximal inhibitory concentration (IC_{50}) values were calculated by probit analysis according to Sebaugh (2011).

3.2.2.6. Statistical Analysis

The results were analysed by Student's *t* test using SPSS version 19.0. Statistical calculations were based on a confidence level ≥ 95% ($P < 0.05$) which was considered statistically significant. All *P* values reported were two-tailed.

3.2.3. Task 3. Reexamining plants with a new look into their defense systems: the search for antibiotic adjuvants to promote effective treatment of drug-resistant *Staphylococcus aureus*

Small description.	The current study was designed to assess 29 selected plants from different families, some of them with traditional use, for their antibacterial activity, and also as adjuvants in antibiotic therapy, against *S. aureus*, including MRSA. Plants have been long scrutinized in the quest for new antibiotics, but no strong antibiotic molecule was ever found. It is believed that most phytochemicals have a regulatory, adjuvant or potentiating effect of other antibacterial compounds, thus promoting an overall greater effect, which can have a pronounced effect when applied in clinical therapies.
Bacterial strains.	*S. aureus* SA1199B, RN4220, XU212, CECT 976, and the MRSA strain MJMC001. Information of bacterial strains can be found in *Section 3.1.4*.
Antibiotics.	Ampicillin, ciprofloxacin, erythromycin, oxacillin and tetracycline. Preparation and specificities of the antibiotics are described in *Section 3.1.5*.
Plant material.	Twenty-nine Portuguese medicinal, invasive and fruit plant species were mostly collected from the region of Trás-os-Montes and Beira Transmontana (Portugal) and characterized by Botanical Garden from Vila Real (Portugal). TABLE 3.5 described the plants tested in this study. Relevant information about the preparation of the plants can be found in *Section 3.1.7*.

3.2.3.1. Extraction of selected plants

The plant materials (5 g) were freeze-dried and extracted with 50 ml of MeOH at 30ºC, stirring at 150 rpm for 60 min. The samples were filtrated and re-extracted with 50 ml MeOH for more 60 min. The resulting extracts were combined and the solvent was evaporated at low temperature (< 40ºC) under reduced pressure. The dried MeOH extracts were prepared in DMSO for antibacterial testing.

3.2.3.2. Antibacterial susceptibility testing

Before testing, the dried MeOH extracts were prepared in DMSO. The MIC of each plant extract was determined by microdilution technique according to CLSI (2015a) guidelines, as described in *Section 3.2.1.2*. Plant extracts did not exceed 5% v v^{-1} of the well. Three independent experiments were performed for each plant extract.

3.2.3.3. Antibiotic-potentiation testing by disk diffusion method

Plant extracts showing no MIC below 4 g l^{-1} were tested for an antibiotic-potentiating activity by disk diffusion method according to CLSI (2015b) guidelines. Samples were applied at several concentrations (between 0.125 to 4 g l^{-1}), in order to define the minimal/optimal concentration causing antibiotic potentiation. Each extract prepared in DMSO was added to MH agar (after autoclaved and cooled) yielding the final concentration desired as described in *Section 3.1.8*. The test was performed as described in *Section 3.2.1.5*. Disks impregnated with plant extracts at the tested concentrations were also applied on simple MH agar plates in order to ensure lack of bacterial growth inhibition. The antibiotic-potentiating activity of each plant extract was categorized into four classes as explained in TABLE 3.6 in *Section 3.2.1.7*.

3.2.3.4. Statistical analysis

For statistical analysis, the *in vitro* results were analysed by Student's *t* test using the statistical program SPSS version 19.0. Statistical calculations were based on a confidence level ≥ 95% ($P < 0.05$) which was considered statistically significant.

3.2.4. Task 4. The potential of *Cytisus striatus* for antibiotic-potentiating activity: isolation and identification of antibiotic adjuvants from this plant

Small description.	Disabling resistance mechanisms may restore the activity of old-generation antibiotics. With this possibility in mind, it was aimed to identify the active potentiating compounds of *Cytisus striatus* using ^{1}H NMR-based metabolomics. This approach, which combines the use of a powerful analytical platform for data collection with multivariate data analysis of the information thus collected can allow the identification of metabolites that may be involved in synergic activities, a major challenge in the discovery of potential drugs derived from natural products. Further investigation concerning the activity of the isolated metabolites on planktonic and biofilm cells and characterization of their mode of action was performed.
Bacterial strains.	*S. aureus* RWW337, M116, RWW50, M82, RN6390, CECT 976 and SA1199B. Information of bacterial strains can be found in *Section 3.1.4*.
Antibiotics.	Ciprofloxacin and erythromycin. Preparation and specificities of the antibiotics are described in *Section 3.1.5*.
Phytochemicals.	Apigenin, chrysin and luteolin, daidzein and genistein. Preparation and specificities of the phytochemicals are described in *Section 3.1.6.* TABLES 3.3 and 3.4 show the chemical structures of the flavonoids and isoflavonoids tested, respectively. Reserpine (Sigma-Aldrich, Sintra, Portugal), a recognized EPI (Gibbons and Udo, 2000; Schmitz et al., 1998), was used as positive control in EtBr accumulation assays and prepared in the same way.
Plant material.	*Cytisus striatus* (Hill) Rothm. (leaves, flowers and twigs). Relevant information about the preparation of this plant can be found in *Section 3.1.7*.

3.2.4.1. Preparation of plant samples and extracts

To study the activity of *C. striatus* leaves, flowers and twigs, 5 g of each of these plant materials were extracted with 50 ml of MeOH at 30ºC, stirring at 150 rpm for 60 min. After filtration, the extracts were taken to dryness and redissolved in 10 ml methanol (MeOH). This extract was partitioned with *n*-hexane (3 × 10 ml) to eliminate lipophilic compounds. The remaining methanolic phase was evaporated using a rotary evaporator and redissolved in

H_2O/MeOH (95:5). The resulting solution was extracted with 3 × 10 ml portions of ethyl acetate (EtOAc) and the extracts were combined and evaporated with a rotary evaporator. These extracts were analysed by ^{1}H NMR and tested for both antibacterial and antibiotic-potentiating activities. The hexane and remnant H_2O/MeOH fractions were also tested in order to ensure that no compound of interest had remained in these extracts.

A multi-extraction strategy was performed for multivariate data analysis. Different combinations of *C. striatus* leaves or flowers with twigs were prepared in order to generate metabolic variation. Thus, *C. striatus* samples were prepared by mixing different percentages of leaves or flowers in the twig [0, 25, 50, 70, 85, 100% ($w_{leaf\ or\ flower}$/ w_{twig})]. Approximately 1 g of each sample was extracted using different conditions of pressure and temperature with a E-916 speed extractor (Büchi, Flawil, Switzerland). The tested conditions were: temperature (30 or 90ºC), pressure (50 or 100 bar), solvent (50, 75 and 100% aqueous MeOH). For all conditions, the extraction was performed using two 4-minute cycles with a flow of 3 ml of solvent min^{-1}. Samples were labelled using a binary numbering system *(x,y)*, where *x* specifies the extraction conditions and *y* the sample composition (TABLE 3.7). In total, 54 different samples were extracted and dried in vacuum (Syncore, Büchi).

TABLE 3.7 | Strategy and conditions used for the multi-extraction of *Cytisus striatus*. Six extractions of different *Cytisus striatus* samples were performed each time, by mixing different percentages of twig in leaves or flowers for 1 g total sample. Nine runs were performed with variation of temperature (30 or 90ºC), pressure (50 or 100 bar) and % MeOH (50-, 75- and 100% in water). Identification of the samples was performed by numbering them as *(x, y)*, where *x* specifies the condition of the extraction and *y* the sample composition

						***y* - Sample composition (% (w/w) twig in leaf/flower)**					
		MeOH (%)	**T (ºC)**	**P (bar)**		**0 % 1**	**15% 2**	**30 % 3**	**50 % 4**	**75 % 5**	**100 % 6**
***x* - Conditions of extraction**	**1**	100	30	50	Leaf	(1,1)	(1,2)	(1,3)	(1,4)	(1,5)	(1,6)
	2		90	100		(2,1)	(2,2)	(2,3)	(2,4)	(2,5)	(2,6)
	3	75	30	50		(3,1)	(3,2)	(3,3)	(3,4)	(3,5)	(3,6)
	4		90	100		(4,1)	(4,2)	(4,3)	(4,4)	(4,5)	(4,6)
	5	50	30	50		(5,1)	(5,2)	(5,3)	(5,4)	(5,5)	(5,6)
	6		90	100		(6,1)	(6,2)	(6,3)	(6,4)	(6,5)	(6,6)
	7	100	30	50	Flower	(7,1)	(7,2)	(7,3)	(7,4)	(7,5)	(7,6)
	8	75	30	50		(8,1)	(8,2)	(8,3)	(8,4)	(8,5)	(8,6)
	9	50	30	50		(9,1)	(9,2)	(9,3)	(9,4)	(9,5)	(9,6)

All the samples were redissolved in 10 mL methanol and submitted to the fractionation scheme described previously. The EtOAc fractions were taken to dryness with a rotary evaporator, analysed by ^{1}H NMR and tested for both antibacterial and antibiotic-potentiating activities.

3.2.4.2. Antibacterial susceptibility testing

The MIC was determined by microdilution techniques according to CLSI (2015a) guidelines as described in *Section 3.2.1.2*. The highest concentration of DMSO remaining after dilution (5% v v^{-1}) caused no inhibition of bacterial growth. At least three independent experiments were performed.

3.2.4.3. Antibiotic-potentiation testing by checkerboard

The checkerboard assay was performed according to CLSI (2015a) guidelines as described in *Section 3.2.1.3*. The antibacterial compounds yielded final concentrations ranging from 2 × MIC to 1/64 × MIC. Compounds with no detectable antibacterial activity were tested at several concentrations between 120 and 0.06 mg l^{-1}. Drug combinations did not exceed 5% v v^{-1} of the well (200 µl). Antibiotic–phytochemical interactions were classified using the FICI as described in TABLE 3.6 in *Section 3.2.1.7*. When the MIC of the phytochemical alone was not detected in the range of tested concentrations, the double of the highest concentration tested was used for FICI determination and if FICI value was ≤ 0.5 this interaction was determined to be a potentiation (Sopirala et al., 2010).

3.2.4.4. Antibiotic-potentiation testing by disk diffusion method

This method was performed as described in *Section 3.2.1.5*. The antibiotic-potentiating activity *C. striatus* extracts was categorized according to TABLE 3.6 in *Section 3.2.1.7*.

3.2.4.5. NMR analysis

Each dried sample was mixed with 500 µl of CD_3OD, vortexed for 10 s, sonicated for 20 min at 42 kHz, and then centrifuged at 13000 rpm at room temperature for 5 min. The supernatant (300 µl) was transferred to a 3 mm micro-NMR tube and analysed. ^{1}H NMR spectra were recorded at 25°C on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton Larmor frequency of 600.13 MHz. Methanol-d_4 was used as the internal lock. ^{1}H NMR experimental parameters were the following: 128 scans requiring 10 min and 26 s acquisition

time, 0.16 Hz point^{-1}, pulse width (PW) = 30° (11.3 μs), and relaxation delay (RD) = 1.5 s. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to CD_3OD_4 at 3.3 ppm, using TOPSPIN 3.2 software (Bruker BioSpin GmbH, Rheinstetten, Germany). 2D-NMR techniques (*J*-resolved, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMBC, and ^{1}H-^{13}C HSQC), were performed when necessary as previously described (Ali et al., 2012; Kim et al., 2010b).

3.2.4.6. Isolation of the bioactive compounds

NMR-guided isolation was used to isolate the bioactive compounds from *C. striatus* leaves for their identification. For this, 55 g of leaves were extracted with 500 ml of MeOH and fractionated with EtOAc as described before. The resulting dry EtOAc fraction (1.2 g) was subjected to medium pressure liquid chromatography (MPLC). The sample was introduced as solid using a pre-Elut column connected just before the main separation column (200 × 35 mm, i.d, Sepacore® Silica 80g, Buchi, Switzerland). Elution was performed using a gradient of chloroform (A) and methanol (B) (B:10 %, 15 min; B:10-30%, 5 min; B: 30%, 20 min; B: 30-50%, 5 min; B:50%, 10 min) at a flow-rate of 20 ml min^{-1}. The eluant was monitored at 220, 254, 280 and 365 nm. Sixty fractions were collected and combined in 6 fractions according to Thin Layer Chromatography (TLC) profile similarities. The TLC was performed on silica gel TLC plates (Merck, Darmstadt, Germany) with $CHCl_3$: MeOH: acetic acid (7.5:2.5:0.2). These six fractions were prepared for ^{1}H NMR analysis and for their antibacterial-potentiating analysis as described previously. One of the fractions (A3) proved to be active according to the disk diffusion method. This fraction (270 mg) was separated on a Sephadex column with methanol at a flow of 3.5 ml min^{-1} and monitored at 220, 254, 280, 365 nm. Sixty sub-fractions were collected and combined into 6 fractions (B1-B6) according to their TLC profile similarities. These were analysed by ^{1}H NMR analysis and tested for their antibacterial-potentiating capacity. Of all these sub-fractions (B), two (B5 and B6) showed activity: B5 proved to have an antibiotic-potentiating activity with both ciprofloxacin and erythromycin and B6 had antibacterial activity against *S. aureus* strains. The fraction B5 (13.5 mg) was purified by semi-preparative reverse-phase high performance liquid chromatography (RP-HPLC) (Luna C_{18} column; 250 × 10 mm, i.d., 5 μm, Phenomex®) and eluted with a gradient of solvent (A) 1% aqueous acetic acid and (B) methanol (B:1-42%, 30 minutes; B: t 42-100%, 10 minutes; B:100%, 2 minutes). The detector was set at 270 nm. Six main fractions were obtained and analysed by ^{1}H NMR analysis for the identification of metabolites, and 2D-NMR techniques were used when necessary. The B6 fraction (8.3 mg) contained only one compound that was identified as luteolin.

3.2.4.7. EtBr accumulation assay by fluorometry

The effect of the compounds isolated from *C. striatus* on EtBr accumulation in *S. aureus* SA1199B and also in MRSA strains was evaluated seeking for efflux pump inhibition. This assay was performed as previously explained in *Section 3.2.2.4.* The MIC of EtBr (prepared in a stock of 10 g l^{-1}) was determined for each strain according to CLSI (2015a) guidelines and described in *Section 3.2.1.2*. Reserpine (20 mg l^{-1}) was used as a positive control as it is a recognized EPI (Holler et al., 2012). To be able to compare the EtBr accumulation experiments, the relative fluorescence (RF) was determined for each assay by applying the equation: $RF_{assay} = (MFI_{assay} - FI_{control})/ FI_{control}$, where MFI_{assay} was the Maximal Fluorescence Intensity obtained in each 60 min assay and $FI_{control}$ was the correspondent Fluorescence Intensity obtained with the DMSO control at the same time. High RF values indicated that cells accumulated more EtBr under the tested conditions than the control and vice-versa for negative values. The experiments were repeated three times, and the RF values presented are the averages of three independent assays.

3.2.4.8. Biofilm control assay

Biofilms of *S. aureus* CECT 976, a control strain usually assessed for biofilm assays (Borges et al., 2012; Saavedra et al., 2010), and SA1199B were developed according to the modified microtiter plate test as described in *Section 3.2.2.1*. Overnight cultures (1×10^8 cells ml^{-1}) were added to sterile 96-well polystyrene microtiter plates to form biofilms during 24 h at 37ºC and 150 rpm. Afterwards, the medium was removed and the biofilms were exposed to the compounds isolated from *C. striatus* (60 g l^{-1}), for 1 h at 37ºC and 150 rpm. Ciprofloxacin and erythromycin at MIC were tested as well. The same protocol was repeated for an exposure time of 24 h. After incubation, biofilms were washed twice with saline solution (0.85% NaCl), scrapped and diluted for CFU counting. The number of CFU cm^{-2} was assessed on MH agar. The results are also presented as CFU reduction (%) from the DMSO control (5% v v^{-1}).

3.2.4.9. Data analysis and statistics

The NMR spectra were bucketed using AMIX 3.9.12 (Bruker BioSpin GmbH, Rheinstetten, Germany). Buckets were obtained by integrating 0.04 ppm intervals and scaling the intensity of individual peaks to the total intensity recorded in the region δ 0.2– 10.02 ppm. The regions of δ 4.85–4.95 and δ 3.2–3.4 were excluded from the analysis because of the residual signal of D_2O and CD_3OD, respectively. To correlate metabolites detected in the ^1H NMR spectra with the bioactivity, OPLS was applied to the data using Y-variable of potentiating activity (IZDs), allowing the removal of spectral X-variables unrelated to the sample classes chosen and filtration

of the biomarkers from the biological varieties. OPLS based on Pareto scaling were performed with the SIMCA-P + software (v. 12.0, Umetrics, Umeå, Sweden). The outliers were not considered. The metabolites contributing to the separation were analysed by an S-plot of the OPLS modeling. For antibacterial-potentiating, EtBr and biofilm assays means and standard deviations were calculated and means comparisons were made with Student's *t*-test (two-tailed based on a confidence level < 0.05).

3.2.5. Task 5. Evaluation of the structure-activity relationship of isoflavonoids as antibiotic adjuvants in clinical therapy

Small description.	Isoflavonoids isolated from *Cytisus striatus* were previously found to have interesting antibiotic-potentiating activities. For SAR evaluation purposes, 22 isoflavonoids were assessed as antibiotic adjuvants. The effect of the isoflavonoids in NorA inhibition was also assessed by fluorometry and flow cytometry. The possible inhibition of Erm methyltransferases was tested. Biofilm eradication activity of isoflavonoids was evaluated within exposure periods of 1 and 24 hours.
Bacterial strains.	*S. aureus* RWW337, M116, RWW50, M82, RN6390, CECT 976, SA1199B and RN4220. Information of bacterial strains can be found in *Section 3.1.4.*
Antibiotics.	Ciprofloxacin and erythromycin. Preparation and specificities of the antibiotics are described in *Section 3.1.5*. Erythromycin (15-μg) and clindamycin (2-μg) commercial disks were obtained from Oxoid (Hampshire, United Kingdom).
Phytochemicals	Biochanin A, calycosin, calycosin-7-*O*-β-D-glucoside, corylifol A, daidzein, daidzin, formonenetin, genistein, genistin, glycitein, glycitin, 3'-hydroxypuerarin, iridin, irigenin, irisflorentin, 3'-methoxypuerarin, neobavaisoflavone, ononin, orobol, puerarin, tectoridin and tectorigenin. Preparation and specificities of the isoflavonoids are described in *Section 3.1.6*. TABLE 3.4 shows the chemical structures of the tested compounds. Reserpine (Sigma-Aldrich, Sintra, Portugal), a recognized EPI (Gibbons and Udo, 2000; Schmitz et al., 1998), was used as positive control in EtBr accumulation assays and prepared in the same way.

3.2.5.1. Antibacterial susceptibility testing

The MIC of each isoflavonoid was determined by microdilution technique according to CLSI (2015a) guidelines described in *Section 3.2.1.2*. The highest concentration of DMSO remaining after dilution (5% v v^{-1}) caused no inhibition of bacterial growth. At least three independent experiments were performed.

3.2.5.2. Antibiotic-potentiation testing by checkerboard

This method was performed for combinations between antibiotics and isoflavonoids as previously described in *Section 3.2.4.3*. Antibiotic–isoflavonoid interactions were classified using the FICI system as described in TABLE 3.6 in *Section 3.2.1.7*. When the MIC of the phytochemical alone was not detected in the range of tested concentrations, the double of the highest concentration tested was used for FICI determination and if FICI value was ≤ 0.5 this interaction was determined to be a potentiation (Sopirala et al., 2010).

3.2.5.3. Antibiotic-potentiation testing by disk diffusion method

This method was performed as described in *Section 3.2.1.5*. The antibiotic-potentiating activity of isoflavonoids was categorized according to TABLE 3.6 in *Section 3.2.1.7*.

3.2.5.4. EtBr accumulation assay by fluorometry

This assay was performed as previously explained in *Section 3.2.2.4*. To be able to compare the EtBr accumulation experiments, RF values were calculated as described previously in *Section 3.2.4.7*. The experiments were repeated three times, and the RF values presented are the averages of three independent assays.

3.2.5.5. EtBr accumulation assay by flow cytometry

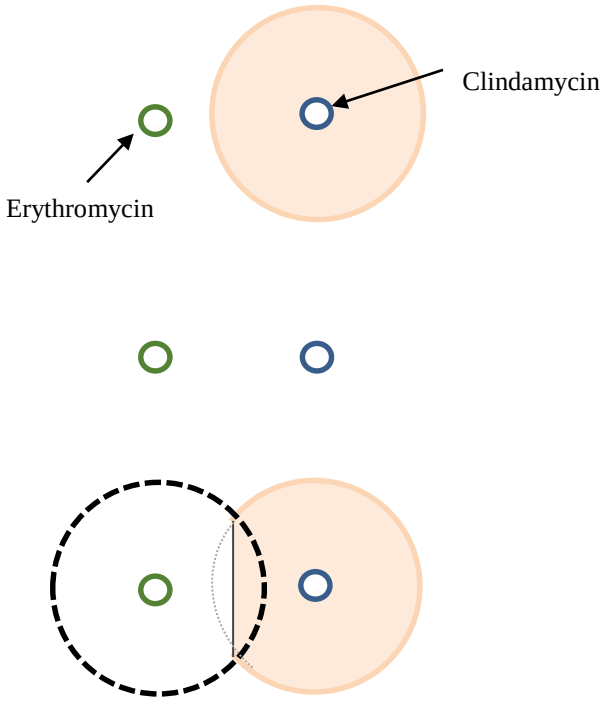
Flow cytometry facilitates the analysis of cells in suspension as they pass through a beam of light at a high rate in a fluid stream. Data acquisition and analysis were performed using a BD Accuri™ C6 Flow Cytometer (Breda, The Netherlands) equipped with three fluorescence detectors: FL1 (515 to 565 nm), FL2 (565 to 605 nm), and FL3 (> 605 nm). Absorption maximum for EtBr is 518 nm and the fluorescence emission maximum is 605 nm. Preparation of *S. aureus* SA1199B was performed as described in the fluorometric method (*Section 3.2.2.4*) and bacterial cultures were distributed in aliquots of 100 µl. EtBr was added at ½ MIC and the isoflavonoids were added at the best concentration defined with the fluorometric method. Positive controls were performed with 20 mg l^{-1} reserpine and negative controls with 1% (v v^{-1}) DMSO. Following incubation at 25°C for 60 minutes, the supernatant was removed and the pellet resuspended in EtBr-free PBS, adjusting the OD_{600} to 0.3. Samples were taken for fluorescence measurement in the BD Accuri™ C6 Flow Cytometer. Data was collected for at least 10,000 events per sample.

3.2.5.6. Biofilm control assay

Biofilms were developed according to the modified microtiter plate test as described in *Section 3.2.2.1*. The exposure of isoflavonoids at 60 mg l^{-1}, except orobol that was tested at 30 mg l^{-1} on biofilms for 1 and 24 h was performed as previously described in *Section 4.4.7*. The number of CFU cm^{-2} was assessed on MH agar and the results are presented as CFU reduction (%) from the DMSO control (5% v v^{-1}).

3.2.5.7. D-test to detect inducible macrolide antibiotic resistance

A major form of resistance to the macrolide-lincosamide-streptogramin type B (MLS_B) antibiotics in pathogenic bacteria results from a base-specific methylation of bacterial 23S ribosomal RNA near or within the macrolide binding site (Gaynor and Mankin, 2003). As a result, the MLS antibiotics lose their ability to bind to the ribosome and no longer exhibit antibacterial activity. RNA methylation occurs through the action of the Erm (erythromycin-resistance methylase) family of methyltransferases (Hajduk et al., 1999). Three resistance phenotypes have been detected to these antibiotics: strains containing $cMLS_B$ (constitutive MLS_B) and $iMLS_B$ (inducible MLS_B), which are resistant to macrolide, lincosamide and streptogramin B antibiotics, and MS, which is only resistant to macrolide and streptogramin B antibiotics. Inducible resistance is obtained following exposure to a macrolide and can be detected by induction tests such as the D-test using erythromycin and clindamycin disks (Fiebelkorn et al., 2003). Despite macrolide antibiotic resistance in *S. aureus* is usually due to ribosomal target modification, active efflux mechanism encoded by *msrA* can also occur. This method was performed as described by Fiebelkorn et al. (2003). The bacterial suspensions were adjusted to 0.5 McFarland standards and seeded over hardened MH agar Petri dishes using a sterilized cotton swab and allowed to set (for 10 to 15 min). MH agar plates prepared with each isoflavonoid yielding the final concentration desired were also prepared, according to *Section 3.1.8*. Control plates had no isoflavonoids incorporated. Erythromycin (15 μg $disk^{-1}$) and clindamycin (2 μg $disk^{-1}$) commercial disks were placed in adjacent positions (15 or 20 mm). After 24 h, the plates were read and analysed according to FIGURE 3.7. The flattening of the clindamycin zone adjacent to the erythromycin disk indicates inducible MLS_B resistance. The characterization of the strains for their susceptibility profiles in D-test was performed according to CLSI (2015b) guidelines.



Phenotype	Inferred genotype
MS phenotype • Erythromycin **R** • Clindamycin **S** (with circular zone of inhibition)	*msrA*
cMLS$_B$ phenotype • Erythromycin **R** • Clindamycin **R** (with circular zone of inhibition if any)	*erm*
iMLS$_B$ phenotype • Erythromycin **R** • Clindamycin **S** (D-shaped zone of inhibition)	*erm*

FIGURE 3.7 | Detecting staphylococci resistance to macrolides and lincosamides by D-test. Disks containing erythromycin (15 μg) and clindamycin (2 μg) are placed 15 to 20 mm apart on an agar plate that has been inoculated with the clinical isolate. In the top scheme, the lack of a zone of inhibition around the erythromycin disk indicates bacterial resistance to macrolides (e.g. perhaps due to MsrA efflux pump); the large clear zone of inhibition around the clindamycin disk indicates sensitivity to clindamycin; the medium scheme indicates constitutive resistance to both erythromycin and clindamycin; the bottom scheme depicts a positive D-test: diffusion of erythromycin from the disk towards the clindamycin disk does not kill bacteria due to *Staphylococcus aureus* resistance to macrolides; however, *S. aureus* contains an erythromycin-inducible methylase (iMLS$_B$) that is encoded by a plasmid-borne gene (*erm*) and when this methylase is induced it alters the binding site on the 23S subunit of the 50S ribosome that both erythromycin and clindamycin bind to, making both antibiotics ineffective (inducing resistance). Resistance phenotypes were identified on the basis of erythromycin- and clindamycin-susceptibility tests and on the basis of clinical implications (CLSI, 2015b; Leclercq, 2002; Prabhu et al., 2011; Woods, 2009). MSB, macrolides and streptogramins B; R, resistant; S, susceptible.

3.2.5.8. Statistical analysis

For statistical analysis, the *in vitro* results were analysed by Student's *t* test using the statistical program SPSS version 19.0. Statistical calculations were based on a confidence level ≥ 95% ($P < 0.05$) which was considered statistically significant.

3.2.6. Task 6. The potential of *Buxus sempervirens* for antibiotic-potentiating activity: isolation and identification of antibiotic adjuvants from this plant

Small description.	*Buxus sempervirens* was chosen for its promising antibiotic-potentiating activity and evaluated for its ability to control (remove and inactivate) biofilms of *S. aureus* CECT 976 within 1 h of exposure. Further phytochemical investigation of *B. sempervirens* was performed. One possible candidate, betulinic acid, along with five similar triterpenoids, were assessed for their antibacterial activity, synergic interaction with the antibiotics by checkerboard technique and, additionally, were also applied against *S. aureus* biofilms.
Bacterial strains.	*S. aureus* CECT 976. Information on this strain can be found in *Section 3.1.4.*
Antibiotics.	Ampicillin, ciprofloxacin, erythromycin and tetracycline. Preparation and specificities of the antibiotics are described in *Section 3.1.5*.
Phytochemicals.	Betulinic acid, lupeol, betulin, hederagenin, ursolic acid and oleanolic acid. Preparation and specificities of the triterpenoids are described in *Section 3.1.6*.
Plant material.	*Buxus sempervirens* L. for. grandiflora (F. Muell.) P. Cout. (leaves). Relevant information about the preparation of this plant can be found in *Section 3.1.7.*

3.2.6.1. Biofilm control assay

Biofilms control experiment was developed as described in *Section 3.2.2.1*. Overnight cultures (~10^6 cells ml^{-1}) were added to sterile 96-well polystyrene microtiter plates to form biofilms at 37ºC and stirring at 150 rpm for 24 h. Afterwards, the medium was removed and the biofilms were exposed to the antibiotics and to the plant extract individually and in combination at 37ºC and stirring at 150 rpm for 1 h. Antibiotic solutions were applied at MIC and 50 × MIC against biofilms. The MeOH extract of *B. sempervirens* was applied at concentrations ranging from 0.05 to 5 g l^{-1}. Drug combinations did not exceed 5% v v^{-1} of the well (200 µl). After incubation, biofilms were washed twice with saline solution (0.85% NaCl), scraped and diluted for CFU counting. The numbers of CFU per unit of adhesion area (CFU cm^{-2}) were assessed in MH agar. Reduction (%) of the number of CFU cm^{-2} (compared with DMSO growth control) was also assessed.

3.2.6.2. Fractionation of active extract of *Buxus sempervirens*

Buxus sempervirens leaves (160 g) were extracted with 500 ml of MeOH following the process described previously (*Section 3.2.3.1*). The MeOH extract was taken to dryness and redissolved in 225 ml of H_2O/MeOH (4:1) and successively partitioned with 3 × 112.5 ml of dichloromethane ($CHCl_2$) and buthanol (*n*-BuOH), respectively. All the fractions were analysed by ^{1}H NMR and tested for both antibacterial and antibiotic-potentiating activities. Afterwards, the *n*-BuOH fraction (1.2 g) was further submitted to phytochemical investigation. The *n*-BuOH fraction was subjected to medium pressure liquid chromatography (MPLC, Sepacore, Büchi, Switzerland) in a silica gel column (80 g, 200 × 35 mm, i.d, Büchi), eluted with a gradient of $CHCl_3$ (A): MeOH + HOAc 2% (B) as follow: 10% B for 15 min; linear increase 10-30% B in 5 min; isocratic elution using 30% B for 20 min; linear increase 30-50% B in 5 min, and finally 50% B for 10 min. The flow rate was 20 ml min^{-1} and the analysis was monitored by UV spectrometer at 220, 254, 280 and 365 nm. Collection was performed by volume where each fraction contained 20 ml, totalizing 53 fractions. The fractions were analysed by analytical TLC and combined into 8 subfractions. TLC was performed using silica gel TLC plates (Merck, Darmstadt, Germany) with $CHCl_3$:MeOH:HOAc (7.5:2.5:0.2). These eight fractions were analysed by ^{1}H NMR and for their antibacterial-potentiating activity.

3.2.6.3. NMR analysis

Five hundred microliters of CD_3OD were added to dried samples, and the resultant mixtures were vortexed for 10 s and sonicated for 20 min at 42 kHz, followed by centrifugation at 3999 g at room temperature for 5 min. Three hundred microliters of the supernatant were transferred to a 3 mm micro-NMR tube and analysed. ^{1}H NMR spectra were recorded at 25 °C on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton frequency of 600.13 MHz. MeOD-d_4 was used as the internal lock. ^{1}H NMR experimental parameters were described in *Section 3.2.4.5.* The resulting spectra were manually phased and baseline corrected, and calibrated to residual CD_3OD at 3.3 ppm, using TOPSPIN 3.2 software (Bruker BioSpin GmbH, Rheinstetten, Germany).

3.2.6.4. Antibacterial susceptibility testing

The MIC of the tested compounds was determined by microdilution techniques according to CLSI (2015a) guidelines as described in *Section 3.2.1.2*. The highest concentration of DMSO remaining after dilution (5% v v^{-1}) caused no inhibition of bacterial growth. At least three independent experiments were performed.

3.2.6.5. Antibiotic-potentiation testing by checkerboard

The checkerboard assay was performed according to CLSI (2015a) guidelines as described in *Section 3.2.1.3* between the phytochemical tested (which had a detectable MIC) and antibiotics. Both antibacterial compounds to be combined yielded final concentrations ranging from 2 × MIC to 1/64 × MIC. Drug combinations did not exceed 5% v v^{-1} of the well (200 µl). Antibiotic–phytochemical interactions were classified using the FICI as described in TABLE 3.6 at *Section 3.2.1.7*.

3.2.6.6. Statistical analysis

For statistical analysis, the *in vitro* results were analysed by Student's *t* test using the statistical program SPSS version 19.0. Statistical calculations were based on a confidence level ≥ 95% ($P < 0.05$), which was considered statistically significant.

Chapter

4

RESULTS AND DISCUSSION

In this section, all the results obtained within this thesis are described, analysed and discussed. Comparison with other important studies and results from relevant bibliographic sources is performed and the novelty of the results obtained is emphasised. A brief conclusion of each task is presented, highlighting the main results obtained and suggestions for future work.

This chapter contains information that was published or has been submitted in:

Abreu, A. C., Serra, S., Borges, A., Salgado, A., Saavedra, M. J., McBain, A. J., Simões, M. (2015) Combinatorial activity of flavonoids with antibiotics against drug-resistant *Staphylococcus aureus*. *Microb Drug Res;* **21**: 600-609 **(Task 1; *Section 4.1*)**.

Abreu, A. C., Serra, S., Borges, A., Salgado, A., Saavedra, M. J., Simões, M. (2014) Evaluation of the best method to assess antibiotic potentiation by phytochemicals against *Staphylococcus aureus*. *Diagn Microbiol Infect Dis;* **9**: 125-134 **(Task 1; *Section 4.1*)**.

Abreu A. C., Saavedra, M. J., Simões, L. C., Simões, M. (2016) Combinatorial approaches with selected phytochemicals to increase antibiotic efficacy against *Staphylococcus aureus* biofilms. *Biofouling;* **32**: 1103-1114 **(Task 2; *Section 4.2*)**.

Abreu A. C., Coqueiro, A., Sultan, A. R., Lemmens, N. Kim, H. K., Verpoorte, R. van Wamel, W. J. B., Simões, M., Choi, Y. H. (2016) Looking to Nature for new concepts in antimicrobial treatments: isoflavonoids from *Cytisus striatus* as antibiotic adjuvants against MRSA. *Submitted.* **(Tasks 4 and 5; *Sections 4.4 and 4.5*)**.

Abreu A. C., Paulet, D., Coqueiro, A., Malheiro, J., Borges, A., Saavedra, M. J., Choi, Y. H., Simões, M. (2016) Antibiotic adjuvants from *Buxus sempervirens* to promote effective treatment of drug-resistant *Staphylococcus aureus* including biofilms. *RSC Advances;* **6**: 95000-95009 **(Task 3 and 6; *Sections 4.3 and 4.6)***.

4.1. TASK 1. EVALUATION OF THE BEST METHOD TO ASSESS ANTIBIOTIC POTENTIATION BY PHYTOCHEMICALS AGAINST *STAPHYLOCOCCUS AUREUS*

Abstract

Selected plant alkaloids and flavonoids were combined with five antibiotics against ten *S. aureus* strains, including those expressing distinct efflux pumps and MRSA. The efficacy of each combination was assessed using the microdilution checkerboard, time-kill, Etest and disk diffusion methods. The methods were optimized for detecting antibiotic-potentiation and the results compared. Reserpine, pyrrolidine, quinine, morin and quercetin were highlighted as antibiotic potentiators. Disk-diffusion and checkerboard methods were considered the best methods to detect antibiotic-potentiation.

4.1.1. Characterization of *S. aureus* strains regarding their resistance profile

The classifications of the *S. aureus* strains according to their antibiotic resistance profile were done based on the comparison of the MICs/IZDs results and the susceptibility breakpoints of CLSI (2015a; 2015b) (TABLE 4.1). Discrepancies between the categorizations were considered insignificant when an isolate found intermediate by one method was susceptible according to another, and no distinction was made in these cases. For *S. aureus* SA1199B, RN4220 and XU212, only ciprofloxacin, erythromycin and tetracycline were tested, respectively. Also, Etest was only done for the antibiotics which achieved good combinatorial results on the checkerboard and disk-diffusion method: ciprofloxacin, tetracycline and erythromycin.

The agreement between the MICs obtained by checkerboard and Etest (within ± 1-$\log_2$ dilutions) was 75% for tetracycline, 88% for erythromycin and only 38% for ciprofloxacin. According to the classifications presented by Reynolds et al. (2003), there was good agreement for erythromycin and poor agreements for tetracycline and ciprofloxacin. The discrepancy results were caused by broth MICs higher than Etest MICs. So, these results demonstrate an apparent increased sensitivity of the agar testing method over the microdilution assay. In the majority of studies, the agreement between the MICs of these two methods varies since these test systems measure different interactions between an antimicrobial agent and a microorganism (Mayrhofer et al., 2008; Serrano et al., 2003; Szekely et al., 1999; van der Heijden et al., 2007). Therefore, it is expected that there will be some minor differences between the systems (Rennie et al., 2012).

TABLE 4.1 | Minimal inhibitory concentration (MIC, for checkerboard and Etest) and inhibition zone diameter (IZD, for disk-diffusion method) of antibiotics against *Staphylococcus aureus*. Strains were classified as susceptible (S), intermediate (I) or resistant (R) to antibiotics according to CLSI (2015b)

S. aureus	**Antibiotic**	**Checkerboard MIC (mg l^{-1})**	**Etest MIC (mg l^{-1})**	**IZD (mm)**	**Classif.**
CECT 976	CIP	1	0.064	33.3 ± 0.6	S
	TET	0.96	0.38	23.7 ± 0.6	S
	ERY	0.24	0.19	26.3 ± 0.6	S
	AMP	1.5	NP	36.0 ± 1.0	S
	OXA	0.48	NP	39.7 ± 0.6	S
SA1199B	CIP	128	4	13.0 ± 0.0	R
XU212	TET	128	32	9.3 ± 0.6	R
RN4220	ERY	256	256	NI	R
MRSA MJMC001	CIP	256	>32	NI	R
	TET	0.5	0.38	26.0 ± 0.0	S
	ERY	96	96	12.5 ± 0.6	R
	AMP	64	NP	NI	R
	OXA	128	NP	NI	R
MRSA MJMC002	CIP	256	>32	NI	R
	TET	0.5	0.38	26.0 ± 0.0	S
	ERY	96	96	12.0 ± 0.0	R
	AMP	64	NP	NI	R
	OXA	128	NP	NI	R
MRSA MJMC004	CIP	256	>32	NI	R
	TET	0.5	0.38	27.0 ± 1.0	S
	ERY	96	96	12.0 ± 0.0	R
	AMP	64	NP	NI	R
	OXA	15.6	NP	NI	R
MSSA MJMC003	CIP	0.5	0.19	36.0 ± 0.0	S
	TET	2	0.38	35.0 ± 1.0	S
	ERY	0.5	0.19	32.3 ± 0.6	S-I*
	AMP	25	NP	10.0 ± 1.0	R
	OXA	1	NP	23.3 ± 0.6	S
MSSA MJMC009	CIP	1	0.19	36.3 ± 0.6	S
	TET	0.5	0.25	35.0 ± 0.0	S
	ERY	2	2	32.0 ± 0.0	S-I*
	AMP	10	Np	10.0 ± 0.0	R
	OXA	1	NP	23.7 ± 0.6	S
MSSA MJMC010	CIP	1	0.19	37.0 ± 1.0	S
	TET	0.5	0.38	37.0 ± 0.0	S
	ERY	0.5	0.25	32.0 ± 0.0	S
	AMP	10	NP	10.0 ± 0.0	R
	OXA	1	NP	23.7 ± 0.6	S

*Discordance between methods; NI, no inhibition; NP, not performed; AMP: ampicillin; OXA: oxacillin; CIP: ciprofloxacin; TET: tetracycline; ERY: erythromycin; Time-kill assays were performed only for *S. aureus* SA1199B, RN4220 and XU212; Etest was only directed for CIP, TET and ERY. Data are means and SD from at least three independent experiments.

According to the analysis of MICs and IZDs by the susceptibility breakpoints of CLSI (2015a) guidelines, the MRSA strains were classified as resistant to ampicillin, oxacillin, erythromycin and ciprofloxacin and only susceptible to tetracycline. MSSA strains were classified as susceptible to all antibiotics, with exception of ampicillin. Broth test, Etest and disk-diffusion method showed 95% agreement on these classifications.

4.1.2. Antibacterial and antibiotic-potentiating activity of alkaloids

Alkaloids are heterocyclic nitrogen compounds (Cowan, 1999). There is an excellent rationale that plant alkaloids should possess antibacterial activity, particularly given the number of cytotoxic drugs and templates from this source (Gibbons, 2004).

All alkaloids were assessed for antibacterial activity using the broth microdilution technique. Only quinine had antibacterial activity, with a MIC of 500 mg l^{-1} against *S. aureus* CECT 976. For the other strains, no MIC was detected for quinine up to 1500 mg l^{-1}. The other alkaloids had no activity against the strains tested for the same concentrations. Reserpine added to the MH broth generated a precipitate, perhaps because of its low solubility in water, causing a blank turbidity, which complicated the detection of its MIC. Therefore, colony counts in MH agar were performed in order to evaluate the growth of the bacteria with the increasing concentrations of reserpine. No growth reduction was verified. No IZD was obtained when each alkaloid was applied alone to the disks at 1500 mg l^{-1}.

As previously mentioned, for detecting RMAs or antibiotic coadjuvants, it is expected to observe a high inactivation of resistant bacteria when the combination of antibiotic/plant compound is applied and only minor or no antibacterial activity of the phytochemical. TABLE 4.2 presents the results of the combinations of antibiotics and alkaloids obtained by checkerboard, Etest, disk-diffusion method and time-kill assay. Etest was only directed for CIP, TET and ERY which achieved good combinatorial results on the checkerboard and disk-diffusion method. Time-kill assay was only performed for the strains with known efflux pumps (*S. aureus* SA1199B, XU212, RN4220) to compare the results obtained with the other methods. Only the combinations that resulted in a significant modification in comparison to antibiotics alone in, at least, one method, have been presented. No increase in the MICs of the antibiotics was obtained in the presence of alkaloids ($P > 0.05$). The IZD of each combination was never smaller than that produced by each drug alone ($P > 0.05$).

TABLE 4.2 | Minimal inhibitory concentrations (MIC)-fold reductions (by checkerboard and Etest), inhibition zone diameters (IZDs) increases (by disk-diffusion) and log_{10} CFU reductions (by time-kill assay) for the combination of antibiotics with alkaloids. Classification of the interaction as potentiation (P), additive (A) or indifferent (I) is made. Total agreement (T) was obtained when all methods give the same classification to the interaction, and minor (m) or major (M) disagreements if one or more than one method, respectively, are in disagreement

S. aureus	**Antib.**	**Alk.**	**Checker-board**	**Etest**	**Disk diffusion**	**Time-kill**	**Final classif.***
CECT 976	ERY	PYR	4.0 (P)	4.0 (P)	6.7 (P)	NP	P (T)
	AMP	RES	NC (I)	NC (I)	9.7 (P)	NP	I (m)
		QUIN	NC (I)	NC (I)	6.0 (P)	NP	I (m)
SA1199B	CIP	RES	2.0 (A)	2.0 (A)	5.0 (A)	0.2 (I)	A (T)
		PYR	2.0 (A)	2.0 (A)	4.3 (A)	2.4 (P)	A (T)
		QUIN	1.3 (I)	8.0 (P)	12.0 (P)	1.3 (A)	P (m)
XU212	TET	RES	4.0 (P)	3.0 (P)	4.0 (A)	2.8 (P)	P (m)
		QUIN	1.3 (I)	2.0 (A)	1.7 (I)	2.0 (P)	I (T)
RN4220	ERY	PYR	4.0 (P)	8.0 (P)	8.7 (P)	0.4 (I)	P (T)
MJMC001	TET	QUIN	2.0 (A)	2.0 (A)	4.7 (A)	NP	A (T)
MJMC002	TET	RES	2.0 (A)	2.0 (A)	NC (I)	NP	A (m)
		QUIN	2.0 (A)	2.0 (A)	NC (I)	NP	A (m)
MJMC003	CIP	RES	2.0 (A)	2.0 (A)	4.0 (A)	NP	A (T)
		PYR	2.0 (A)	2.0 (A)	NC (I)	NP	A (m)
		QUIN	2.0 (A)	2.0 (A)	NC (I)	NP	A (m)
	TET	PYR	2.0 (I)	NC (I)	6.0 (P)	NP	I (m)
		QUIN	2.0 (A)	2.0 (A)	NC (I)	NP	A (m)
	ERY	RES	2.0 (A)	2.0 (A)	NC (I)	NP	A (m)
		PYR	4.0 (P)	4.0 (P)	12.0 (P)	NP	P (T)
		QUIN	2.0 (I)	NC (I)	NC (I)	NP	I (T)
	AMP	RES	7.0 (P)	NP	NC (I)	NP	?
MJMC009	CIP	RES	2.0 (A)	2.0 (A)	8.7 (P)	NP	A (m)
		PYR	8.0 (P)	2.0 (A)	8.0 (P)	NP	P (m)
		QUIN	4.0 (P)	2.0 (A)	10.0 (P)	NP	P (m)
	TET	RES	8.0 (P)	NC (I)	5.0 (A)	NP	? (M)
		PYR	2.0 (A)	NC (I)	5.0 (A)	NP	A (m)
		QUIN	8.0 (P)	NC (I)	5.0 (A)	NP	? (M)
	ERY	RES	NC (I)	2.0 (A)	9.0 (P)	NP	? (M)
		PYR	2.0 (A)	4.0 (P)	8.3 (P)	NP	P (m)
		QUIN	2.0 (A)	NC (I)	5.0 (A)	NP	A (m)
	AMP	RES	7.0 (P)	NP	NC (I)	NP	?
		PYR	14.0 (P)	NP	NC (I)	NP	?
		QUIN	7.0 (P)	NP	NC (I)	NP	?
MJMC010	CIP	RES	8.0 (P)	4.0 (P)	6.0 (P)	NP	P (T)
		PYR	4.0 (P)	3.0 (P)	6.0 (P)	NP	P (T)
	TET	RES	8.0 (P)	6.0 (P)	NC (I)	NP	P (m)
		QUIN	8.0 (P)	1.5 (I)	NC (I)	NP	I (m)

ERY	RES	NC (I)	2.0 (A)	9.0 (P)	NP	? (M)
	PYR	2.0 (A)	4.0 (P)	NC (I)	NP	? (M)
	QUIN	2.0 (A)	4.0 (P)	4.0 (A)	NP	A (m)

NP, not performed; NC, no change; (?) contradictory results. CAF: caffeine; RES: reserpine; PYR: pyrrolidine; THEO: theophylline; QUIN: quinine; AMP: ampicillin; OXA: oxacillin; CIP: ciprofloxacin; TET: tetracycline; ERY: erythromycin. Only the combinations that were significantly different from the antibiotic control in, at least, one method are presented. Additive interactions were distinguished from indifference when two or more methods indicated that same categorical result. Time-kill assays were performed for *S. aureus* SA1199B, RN4220 and XU212; Etest was directed for CIP, TET and ERY. *Final classification considers the results of checkerboard, Etest and disk diffusion method. Data are means and SD from at least three independent experiments.

FIGURE 4.1 shows the resume of time-kill assay after 24 h of cell incubation with the combination of antibiotics with the alkaloids. The $\log_{10}$ CFU increase or decrease obtained for each combination over the single activity of the antibiotic alone can be visualised.

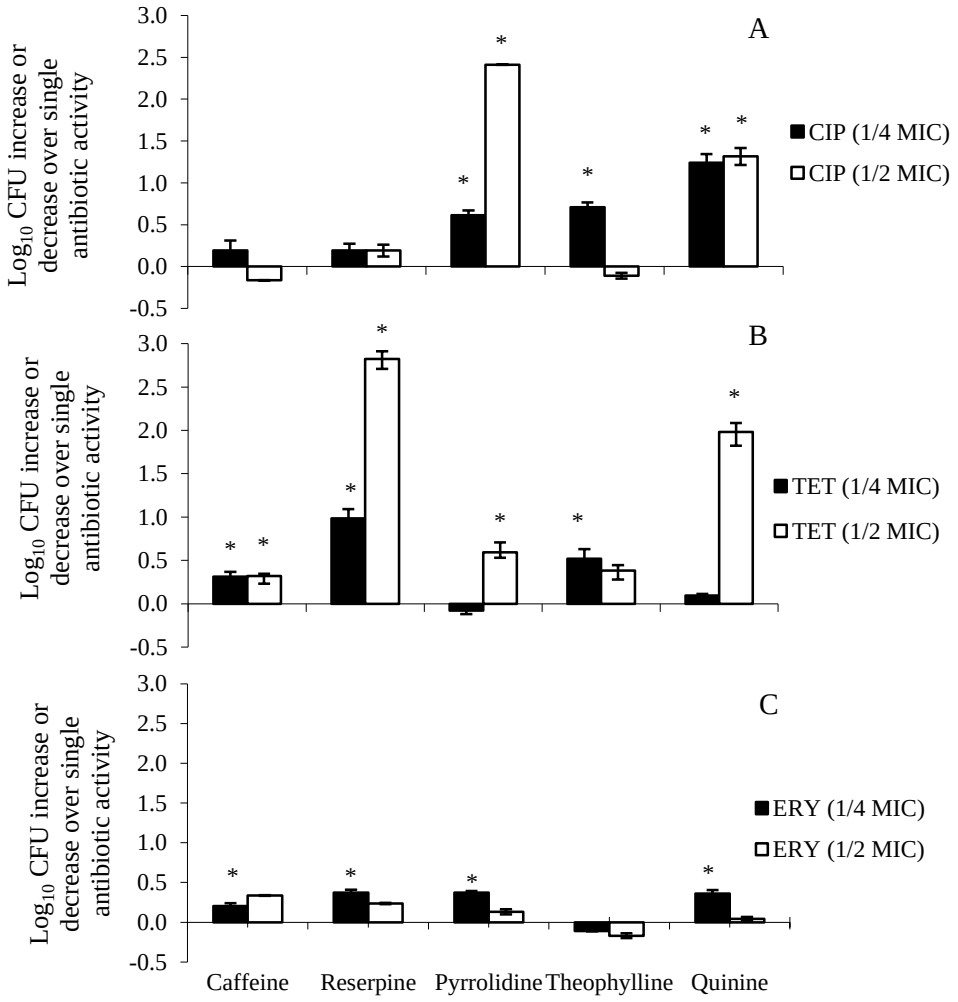


FIGURE 4.1 | Time-kill assay after 24 h incubation of *Staphylococcus aureus* SA1199B (A), XU212 (B) and RN4220 (C) with the alkaloids combined with ciprofloxacin (CIP), tetracycline (TET) and erythromycin (ERY), respectively. Bars with (*) are statistically different from the antibiotic controls ($P < 0.05$).

No CFU reduction was observed with the alkaloids alone for the concentrations tested ($P > 0.05$, data not shown). The effect of the combination between pyrrolidine and ciprofloxacin (½ MIC) against *S. aureus* SA1199B caused 2.4 $\log_{10}$ decrease in CFU ml^{-1} (with an initial inoculum of 6.5 ± 0.53 $\log_{10}$ CFU ml^{-1} to undetectable levels) after 24 h of incubation when compared with ciprofloxacin alone. Also, the combination between quinine and ciprofloxacin (½ MIC) caused a 1.3 $\log_{10}$ decrease in CFU ml^{-1} after the same time over antibiotic activity alone. Tetracycline at ½ MIC combined with reserpine and quinine against *S. aureus* XU212 achieved $\log_{10}$ reduction of 2.8 and 2.0 in CFU ml^{-1}, respectively, comparing with that promoted by tetracycline alone. No effect was observed with the combination of alkaloids and erythromycin against *S. aureus* RN4220 ($P > 0.05$).

Results from each test were classified based on the interpretation of an interaction as negative/indifferent/additive/potentiation and compared between each other (TABLE 4.2). Indifference was most common among all three methods, being found in 157 of 190 combinations (82.6%), considering the cases showing agreement or minor disagreement. Additive interactions were found in 14 of 190 combinations (7.4%). Potentiating activities were found in 11 of 190 combinations (5.8%), also considering both the total agreement and minor disagreement cases. No negative interactions were detected. Relevant potentiation or additive interactions were observed with:

- reserpine (100 mg l^{-1}) when combined with ciprofloxacin against SA1199B, MSSA strains (3/3), and with tetracycline against XU212, one MSSA strain (1/3) and one MRSA strain (1/3);
- pyrrolidine (500 mg l^{-1}) when combined with erythromycin against strains CECT 976, RN4220, and MSSA strains (2/3), and with ciprofloxacin against MSSA strains (3/3) and SA1199B;
- quinine (100 mg l^{-1}) when combined with ciprofloxacin against SA1199B and two MSSA strain (2/3).

No effect was observed with the combination between β-lactams and alkaloids. Also, caffeine and theophylline did not potentiate any antibiotic against any strain.

4.1.3. Antibacterial and antibiotic-potentiating activity of flavonoids

Flavonoids are hydroxylated phenolics commonly found in leaves, fruits and flowers. The existence, number, position and degree of substitution of hydroxyl or methyl groups on the benzene ring provide much of the structural variation found in flavonoids (Bohm, 1998) and they consequently have a diverse range of pharmacological properties, including anti-inflammatory,

oestrogenic, anti-allergic, antifungal, antimicrobial and antioxidant (Cowan, 1999; Harborne and Williams, 2000; Martini et al., 2004). Importantly, several flavonoids were found to potentiate some antibiotics due to a possible activity as inhibitors of MDR mechanisms, such as efflux pumps (Musumeci et al., 2003). Quercetin, rutin, hesperidin and (+)-catechin were reported to have a synergistic activity when combined with oxacillin against VISA (Basri et al., 2008). It was aimed to evaluate if these compounds were able to potentiate other antibiotics against other *S. aureus* resistant strains. Morin, an isomer of quercetin, was also included in the tests.

No MIC was detected for any flavonoid for concentrations lower than 1500 mg l^{-1}. Also, no IZD was obtained with the flavonoids for the same amount of compound (22.5 μg $disk^{-1}$).

TABLE 4.3 presents the results of the combinations of antibiotics and flavonoids obtained by checkerboard, Etest, disk-diffusion method and time-kill assay. Only the combinations that resulted in a significant modification in comparison with antibiotics alone in, at least, one method have been presented.

TABLE 4.3 | Minimal inhibitory concentrations (MIC)-fold reductions (by checkerboard and Etest), inhibition zone diameters (IZDs) increases (by disk-diffusion) and log_{10} CFU reductions (by time-kill test) for the combination of antibiotics with flavonoids. Classification of the interaction as potentiation (P), additive (A) or indifferent (I) is made. Total agreement (T) is obtained when all methods give the same classification to the interaction; minor disagreement (m) if one method is in disagreement

S. aureus	**Antib.**	**Flav.**	**Checker-board**	**Etest**	**Disk diffusion**	**Time-kill**	**Final classif.***
CECT 976	CIP	MOR	2.0 (A)	NC (I)	4.4 (A)	NP	A (m)
		QUERC	4.0 (P)	3.0 (P)	20.0 (P)	NP	P (T)
	TET	MOR	4.0 (P)	4.0 (P)	9.3 (P)	NP	P (T)
		QUERC	4.0 (P)	12.0 (P)	24.0 (P)	NP	P (T)
		RUT	NC (I)	3.0 (P)	7.0 (P)	NP	P (m)
	ERY	MOR	2.0 (I)	NC (I)	6.4 (P)	NP	I (m)
		QUERC	4.0 (P)	2.0 (I)	14.0 (P)	NP	P (m)
	AMP	MOR	1.5 (I)	NP	9.7 (P)	NP	-
SA1199B	CIP	MOR	NC (I)	3.0 (P)	13.7 (P)	-1.6 (I)	P (m)
		QUERC	4.0 (P)	3.0 (P)	18.9 (P)	-1.2 (I)	P (T)
XU212	TET	MOR	NC (I)	NC (I)	NC (I)	1.2 (I)	I (T)
		QUERC	NC (I)	NC (I)	NC (I)	2.3 (P)	I (T)
MJMC001	TET	MOR	4.0 (P)	3.0 (P)	6.0 (P)	NP	P (T)
		QUERC	8.0 (P)	8.0 (P)	6.1 (P)	NP	P (T)
MJMC002	TET	MOR	4.0 (P)	6.0 (P)	9.0 (P)	NP	P (T)
		QUERC	8.0 (P)	6.0 (P)	18.0 (P)	NP	P (T)
		RUT	NC (I)	NC (I)	8.3 (P)	NP	I (m)

MJMC003	CIP	MOR	2.0 (I)	6.0 (P)	8.3 (P)	NP	P (m)
		QUERC	2.0 (I)	NC (I)	15.0 (P)	NP	I (m)
	TET	MOR	4.0 (P)	12.0 (P)	10.7 (P)	NP	P (T)
		QUERC	2.0 (A)	8.0 (P)	4.3 (A)	NP	A (m)
	ERY	MOR	2.0 (A)	2.0 (A)	15.0 (P)	NP	A (m)
		QUERC	NC (I)	NC (I)	5.0 (I)	NP	I (T)
MJMC004	TET	MOR	4.0 (P)	4.0 (P)	9.0 (P)	NP	P (T)
		QUERC	8.0 (P)	8.0 (P)	6.0 (P)	NP	P (T)
MJMC009	CIP	MOR	NC (I)	6.0 (P)	9.7 (P)	NP	P (m)
		QUERC	NC (I)	NC (I)	6.3 (P)	NP	I (m)
	TET	MOR	8.0 (P)	8.0 (P)	6.0 (P)	NP	P (T)
		QUERC	4.0 (P)	3.0 (P)	11.7 (P)	NP	P (T)
	ERY	MOR	2.0 (I)	16.0 (P)	18.1 (P)	NP	P (m)
		QUERC	NC (I)	3.0 (P)	16.3 (P)	NP	P (m)
MJMC010	CIP	MOR	2.0 (I)	16.0 (P)	6.0 (P)	NP	P (m)
	TET	MOR	8.0 (P)	8.0 (P)	10.3 (P)	NP	P (T)
		QUERC	2.0 (I)	8.0 (P)	6.0 (P)	NP	P (m)
	ERY	MOR	2.0 (I)	3.0 (P)	10.1 (P)	NP	P (m)
		QUERC	2.0 (A)	2.0 (A)	13.0 (P)	NP	A (m)
	AMP	QUERC	NC (I)	NP	3.2 (I)	NP	I (-)

NP, not performed; NC, no change. MOR: morin; QUERC: quercetin; RUT: rutin; HESP: hesperidin; CAT: catechin; AMP: ampicillin; OXA: oxacillin; CIP: ciprofloxacin; TET: tetracycline; ERY: erythromycin. Only the combinations that were significantly different from the antibiotic control in, at least, one method are presented. An additive interaction was only distinguished from indifference when two or more methods indicated that same categorical result. Time-Kill assays were only performed for *S. aureus* SA1199B, RN4220 and XU212; Etest was only directed for CIP, TET and ERY. *Final classification considers the results of checkerboard, Etest and disk diffusion method. Data are means and SD from at least three independent experiments.

Checkerboard and Etest methods revealed MIC reductions of the antibiotics, especially in the presence of morin and quercetin at 500 mg l^{-1}. No increase on the MIC was obtained for antibiotics with the addition of flavonoids, which proves there was no antagonism detected in the combinations. The same behavior was observed by disk-diffusion method, where the higher IZD increases were obtained especially with morin and quercetin. Also, the IZD of each combination was never smaller than that produced by each drug alone ($P > 0.05$).

Time-kill assays were performed only for *S. aureus* SA1199B, RN4220 and XU212 since this method was done on combinations selected on the basis of their performance, assessed by the other three tests. FIGURE 4.2 presents the $\log_{10}$ CFU ml^{-1} results *vs* time for each combination. No CFU reduction was observed with the flavonoids alone for the concentrations tested comparing to the growth control ($P > 0.05$, data not shown).

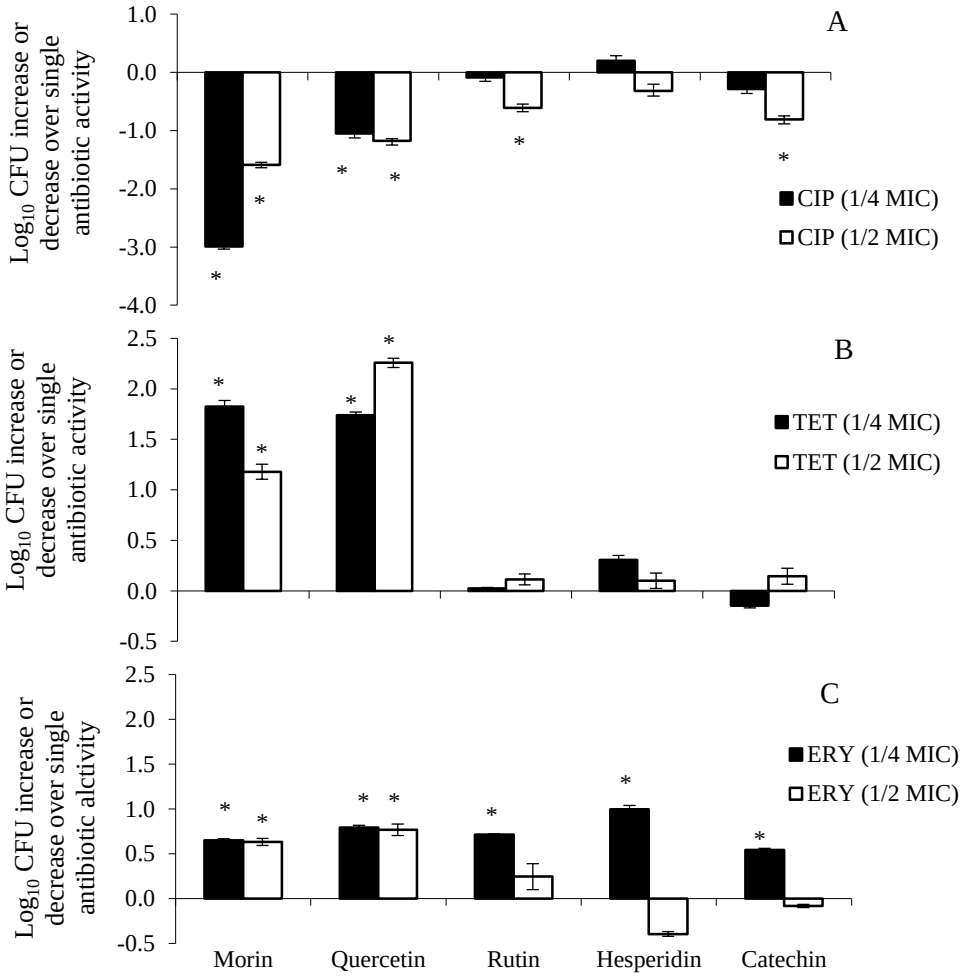


FIGURE 4.2 | Time-kill assay after 24 h incubation of *Staphylococcus aureus* SA1199B (A), XU212 (B) and RN4220 (C) with flavonoids combined with ciprofloxacin (CIP), tetracycline (TET) and erythromycin (ERY), respectively. Bars with (*) are statistically different from the antibiotic controls ($P < 0.05$).

Log_{10} CFU ml^{-1} increases were obtained for *S. aureus* SA1199B when ciprofloxacin (½ MIC) was combined with morin and quercetin (500 mg l^{-1}), comparing with ciprofloxacin alone (1.6 and 1.2 log_{10} increases of CFU ml^{-1}, respectively). The other methods provided potentiating effects for these combinations (TABLE 4.3). For *S. aureus* XU212, tetracycline combined with morin and quercetin caused 1.8 and 1.7 log_{10} CFU ml^{-1} reduction, respectively, when compared with tetracycline (¼ MIC); and 1.2 and 2.6 log_{10} CFU ml^{-1} reduction, respectively, compared with tetracycline (½ MIC). However, indifferent results were obtained for these combinations with the other three methods (TABLE 4.3). For *S. aureus* RN4220, time-kill assay and the other methods provided indifferent results.

The combinations tested with the four methods were classified as potentiation, additive or indifferent as previously described (TABLE 4.3). No antagonistic effects were observed. Considering the total agreement and minor disagreement cases (for checkerboard, Etest and disk-diffusion method), potentiation was found for 24 of 190 combinatorial cases (12.6%) and additive interactions only for 4 of 190 combinations (2.1%). The majority of the combinations (84.2%) were indifferent. Potentiation or an additive effect were obtained for the combination of:

- Morin (500 mg l^{-1}) with ciprofloxacin against SA1199B, MSSA strains (3/3) and CECT 976, tetracycline against CECT 976, MRSA (3/3) and MSSA strains (3/3), and erythromycin against MSSA (3/3);
- Quercetin (500 mg l^{-1}) with ciprofloxacin against CECT 976, SA1199B, and MSSA (1/3), tetracycline against CECT 976, MRSA (3/3) and MSSA (3/3) strains, and erythromycin against CECT 976 and MSSA (2/3);
- Rutin (500 mg l^{-1}) with tetracycline against CECT 976.

No additive or potentiating effects were obtained with catechin or hesperidin. Furthermore, no flavonoid potentiated the activity of neither β-lactam (ampicillin and oxacillin).

4.1.4. Overall agreement between the methods

As previously explained, total agreement between methods was defined as all methods having the same interpretative category, minor disagreement as only one method displaying disagreement results and major disagreement was defined when two or more methods display disagreement results. Time-kill assay results showed 66.7% of agreement when compared with the other methods for SA1199B, XU212, RN4220 (indeed, this method disagrees with the others in 5 out of the 6 cases showed in TABLE 4.2) for combinations with alkaloids. For combination with flavonoids, the agreement was higher (80%). However, time-kill assay usually failed to agree with the other methods for the most promising combinations (with morin and quercetin) against *S. aureus* SA1199B and XU212. Agreement between checkerboard, Etest and disk-diffusion method was obtained in 77.5% of combinatorial cases with alkaloids and 85% with flavonoids. So, considering the total population, agreement between these three methods was observed for 195 of 240 cases (81%). Minor disagreements were obtained in 41 of 240 cases (17.0%) and major disagreements were found in only 5 of 240 combinations (2.1%) and only for combinations involving alkaloids. About 214 of 240 cases (89.2%) showed agreement between checkerboard and Etest, 338 of 380 cases (88.9%) between checkerboard and disk-diffusion method and 210 of 240 cases (87.5%) demonstrated agreement between Etest and disk-diffusion method.

4.1.5. Relevant remarks

Regarding the alkaloids, despite the minimal antimicrobial activity observed, MIC reductions were obtained with reserpine, pyrrolidine or quinine combined with ciprofloxacin, tetracycline or erythromycin. Of all 190 combinations performed in this study, only 5.8% and 7.4% corresponded to potentiation and additive interactions, respectively, while the remaining were considered indifferent. No potentiating activity was obtained against MRSA strains; only additive effects were observed between tetracycline and quinine (against 2/3 MRSA strains) and reserpine (against 1/3 MRSA). MSSA strains and, less extensively, SA1199B, XU212 and RN4220 strains showed to be much more amenable to potentiation with the alkaloids than the MRSA strains. With the aim of finding new therapy options in mind, this is not the kind of result one would hope to see. The success of a potentiation may depend on the virulence, persistence and resistance profiles of the strains.

Reserpine, originally extracted from *Rauwolfia serpentina*, was already studied by several authors due to its properties as EPI (Aeschlimann et al., 1999; Gibbons and Udo, 2000; Markham et al., 1999; Schmitz et al., 1998). In this work, this alkaloid showed a potentiating/additive activity when combined with tetracycline against XU212, one MRSA and one MSSA strains and with ciprofloxacin against SA1199B, one MRSA and two MSSA strains. In general, this fact supports previous studies (Gibbons et al., 2003a; Gibbons and Udo, 2000; Schmitz et al., 1998). Reserpine appears to be involved especially in the inhibition of NorA and also TetK efflux pumps but other efflux pumps may probably be implicated. While reserpine had effect on some *S. aureus* strains, for others no effect was observed. This can be associated to the fact that strains could vary in the extent to which reserpine is able to block NorA or TetK or, also, due to the varying rates of transcription or expression of the *norA* and *tetK* genes or differences in the stability of its mRNA (Schmitz et al., 1998).

Pyrrolidine, found naturally in the leaves of tobacco and carrot, potentiated or had an additive effect on the activity of erythromycin against *S. aureus* RN4220, CECT 976 and one MSSA strain and of ciprofloxacin against all MSSA strains. This compound has never been studied for its synergistic activity.

Quinine occurs naturally in the bark of *Cinchona* tree and it is a well-known compound with a long history of use due to their efficacy as an anti-malaria agent. Despite major toxicity concerns and adverse reactions among patients, quinine remains one of the most commonly used antimalarial for therapy (van Vuuren and Viljoen, 2011). Moreover, this alkaloid was reported for the inhibition of the invasive ability of some bacteria, namely staphylococci, which would protect the immunocompromised patients of being infected during the antimalarials treatment (Wolf et al., 2002; Wolf et al., 2006). In this study, quinine potentiated or had an additive effect

on ciprofloxacin against SA1199B and MSSA 3 and 9. Several additive combinations were also observed with quinine combined with tetracycline and erythromycin.

Concerning the flavonoids, potentiating and additive interactions were found in 24 and 4 out of 190 combinatorial cases (12.6 and 2.1%), respectively, and mainly for combinations involving morin and quercetin. Both morin and quercetin increased the activity of ciprofloxacin and tetracycline against *S. aureus* CECT 976; of ciprofloxacin against SA1199B and of tetracycline against all MRSA and MSSA strains. Also, some potentiation/additive interactions were found with morin and quercetin combined with erythromycin. Rutin only caused potentiation when combined with tetracycline against *S. aureus* CECT 976. While some potentiating results were found on some *S. aureus* strains, for others this was not observed.

Quercetin is the most abundant flavonol found in the human diet (Duthie and Dobson, 1999). Morin is an isomer of quercetin and has also been found to possess antibacterial activity. Rutin is the glycosidic form of quercetin (Andlauer et al., 2001). Reports on the antibacterial activity of these flavonoids are conflicting, especially regarding their MICs, probably owing to inter and intra-assay variation in susceptibility testing or due to the difference in genetic variation of the strain (Basri et al., 2008; Cushnie and Lamb, 2005; Hirai et al., 2010; Rattanachaikunsopon and Phumkhachorn, 2010; Rauha et al., 2000). Quercetin (15 mg l^{-1}) was found to exert antibacterial activity against *S. aureus*, including MRSA, and to potentiate the activity of erythromycin, oxacillin, ampicillin, vancomycin and gentamicin against MRSA (Hirai et al., 2010). Other investigation showed that 87.3% of the combinations between eight flavonoids (including catechin, hesperidin, quercetin and rutin) and oxacillin were synergistic against VISA (Basri et al., 2008). However, in this study, no synergy was obtained with oxacillin against the tested *S. aureus* strains. Eumkeb et al. (2010) also demonstrated that quercetin, galangin and baicalein exhibited the potential to reverse bacterial resistance to β-lactam antibiotics against penicillin-resistant *S. aureus* (PRSA) apparently due to interaction with penicillinase, cytoplasmic membrane damage, inhibition of protein synthesis and changes on the PBP2a.

Among flavonoid-antibiotic combinations, the only potentiation of an antibiotic against a strain resistant to that antibiotic was observed for SA1199B with the combination of morin or quercetin with ciprofloxacin. In the other combinations, potentiation occurred only with strains that were susceptible to the antibiotics. Therefore, with the exception of the first situation which may be due to efflux pump inhibition, these activities are in general probably not related to a resistance–modifying activity. In fact, several studies have examined the potential of quercetin to inhibit various bacterial enzymes, explaining why this molecule has such a diverse applicability (including anti-tumor therapy, neuroprotection, cardiovascular disease prophylaxis, inflammation, diabetes mellitus, infection, etc.) (Ferry et al., 1996; Guardia et al., 2001; Scambia

et al., 1994). However, it is clear that further research is necessary to understand morin/quercetin potentiation of ciprofloxacin, tetracycline and erythromycin. One of the proposed mechanisms of action of quercetin was the inhibition of gyrases through two different mechanisms based either on interaction with DNA or with ATP binding site of gyrase (Plaper et al., 2003). Since ciprofloxacin also functions by inhibiting DNA gyrase, the presence of a second compound with the same target could inhibit or potentiate its activity. In fact, in this study, it was found potentiation of ciprofloxacin by quercetin.

In this study, four distinct methods were used to evaluate the potentiating activity of alkaloids and flavonoids when combined with common antibiotics. It is not new that experimentation on drug interactions can lead to opposite conclusions by different methodologies. The various tests used measure quite distinct effects of antibiotic interactions against bacteria and use different endpoints (inhibition or killing) and medium state (broth *vs* agar) (Bonapace et al., 2000; Eliopoulos and Eliopoulos, 1988; White et al., 1996). However, with this strategy, it was obtained an agreement of 81% between checkerboard, Etest and disk-diffusion method. Also, the agreements of 89.2% between checkerboard and Etest, 88.9% between checkerboard and disk-diffusion method and 87.5% between Etest and disk-diffusion method were also very good. On the contrary, the agreement of time-kill assay with the other three methods was lower. In general, the agreements obtained in the literature are diverse (Bonapace et al., 2000; Cantón et al., 2005; Lewis et al., 2002; Martin, 2010; Orhan et al., 2005; White et al., 1996). However, a variety of investigators found disagreement between checkerboard and time-kill results (Cappelletty and Rybak, 1996; Chang et al., 2007; Visalli et al., 1988; White et al., 1996).

Regarding the performance of all methods, Etest represents, apparently, the ideal testing methodology for the clinical microbiology laboratory since it is a simple and fast method with excellent reproducibility. However, it is somewhat expensive. Disk-diffusion method seems to be as an excellent strategy because it is also very easy to perform, has good agreement results with the others methods and it is much less expensive. In fact, agar methods showed to be suitable for tests with phytochemicals (and plant extracts). Due to their low solubility in broth medium, the phytochemicals may precipitate when added to the medium even at low concentrations. When one is performing checkerboard, this precipitation will difficult the reading of the endpoint (i.e., the complete inhibition of growth). The same does not happen in agar testing since the inhibition zones can be read anyway and bacterial growth visualised. However, checkerboard allows to test several concentrations and define minimal concentrations causing antibiotic potentiation. Time-kill assay is very laborious if a high number of combinations are required, and the results are greatly influenced by differences on the inoculum size (White et al., 1996). Also, methods for the interpretation of kill-kinetic studies vary and synergy has been defined by some authors as at least

a 100-fold increase in killing at 24 h (Bonapace et al., 2000; Jung et al., 2004; Kiraz et al., 2009; Mayer and Nagy, 1999), while other authors consider a 200-fold increase (Guo et al., 2008; Leonard, 2012; Tan et al., 2011).

Considering the advantages and disadvantages of each method, it can be considered that the diffusion method can be easily used to detect antibiotic potentiation promoted by phytochemicals or plant extracts, but confirmation of potentiation using checkerboard should be done (also to determine ideal concentrations for cotherapy). For combinations between two antibacterial compounds, checkerboard is the most preeminent method and synergy is defined taking into account FICI calculations.

4.2. TASK 2. COMBINATORIAL APPROACHES WITH SELECTED PHYTOCHEMICALS TO INCREASE ANTIBIOTIC EFFICACY AGAINST *STAPHYLOCOCCUS AUREUS* BIOFILMS

Abstract

Combinations of selected phytochemicals (reserpine, pyrrolidine, quinine, morin and quercetin) with antibiotics (ciprofloxacin, tetracycline and erythromycin) were tested on the prevention and control of *S. aureus* biofilms. The phytochemicals were also studied for their ability to avoid antibiotic adaptation and to inhibit antibiotic efflux pumps. Morin, pyrrolidine and quercetin at subinhibitory concentrations had significant effects in biofilm prevention and/or control when applied alone and combined with antibiotics. Synergy between antibiotics and phytochemicals was found especially against biofilms of NorA overexpressing strain *S. aureus* SA1199B. This strain growing with subinhibitory concentrations of ciprofloxacin developed increased tolerance to this antibiotic. However, this was successfully reversed by quinine and morin. Besides reserpine, quercetin also showed significant efflux pump inhibition. The overall results demonstrate the role of phytochemicals in co-therapies to promote more efficient treatments and decrease bacterial resistance to antibiotics.

4.2.1. Effect of phytochemicals for biofilm control within 1 and 24 h exposure

Phytochemicals have been extensively studied as antibacterial products and, more recently, as potentiators/adjuvants of antibiotics (Markham et al., 1999). However, few data can be found on their effects against biofilms, even though most infections are biofilm-related. Biofilms are recognized for their insusceptibility to current therapeutic approaches (Römling and Balsalobre, 2012; Smith, 2005). In biofilms, *S. aureus* expresses a different set of genes such as *ica*, *bap* and *agr* types, with an increased potential to induce and develop recurrent infections (Gogoi-Tiwari et al., 2015). Therefore, new biofilm control strategies are required.

The ability of phytochemicals and antibiotics to control 24 h-old biofilms formed in sterile 96-well polystyrene microtiter plates within 1 and 24 h of exposure was evaluated. Antibiotics were first applied alone at MIC, 10 × MIC and 50 × MIC. As no significant differences ($P > 0.05$) in biofilm $\log_{10}$ CFU cm^{-2} reduction were obtained, the combinations were performed with antibiotics at MIC. FIGURE 4.3 presents the number of CFU cm^{-2} in the biofilm after the incubation with antibiotics at their MIC and phytochemicals individually for 1 h.

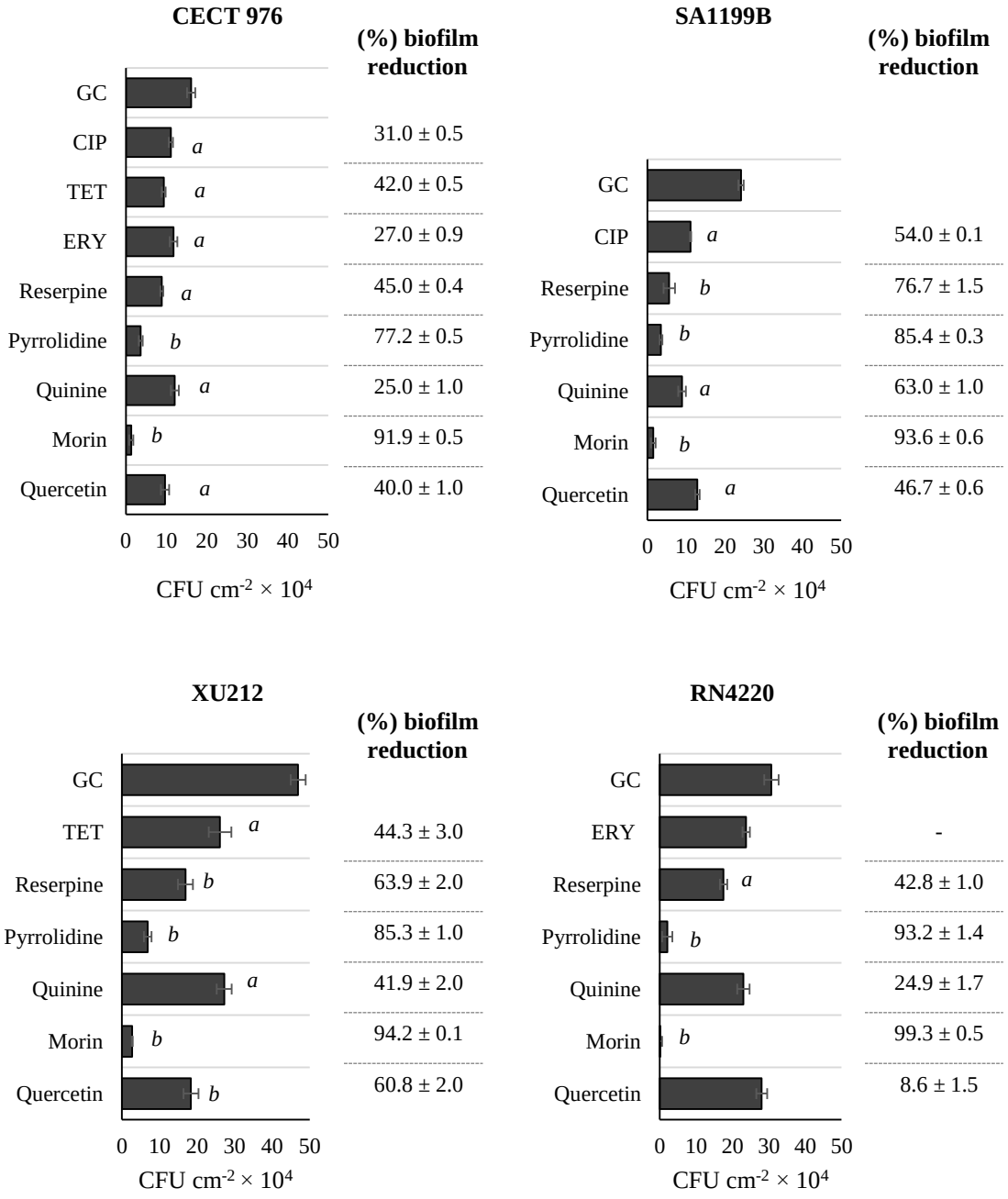


FIGURE 4.3 | CFU cm^{-2} of 24 h-old biofilms of *Staphylococcus aureus* strains that remained active after exposure to selected phytochemicals and antibiotics for 1 h. Strains SA1199B, XU212 and RN4220 were only exposed to the antibiotic they are resistant to: ciprofloxacin (CIP), tetracycline (TET) and erythromycin (ERY), respectively. Antibiotics were applied at minimal inhibitory concentration (MIC); reserpine and quinine at 100 mg l^{-1}; pyrrolidine, morin and quercetin at 500 mg l^{-1}. (*a*) when statistically lower than GC ($P < 0.05$); (*b*) when statistically lower than GC and the antibiotics ($P < 0.05$). GC – growth control (5% v v^{-1} DMSO).

The number of CFU cm^{-2} of the biofilms after the incubation with antibiotics at their MIC and phytochemicals individually for 24 h is presented in FIGURE 4.4.

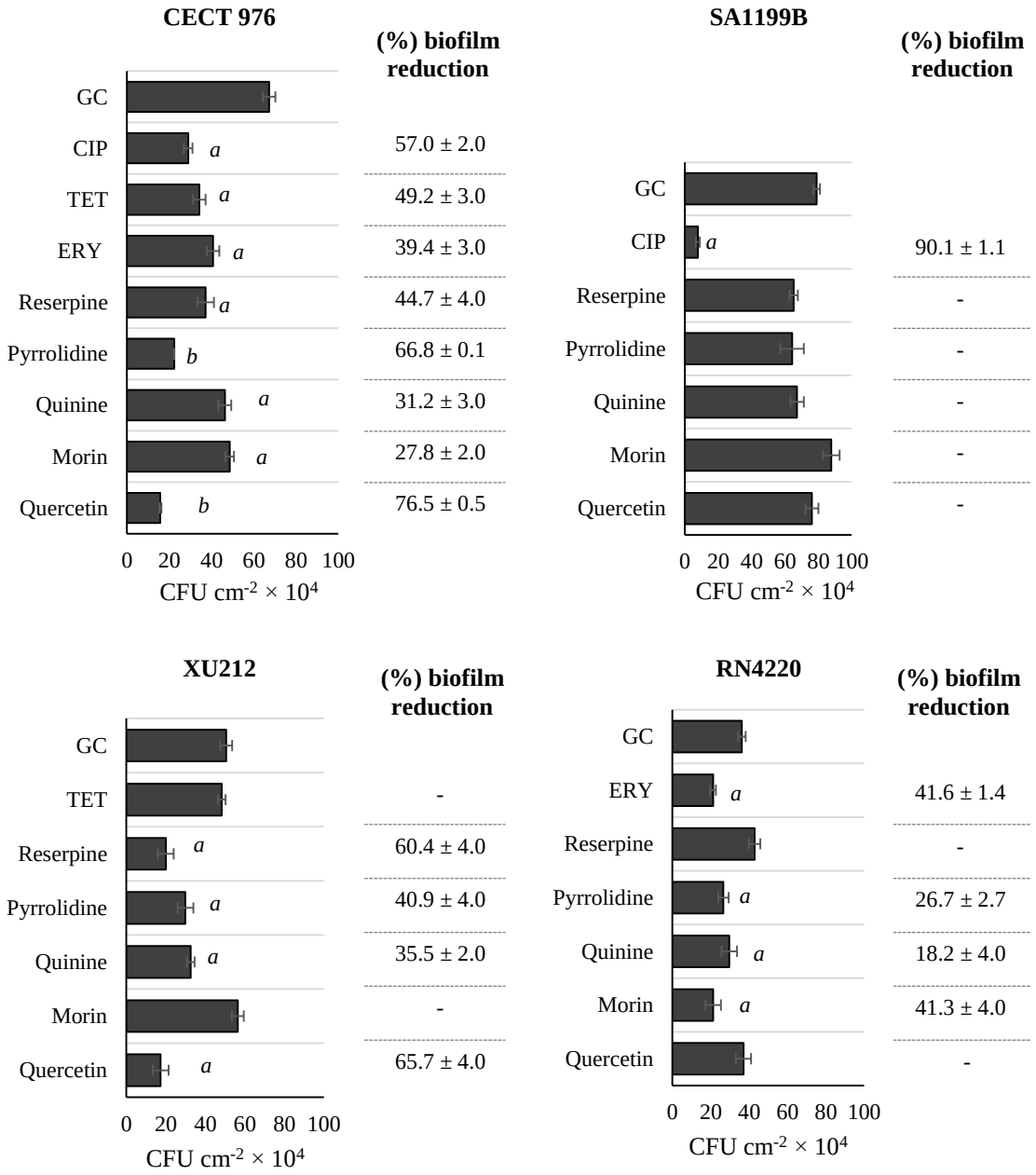


FIGURE 4.4 | CFU cm^{-2} of 24 h-old biofilms of *Staphylococcus aureus* strains that remained active after exposure to selected phytochemicals and antibiotics for 24 h. Strains SA1199B, XU212 and RN4220 were only exposed to the antibiotic they are resistant to: ciprofloxacin (CIP), tetracycline (TET) and erythromycin (ERY), respectively. Antibiotics were applied at minimal inhibitory concentration (MIC); reserpine and quinine at 100 mg l^{-1}; pyrrolidine, morin and quercetin at 500 mg l^{-1}. (*a*) when statistically lower than GC ($P < 0.05$); (*b*) when statistically lower than GC and the antibiotics ($P < 0.05$). GC – growth control (5% v v^{-1} DMSO).

Morin at 500 mg l^{-1} applied for 1 h caused the highest CFU reductions (log_{10} CFU cm^{-2} reductions of 1.2 - 2.1 for all strains, which is equivalent to 92 - 99% of biofilm eradication), followed by pyrrolidine (500 mg l^{-1}, log_{10} CFU cm^{-2} reductions of 0.7 - 1.1; 77 - 93% of biofilm eradication). These reductions were higher ($P < 0.05$) than those obtained with antibiotics applied at MIC (log_{10} CFU cm^{-2} reductions of 0.08 - 0.4; 23 - 54 % of biofilm eradication). These results proposed that the selected phytochemicals caused biofilm disruption as they did not show antimicrobial activity by themselves.

Despite being effective when applied for 1 h, morin and pyrrolidine were not so effective ($P < 0.05$) in disturbing biofilms when exposed for 24 h, for which maximal log_{10} CFU cm^{-2} reductions were 0.3 and 0.5, respectively (biofilm eradication of 41 and 67%, respectively). In fact, the decreased susceptibility to prolonged exposure periods was generally observed for almost all the strains. This is probably due to the effects of longer incubation periods. The fast growing cells will die readily when exposed to the antibiotics but the dormant cells fraction can survive and replicate (Shafahi and Vafai, 2010). The exception is verified with strain SA1199B when exposed to ciprofloxacin, which log_{10} CFU cm^{-2} reduction increased with the longer exposure from 0.4 to 1.1 (promoting 90% of biofilm reduction).

4.2.2. Effect of selected phytochemicals to prevent biofilm formation

Additionally, in order to assess the ability of the phytochemicals to prevent bacterial adhesion and biofilm formation, tests with sessile cells were performed in the presence of the phytochemicals for 24 h. FIGURE 4.5 presents the number of CFU cm^{-2} that was able to adhere to polystyrene when grown in the presence of the antibiotics and phytochemicals for 24 h.

All antibiotics at MIC caused high prevention of biofilm formation - reduced number of CFU cm^{-2} compared to growth control ($P < 0.05$). Morin was able to reduce biofilm formation of all strains ($P < 0.05$), especially of RN4220 (1.4 log_{10} CFU cm^{-2} reductions; 98% of biofilm reduction). Additionally, quercetin at 500 mg l^{-1} also had a considerable effect in preventing biofilm formation ($P < 0.05$), particularly of strains SA1199B and XU212 (approximately 1 log_{10} CFU cm^{-2} reductions; 88% of biofilm reduction). Pyrrolidine was unable to prevent biofilm formation, as it did not affect biofilm removal when applied for 24 h, despite showing significant effects when applied for 1 h ($P > 0.05$). No antibiotic or phytochemical caused total biofilm control or prevention. This reinforces the higher tolerance of biofilm cells compared to their planktonic counterparts. Studies report that even cells that are merely adhered to a surface have more resistant phenotypes (Bridier et al., 2011). However, it seems that, among all phytochemicals, morin is particularly effective in controlling (within 1 h of incubation) and

inhibiting biofilm formation. This provides morin to the reduced list of phytochemicals with potential to interfere with the process of biofilm formation and development (Simões et al., 2009).

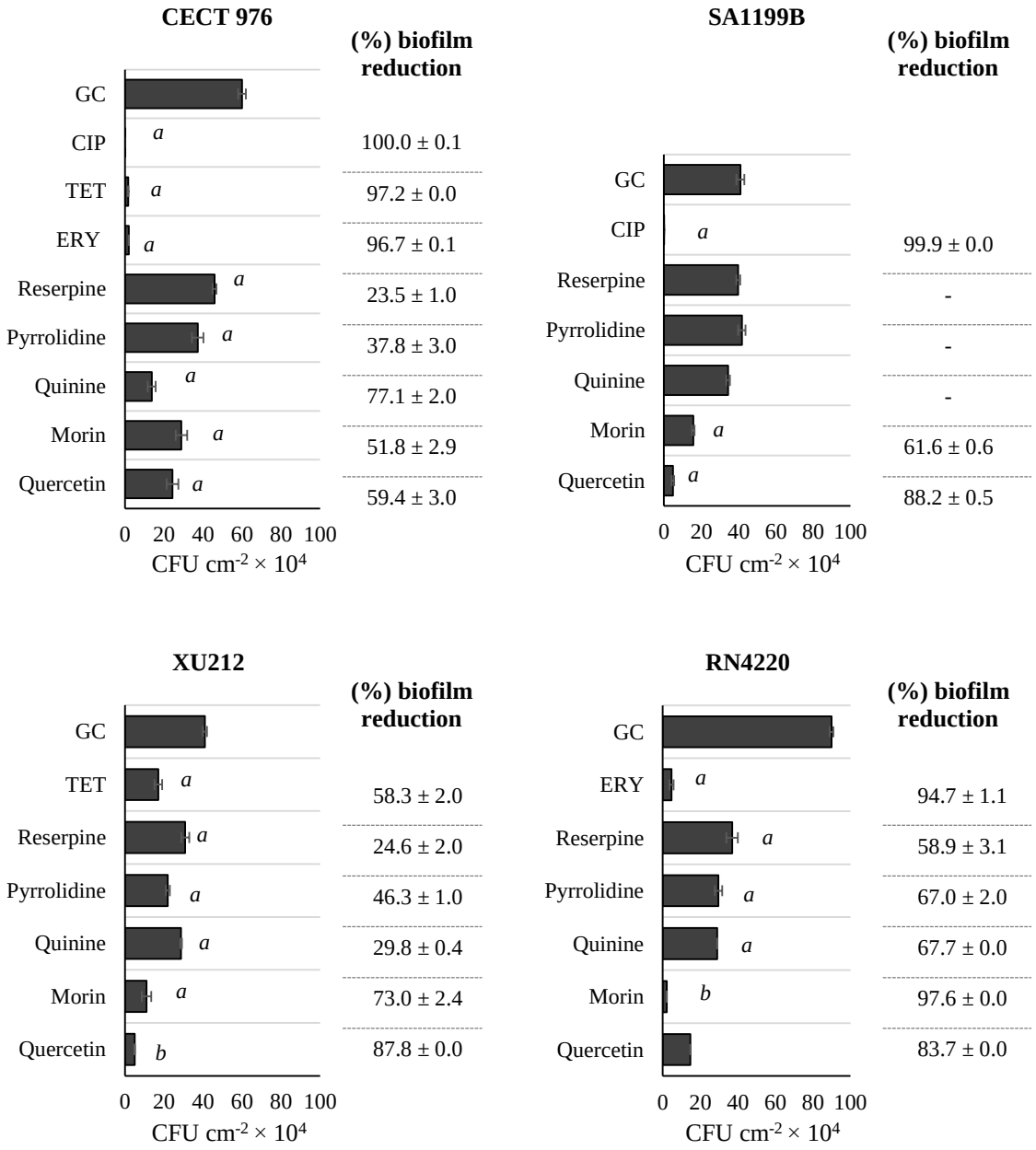


FIGURE 4.5 | CFU cm^{-2} of biofilms of *Staphylococcus aureus* strains formed during 24 h in 96-well microtiter plates in the presence of selected phytochemicals or antibiotics. Strains SA1199B, XU212 and RN4220 were only exposed to the antibiotic they are resistant to: ciprofloxacin (CIP), tetracycline (TET) and erythromycin (ERY), respectively. Antibiotics were applied at minimal inhibitory concentration (MIC); reserpine and quinine at 100 mg l^{-1}; pyrrolidine, morin and quercetin at 500 mg l^{-1}. (*a*) when statistically lower than GC ($P < 0.05$); (*b*) when statistically lower than GC and the antibiotics ($P < 0.05$). GC – growth control (5% v v^{-1} DMSO).

4.2.3. Effect of combinations antibiotic-phytochemical in biofilm control/prevention

TABLE 4.4 presents the log_{10} CFU cm^{-2} reductions caused by the combinations phytochemical-antibiotic towards *S. aureus* biofilm control and prevention.

TABLE 4.4 | Log_{10} CFU cm^{-2} reduction after biofilm exposure to the drugs combinations. A – biofilm exposure to antibiotics and phytochemicals for 1 h; B – biofilm exposure to antibiotics and phytochemicals for 24 h; C – biofilm grown in the presence of antibiotics and phytochemical/biofilm prevention

Strains	Antibiotic	Reserpine	Pyrrolidine	Quinine	Morin	Quercetin
A - Biofilm control (1 h)						
CECT 976	CIP	0.29	0.91	0.36	1.29	0.45
	TET	0.49	0.81	0.42	0.99	0.05 (A)
	ERY	0.28	**1.44 (S)**	0.26	0.64 (A)	0.28
SA1199B	CIP	0.37	1.10	0.53	0.98 (A)	0.57
XU212	TET	0.33	0.93	0.29	0.92 (A)	0.37
RN4220	ERY	0.01	1.08	0.24 (S)	1.03 (A)	0.47 (S)
B - Biofilm control (24 h)						
CECT 976	CIP	0.33	0.52	0.48	0.05 (A)	0.03 (A)
	TET	0.28	0.24 (A)	0.25	0.04 (A)	0.04 (A)
	ERY	0.49	0.47	0.48	0.18	0.28 (A)
SA1199B	CIP	**1.30 (S)**	**1.71 (S)**	**2.10 (S)**	1.05	0.92
XU212	TET	0.23	0.16	0.22	0.33 (S)	0.11
RN4220	ERY	0.36	0.39	0.49	0.29	0.05 (A)
C - Biofilm prevention						
CECT 976	CIP	3.21	3.34	3.51	1.42 (A)	1.13 (A)
	TET	1.43	0.97 (A)	1.41	1.33	1.06 (A)
	ERY	1.51	**2.09 (S)**	1.50	1.17	1.18
SA1199B	CIP	**3.52 (S)**	2.7	3.05	3.43	2.11
XU212	TET	0.70 (S)	**1.55 (S)**	0.48	**1.12 (S)**	1.16
RN4220	ERY	1.08	1.03	1.07	1.26	0.84

The combination was considered synergic (S) when log_{10} CFU cm^{-2} reduction of the combination treatment was significantly higher ($P < 0.05$) than the sum of log_{10} CFU cm^{-2} reductions of individual treatments (Monzón et al., 2001). An antagonistic (A) combination was obtained when the log_{10} CFU cm^{-2} reduction of the combination was significantly lower ($P < 0.05$) than that obtained with the most effective product. Bold values represent synergic interactions with log_{10} CFU cm^{-2} reduction ≥ 1.0.

The maximum CFU reduction was obtained after 24 h exposure to the synergic combination of ciprofloxacin and quinine (log_{10} CFU cm^{-2} reduction of 2.1) against SA1199B biofilms. Additionally, the combination between ciprofloxacin with reserpine, also considered synergic,

was the most efficient in preventing biofilm formation (log_{10} CFU cm^{-2} reduction of 3.5) of strain SA1199B. Other synergic antibiotic-phytochemical combinations were found, being highlighted the following ones causing ≥ 1 log_{10} reduction in CFU cm^{-2}: ciprofloxacin combined with pyrrolidine and reserpine (log_{10} CFU cm^{-2} reduction of 1.7 and 1.3, respectively) to control SA1199B biofilms exposed for 24 h; erythromycin combined with pyrrolidine to control biofilms of CECT 976 exposed for 1 h (log_{10} CFU cm^{-2} reduction of 1.4) and also to prevent their formation (log_{10} CFU cm^{-2} reduction of 2.1); tetracycline combined with pyrrolidine and morin to prevent biofilm formation of XU212 (log_{10} CFU cm^{-2} reduction of 1.6 and 1.1, respectively).

The majority of these synergic results corroborate previous studies with planktonic cells (*Section 4.1*). Some antagonistic interactions were obtained, especially for combinations involving the phenolic compounds. It seems that the presence of antibiotics disturbs the activity of morin, which was found to be effective when applied individually for 1 h. Indeed, biofilms facilitate the spread of antibiotic resistance by promoting horizontal gene transfer and cells can switch to more tolerant phenotypes upon environmental stresses (Fux et al., 2005). Antagonistic combinations were also found with quercetin when combined with antibiotics mainly against CECT 976 biofilms. Other discrepancies in the effects against the diverse strains were found, such as the fact that combinations of tetracycline with morin and tetracycline with pyrrolidine were synergic against XU212 but antagonist against CECT 976 in biofilm control for 24 h and biofilm prevention, respectively. This strain-dependent susceptibility can be related to the impact of combined stresses on their susceptibility/resistance patterns and it would need a more integrated approach in order to be fully understood. Antibiotic-mediated interactions may trigger multicellular behaviors in bacteria, which turns impossible the prediction of cell responses. The rest of the combinations tested had indifferent effects in biofilm prevention and control.

4.2.4. Effect of phytochemicals on preventing *Staphylococcus aureus* SA1199B adaptation to ciprofloxacin

Since most synergic interactions were obtained against strain SA1199B (the NorA overexpressing strain), a further experiment was conducted to force adaptation of SA1199B cells to ciprofloxacin. The phytochemicals were also added to understand their effects in reversing bacterial adaptation to this antibiotic ciprofloxacin after long exposure times.

FIGURE 4.6 shows the results obtained in the adaptation assay with SA1199B to ciprofloxacin (the treatment was performed as previously indicated in *Section 3.2.2.2* in FIGURE 3.6).

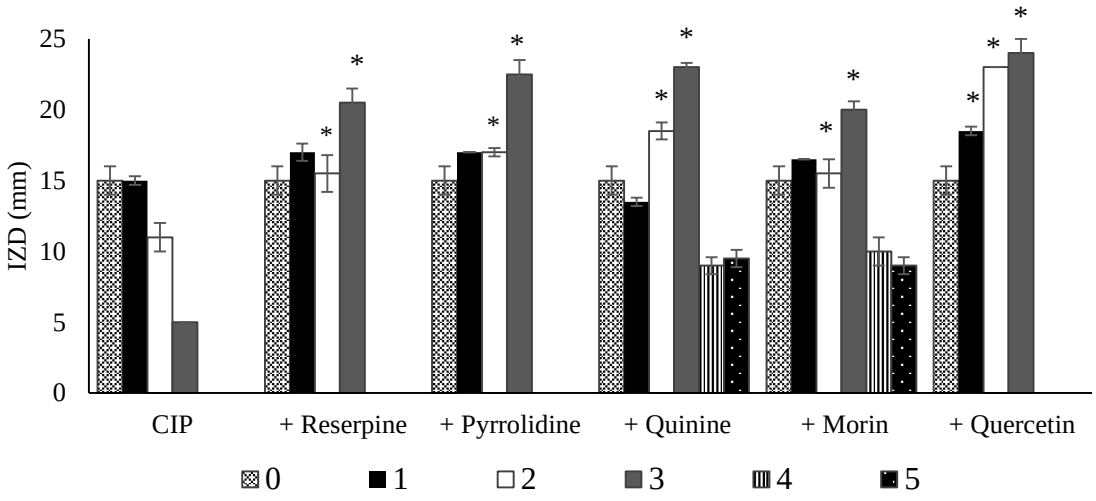


FIGURE 4.6 | Inhibition zone diameters (IZDs) promoted by ciprofloxacin (CIP) against *Staphylococcus aureus* SA1199B growing during 15 days in the presence of sub-inhibitory doses of this antibiotic and/or the five selected phytochemicals. Results are given for the five different phases of this assay by increasing ciprofloxacin doses every three days (from 1/32 MIC to 1/2 MIC) in a total of 15 days, according to FIGURE 3.6 shown in *Section 3.2.2.2*. Reserpine and quinine were applied at 100 mg l^{-1}; pyrrolidine, morin and quercetin at 500 mg l^{-1}. Bars with (*) presented significantly higher inhibition zone diameter ($P < 0.05$) comparing to ciprofloxacin control population for each phase.

By growing this strain with sub-inhibitory concentrations of ciprofloxacin (control assay), it was possible to observe reduction in the IZDs until day 9 (phase 3), and then no growth inhibition was detected (phases 4 and 5), proposing that bacteria became more resistant to ciprofloxacin. In fact, biological responses induced in bacteria when antibiotics are applied at sub-inhibitory concentrations can affect various cellular responses or alter gene expression leading to different adaptive responses impacting antibiotic resistance/tolerance (Bernier and Surette, 2013; Kaplan et al., 2012). A potential increased NorA overexpression could explain the improved tolerance to ciprofloxacin observed by strain SA1199B.

In phases 2 and 3, it is possible to observe that the populations growing in the presence of all phytochemicals were more susceptible, since IZDs promoted by ciprofloxacin against these populations were significantly higher ($P < 0.05$) than IZDs obtained when bacteria were growing only in the presence of ciprofloxacin. In fact, IZDs obtained in phase 3 allowed to characterize bacterial cultures as susceptible to ciprofloxacin and not resistant anymore, according to CLSI (2015b) guidelines. This susceptibility was observed earlier for quercetin growing-population (in phase 1). This means that until day 9, all the phytochemicals were able to reverse the bacterial

resistance mechanisms. However, by increasing ciprofloxacin concentrations to ¼ and ½ MIC (phases 4 and 5, respectively), all populations were resistant to ciprofloxacin similarly to the control (no IZD detected), with the exception of bacteria growing in the presence of quinine (100 mg l^{-1}) and morin (500 mg l^{-1}). The results propose that both phytochemicals were successful in preventing higher tolerance of SA1199B to ciprofloxacin ($P < 0.05$).

4.2.5. Effect of selected phytochemicals on EtBr accumulation

A variety of methods have been used to identify active efflux systems in bacteria, such as the use of radiolabeled substrates, fluorometric assays or the determination of the MIC for different substrates in the presence of compounds known to modulate the activity of efflux pumps (EPIs) (Costa et al., 2011). The potential of the phytochemicals to inhibit NorA was tested using EtBr, a substrate widely applied for detecting efflux activity in *S. aureus* strains (Costa et al., 2013). EtBr form complexes with double stranded DNA and RNA by intercalating between base pairs (Walberg et al., 1999). The accumulation of EtBr has been observed to increase in bacterial cells in the presence of an EPI, such as reserpine that inhibits EtBr efflux activity of Gram-positive bacteria (Holler et al., 2012; Mullin et al., 2004). This accumulation can be measured fluorometrically in strain SA1199B cells due to the retention of fluorescence over time. Also, in order to understand if overexpression of NorA is the reason for the increased tolerance of bacterial cells growing with ciprofloxacin for 15 days, this population, named SA1199B(r) (recovered as described in *Section 3.2.2.2*), was included in the experiments. The susceptible strain CECT 976 was also tested as negative control.

The MIC of EtBr was first determined for each strain according to CLSI (2015a) guidelines as described in *Section 3.2.1.2*: 5 mg l^{-1} for CECT 976 and 40 mg l^{-1} for SA1199B and SA1199B(r). Due to the good sensitivity of the fluorometric method, the demonstration of the effects of reserpine on the accumulation of EtBr in strain SA1199B over time was readily made. FIGURE 4.7 shows the EtBr accumulation results in SA1199B. FIGURE 4.7-A shows the comparison between EtBr accumulation in strains CECT 976, SA1199B and SA1199B(r). CECT 976 can accumulate more EtBr ($P < 0.05$) contrarily to SA1199B and SA1199B(r), both overexpressing NorA. There were no significant differences between EtBr accumulation in SA1199B and SA1199B(r) ($P > 0.05$), proposing that the higher resistance of this strain to ciprofloxacin may be due to the expression of resistance mechanisms other than overexpression NorA efflux pump. FIGURE 4.7-B shows the effect of several concentrations of reserpine in EtBr accumulation. Reserpine at 20 mg l^{-1} is usually used as reference by diverse authors (Couto et al., 2008; Schmitz et al., 1998).

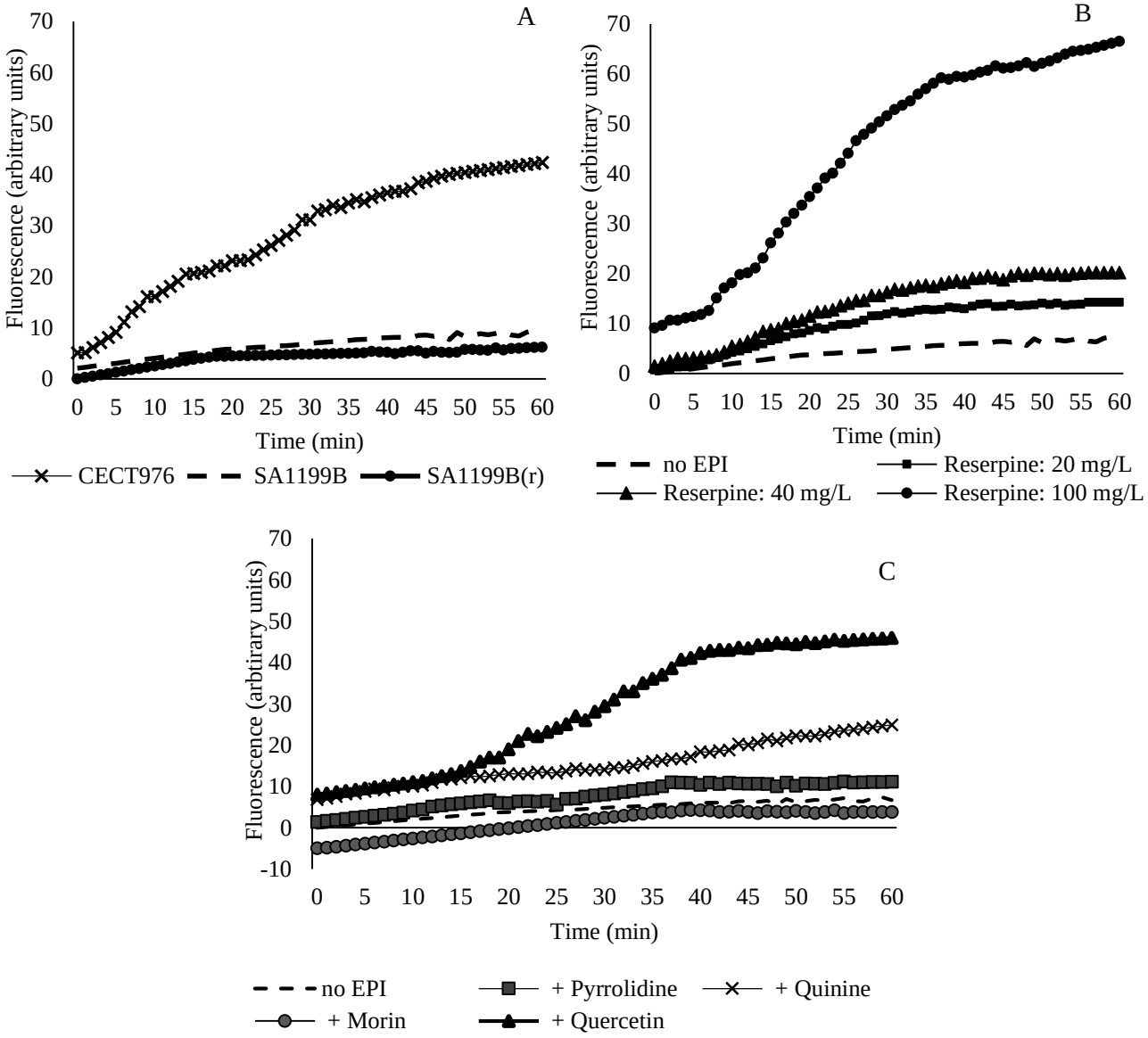


FIGURE 4.7 | Detection of the effect of the selected phytochemicals on ethidium bromide accumulation for 60 min in *Staphylococcus aureus* strains by fluorometry. (A) Fluorescence as a measure of EtBr accumulation in *Staphylococcus aureus* CECT 976, SA1199B and SA1199B(r) for 60 min; (B) effects of reserpine at 20, 40 and 100 mg l^{-1} and (C) of the other phytochemicals on EtBr accumulation in *S. aureus* SA1199B. The bacteria were loaded with EtBr at ½ MIC in the presence of different phytochemicals for a period of 60 minutes at 37°C. Mean values of three replicates are shown.

FIGURE 4.7-C shows the accumulation of EtBr in SA1199B in the presence of the phytochemicals. Quercetin (500 mg l^{-1}) showed the highest EtBr accumulation in SA1199B ($P <$

0.05). Quinine (100 mg l^{-1}) also improved EtBr accumulation ($P < 0.05$) proposing that this compound is inhibiting NorA efflux pump, which could have helped preventing SA1199B to become more tolerant to ciprofloxacin. Contrarily, morin (500 mg l^{-1}) had no effect on EtBr accumulation, but it was also able to prevent bacterial adaptation to ciprofloxacin, suggesting that this product is apparently involved in the inhibition of other mechanism, excluding NorA efflux pump, or that this effect may be related to its ability to control biofilms (within 1 h) and to prevent their formation. Pyrrolidine did not improve EtBr accumulation neither was able to prevent higher tolerance of SA1199B to ciprofloxacin ($P > 0.05$). However, it caused biofilm removal when applied for 1 h.

4.2.6. Cytotoxicity results of the selected phytochemicals

To assess the suitability of the tested phytochemicals for antimicrobial therapy, cytotoxicity tests were carried out by MTS tests with L929 cells. The positive growth control produced large amounts of a brown formazan product after incubation, which is an indicator of normal metabolism and that cells were able to metabolize MTS. The DMSO control presented a similar viability to the growth control ($P < 0.05$) indicating that this compound (at 2% v v^{-1}) was not toxic for the cells. From the dose-response curves obtained, IC_{50} values were calculated by probit analysis. Quinine demonstrates a high toxicity to mammalian cells, being the most toxic alkaloid ($IC_{50} = 25 \pm 2.2$ mg l^{-1}). The concentration of reserpine used to potentiate antibiotics, 100 mg l^{-1}, was inferior to its IC_{50} (627 ± 57 mg l^{-1}). The other alkaloids were used at concentrations above their IC_{50}, which was 352 ± 28, 274 ± 18, and 100 ± 4.7 mg l^{-1} for caffeine, pyrrolidine and theophylline, respectively. The flavonoids quercetin, morin and hesperidin presented low values of IC_{50} (41.8, 67.5, and 50.4 mg l^{-1}, respectively) after 72 h incubation with L929 cells. Catechin had a IC_{50} of 302.5 mg l^{-1} and rutin showed the highest value (1267.5 mg l^{-1}).

4.2.7. Relevant remarks

With this study, known phytochemicals are highlighted for their important activities in cotherapies with known antibiotics against *S. aureus* biofilms. Despite being structurally different and presenting diverse activities, all phytochemicals showed interesting results, highlighting the use of phytochemicals as adjuvants of antimicrobial therapy against planktonic and sessile *S. aureus*.

Several significant synergic combinations of antibiotics with the phytochemicals were obtained being highlighted the combinations between ciprofloxacin and quinine to control SA1199B biofilms (24 h exposure with $\log_{10}$ CFU cm^{-2} reduction of 2.1) and between

ciprofloxacin with reserpine to prevent SA1199B biofilm formation (log_{10} CFU cm^{-2} reduction of 3.5). These effects depend on the incubation time as with longer incubation and at non-lethal antibiotic doses, bacteria showed increased antibiotic tolerance. Unfortunately, reserpine cannot be used to potentiate the activities of fluoroquinolones because of its neurotoxicity at the concentrations required for NorA inhibition (Markham et al., 1999).

Morin applied for 1 h showed the best biofilm reduction and significantly inhibited biofilm formation for all strains. Interestingly, morin has been studied in other fields as an inhibitor of amyloid β-peptide aggregation, whereas other hydroxyflavones such as quercetin failed to be effective (Lemkul and Bevan, 2012; Noor et al., 2012). Pyrrolidine had a significant effect when exposed for 1 h against biofilms, while quercetin was highlighted for preventing biofilm formation. Quercetin promoted the highest EPI activity in strain SA1199B.

Interestingly, the difference in the structures between morin and quercetin is that the first is 2',4'-dihydroxylated in B ring while the second is 3',4'-dyhydroxylated (as can be visualised in TABLE 3.3 in *Section 3.1.6*). However, it is already known that different substitutions on phenolic rings can promote significantly different activities (Cushnie and Lamb, 2005; Kumar and Pandey, 2013). Indeed, Tsuchiya et al. (1996) indicated that 2′,4′- or 2′,6′-dihydroxylation of the B ring and 5,7-dihydroxylation of the A ring in the flavanone structure was important for anti-MRSA activity. Previous findings of flavonols, such as fisetin, quercetin, and kaempferol state an anti-biofilm activity against *S. aureus* (Lee et al., 2013). In addition, it was found previously that several flavonoids, such as quercetin, reduce hemolysis by *S. aureus*, which *w*ould reduce the virulence of *S. aureus* and the ability of the bacterium to form biofilms (Caiazza and O'Toole, 2003). Moreover, due to their extensive functional group chemistry and chirality those products are potentially interesting scaffolds for the discovery and development of antibacterial therapeutic approaches.

It has been suggested that flavonoids are likely to have limited toxicity because they are widely distributed in edible plants and beverages and have previously been used in traditional medicine (Bylka et al., 2004). Indeed, in the U.S., the daily dietary intake of mixed flavonoids is estimated to be in the range of 500-1000 mg (Skibola and Smith, 2000). Quercetin has also been used as a dietary supplement (250-500 mg three times per day) for therapeutic purposes. However, in this study, quercetin presented low values of IC_{50} after 72 h incubation with L929 cells (41.8 mg l^{-1}). This value is in accordance with some other reports. Ngomuo and Jones (1996) carried out the cytotoxic effects of quercetin and other compounds and they reported inhibition of cell growth by 50% after 48 h of incubation for quercetin at 24 mg l^{-1} for Chinese hamster ovary cells, 36 mg l^{-1} for mouse fibroblast cells (3T3) and 21 mg l^{-1} for normal rat kidney cells. Pawlikowska-Pawlega and Gawron (1995) reported that quercetin caused partial growth inhibition of mouse fibroblast

cells when used at 10 mg l^{-1}, and almost complete growth inhibition when applied at 50 mg l^{-1}. However, based on the information provided by Quercegen Pharmaceutics concerning toxicological safety studies (personal communication), human clinical studies corroborating epidemiological studies, human pharmacokinetic studies, as well as other information available to FDA, quercetin received GRAS status for its intended use as ingredient in beverages, grain products and pastas, processed fruits and fruit juices, and soft candies at levels up to 500 milligrams per serving. FDA has not, however, made its own determination regarding the GRAS status of the subject use of quercetin.

There are tens of reports in the literature concerning the potential benefits of quercetin for various applications, with various purported modes of action, while others refer to toxicity issues (Ferry et al., 1996; Guardia et al., 2001; Scambia et al., 1994). Although quercetin has tested positive for mutagenicity and genotoxicity *in vitro* in some reports, other *in vitro* studies suggest that quercetin is protective against genotoxicants, and regarded as antimutagenic (Okamoto, 2005). The implications of these conflicting findings in an assessment of human safety have not been established (Harwood et al., 2007). Therefore, the mechanisms underlying its biological effects remain obscure and understanding how quercetin causes either protection or cell death in the same model is a research priority (Ossola et al., 2008). Considering the considerable number of studies reporting the potentially beneficial effects of quercetin on health, little is known about its bioavailability (Erlund et al., 2000). At present, a number of studies have been carried out both in animals and humans. However, *in vivo* data on the disposition, absorption, bioavailability, and metabolism of quercetin are scarce and contradictory. The low bioavailability of flavonoids has been a concern. However, it can be improved by using food matrix components or particular delivery forms (Bischoff, 2008).

4.3. TASK 3. REEXAMINING PLANTS WITH A NEW LOOK INTO THEIR DEFENSE SYSTEMS - THE SEARCH FOR ANTIBIOTIC ADJUVANTS TO PROMOTE EFFECTIVE TREATMENT OF DRUG-RESISTANT *STAPHYLOCOCCUS AUREUS*

Abstract

It becomes apparent that plants adopt a different paradigm – synergy – to combat infections. The current study assessed 29 plants from different families for their antibacterial activity, and also as adjuvants in antibiotic therapy, against *S. aureus*, including one MRSA strain. *Eucalyptus globulus*, *Castanea sativa*, *Agrimonia eupatoria* and *Fraxinus excelsior* methanolic extracts showed antibacterial activity with MICs of 0.125-0.5, 0.5-1.0, 1.0-2.0, and 2.0-4.0 g l^{-1}, respectively. Non-antibacterial plants were assessed in combination with ampicillin, oxacillin, ciprofloxacin, erythromycin and tetracycline by disk diffusion test. Methanolic extracts of *Acacia dealbata*, *Prunus* spp. plants, *Centaurea nigra*, *Eupatorium cannabium* and *Buxus sempervirens* showed a potentiating effect mostly of ciprofloxacin, erythromycin and tetracycline.

4.3.1. Determination of antibacterial properties of selected plants

During the last few years, the increasing incidence of drug-resistant pathogens has drawn the attention of the pharmaceutical and scientific communities towards studies on the potential antimicrobial activity of plant-derived substances. This allowed a resurgence in the use of herbal medicines worldwide (Yap et al., 2014). Plant-based systems continue to play an essential role in healthcare, and their use by different cultures has been extensively documented (Cragg and Newman, 2013).

Regardless of their medicinal uses, all plants have their own defense mechanisms from pathogens, producing a wide range of different chemicals for that purpose. In this study, 29 plants were selected, among different families, mainly from Rosaceae (9 plants), Fabaceae (3 plants), Asteraceae, Solanaceae and Apocynacea (with 2 plants of each family), in order to be able to test a large variety of extracts and metabolites. Even common and well-studied plants need to be continuously investigated from different perspectives to achieve a deeper understanding of their defense mechanisms and to ascertain their potential biotechnological applications. The plants

selected include some typical invasive species existent in Portugal, common fruit plants and others considered medicinal for their wide range of therapeutic applications. TABLE 3.5 in the *Section 3.1.7* described the plants tested in this study and TABLE A.2 in Appendix A2 described their ethnopharmacological relevance according to the Plants For A Future (PFAF, 2016) online database. The selected plants were assessed for their antibacterial activities against diverse *S. aureus* strains. The MIC for all methanolic extracts was determined for concentrations below 4 g l^{-1}. Only four plant extracts showed a detectable MIC, as shown in TABLE 4.5.

TABLE 4.5 | Ranges of minimal inhibitory concentrations (MIC, g l^{-1}) for the plant methanolic extracts that exhibited antibacterial activity for concentrations lower than 4 g l^{-1} against four *Staphylococcus aureus* strains (CECT 976, SA1199B, XU212, RN4220 and one MRSA strain MJMC001)

Plant	**MIC (g l^{-1})**
E. globulus	0.125 - 0.5
C. sativa	0.5 - 1.0
A. eupatoria	1.0 - 2.0
F. excelsior	2.0 - 4.0

Eucalyptus globulus presented the highest antibacterial activity with MIC between 0.125 and 0.25 g l^{-1} against the diverse *S. aureus* strains (CECT 976, SA1199B, XU212, RN4220 and one MRSA strain MJMC001). This activity is in accordance with its therapeutic use (PFAF, 2016). Other studies reported that the essential oils from the leaves and the flowers of *E. globulus* inhibited the growth of *E. coli*, *S. aureus* (Bachir and Benali, 2012), MRSA and the VRE *Enterococcus faecalis* (Srinivasan et al., 2001). Aromadendrene was described as the major constituent causing antimicrobial activity (Srinivasan et al., 2001). The aqueous extract of *E. globulus* leaves was also found to inhibit a wide range of bacteria and fungi (Mulyaningsih et al., 2010).

Concerning the other antibacterial plants, other studies were in agreement with the results obtained. Basile et al. (2000) found a MIC in the range of 64–256 mg l^{-1} of the aqueous extract of *Castanea sativa* (pH 3.0) against several bacteria including *S. aureus.* Rutin, hesperidin, quercetin, apigenin, morin, naringin, galangin and kaempferol were already identified in this plant extract (Basile et al., 2000). *Agrimonia eupatoria* was reported for its inhibitory effects against *S. aureus* (Copland et al., 2003; Dulgerm and Gonuz, 2004; Watkins et al., 2012). Several constituents were described for *A. eupatorium*: apigenin, luteolin, quercetin, kaempferol, tiliroside, triterpene glycosides, such as euscapic acid and tormentic acid, phenolic acids and

tannins (Watkins et al., 2012). Middleton et al. (2005) reported antibacterial activity of *n*-hexane and $CHCl_2$ extracts of *Fraxinus excelsior* against *S. aureus* (MIC of 0.125 and 0.25 g l^{-1}, respectively) and MRSA strains (MIC of 0.5 and 1.0 g l^{-1}, respectively). To date, various classes of compounds, including coumarins, benzoquinones, flavonoids, phenylethanoids, secoiridoid glucosides, indole derivatives and simple phenolics have been reported from *F. excelsior* (Middleton et al., 2005), and isolated compounds included 7-β-1-D-glucopyranosyl 11-methyloleoside, oleoside dimethyl ester, excelsioside and ligustroside (Soler-Rivas et al., 2000).

4.3.2. Evaluation of antibiotic-potentiating effect by plant extracts

The plant extracts that did not show any detectable antibacterial activity were further evaluated for antibiotic-potentiating activity with five antibiotics by disk diffusion method. First, the classification of *S. aureus* strains according to their resistance profile was performed based on the comparison of the MICs/IZDs results and the susceptibility breakpoints of CLSI (2015b) guidelines, as shown previously in TABLE 4.1 (*Section 4.1.1*): CECT 976 is susceptible to all antibiotics, while *S. aureus* SA1199B, RN4220 and XU212 are resistant to ciprofloxacin, erythromycin and tetracycline, respectively, and the MRSA MJMC001 is resistant to all antibiotics, excepting tetracycline. TABLE 4.6 shows the antibiotic-potentiation results obtained for the plant extracts. Only extracts showing potentiation of at least one antibiotic were included.

TABLE 4.6 | Classification of the combination between the antibiotics and the plant methanolic extracts against *Staphylococcus aureus* strains by disk diffusion method as indifferent (+), additive (++) and potentiation (+++)

		CECT 976					**SA1199B**	**XU212**	**RN4220**	**MJMC001**				
Plant	**g l^{-1}**	**AMP**	**OXA**	**CIP**	**TET**	**ERY**	**CIP**	**TET**	**ERY**	**AMP**	**OXA**	**CIP**	**TET**	**ERY**
C. striatus	0.5	+	+	+++	++	+++	+++	+	+++	+	+	+++	+	+++
A. dealbata	2.0	+	+	+++	+	+++	+++	+	++	+	+	+	+	++
P. communis	4.0	+	+	+	+++	+++	+	++	+	+	+	+	+	+
P. avium	4.0	+	+	+	+++	+++	+++	++	+++	+	+	+	+	++
P. domestica	4.0	+	+	+	+++	+++	+++	++	+++	+	+	+	+	++
P. persica	4.0	+	+	++	+++	++	+++	++	+++	+	+	++	+	++
C. nigra	1.0	+	+	+++	+	+++	+++	+	+	+	+	+	+	+
E. cannabinum	1.0	+	+	+	+++	+++	+	+	+++	+	+	+	+	+++
F. carica	2.0	+	+	+	++	+	+++	+	+	+	+	+	+	+
B. sempervirens	1.0	++	++	+++	+++	+++	+++	+	+++	+	+	+	+	+++

The concentrations described for each plant extract are the minimal/optimal concentrations causing potentiation of the antibiotics. Only the plant extracts that potentiate at least one antibiotic are presented. No antagonistic interactions between antibiotics and plant extracts were obtained.

No IZD originated by the combinations between plant extracts and antibiotics was ever inferior to that promoted by the antibiotic alone ($P > 0.05$). Potentiating results were obtained with plants from Fabaceae family, especially *Cytisus striatus* and *Acacia dealbata*, and Rosaceae family, including all *Prunus* spp. and *Pyrus communis*. Also, both plants from Asteraceae family, *Centaurea nigra* and *Eupatorium cannabium*, showed good potentiating results. *Buxus sempervirens* was also highlighted for its high antibiotic-potentiating activity. No plant extract significantly potentiated β-lactam antibiotics (ampicillin and oxacillin), but additive effects were found with *B. sempervirens*.

The MeOH leaf extracts from both invasive Fabaceae plants, *C. striatus* (0.5 g l^{-1}) and *A. dealbata* (2 g l^{-1}), showed similar potentiating effects. Both extracts potentiated ciprofloxacin against *S. aureus* CECT 976 and SA1199B and erythromycin against CECT 976, RN4220 and MJMC001 (*A. dealbata* only showed additive interactions against these last two strains). *C. striatus* leaves extract also potentiated ciprofloxacin against MJMC001, being thus highlighted from the other extracts. More studies should be performed in order to understand what metabolites exist in common between these two plants that could cause similar antibiotic-potentiating activities. The presence of isoflavonoids is common among Fabaceae family. Taguri et al. (2006) found generally weak activity of *A. dealbata* extract against many different bacteria. Olajuyigbe and Afolayan (2012) found synergistic interactions between one *Acacia* sp. plant, *Acacia mearnsii*, and erythromycin, metronidazole, amoxicillin, chloramphenicol and kanamycin against *S. aureus*. No reports of antibiotic potentiation were found for *C. striatus*.

Prunus spp. MeOH extracts showed interesting potentiating results though only at high concentrations (4 g l^{-1}). Results were very similar between the three plant extracts. Potentiation/additive effects were mainly found with ciprofloxacin against SA1199B, tetracycline against CECT 976 and erythromycin against CECT 976, RN4220 and MJMC001. No study about antibiotic-potentiating activity of these *Prunus* spp. was previously reported. Yet, some main compounds from these plants were reported: hentricontane, ethyl hexadecanoate and linoleic acid were found from *P. domestica* oil (Mahmood et al., 2009); tectochrysin and (-)-sakuranetin were isolated from $CHCl_3$ extract of *P. avium*, while from the MeOH extract (+)-catechin, (+)-naringenin [which has antibacterial activity according to Alzoreky and Nakahara (2003)] (−)-dihydrowogonin and (+)-dihydrokaempferol were identified (McNulty et al., 2009).

B. sempervirens (1 g l^{-1}) is also highlighted since its methanolic extract promoted several additive and potentiating effects when combined with all antibiotics against CECT 976, with ciprofloxacin against SA1199B and erythromycin against RN4220 and MJMC001. So, these extracts seem to act in a more "general" way to potentiate the antibiotics tested. No other similar reports about antibiotic-potentiating activity promoted by this plant were found in bibliography.

Other MeOH extracts showed in TABLE 4.6 presented promising results, being highlighted the following combinations: *C. nigra* (1 g l^{-1}) potentiated ciprofloxacin against CECT 976 and SA1199B and erythromycin against CECT 976; *E. cannabium* (1 g l^{-1}) had a potentiating effect on tetracycline against CECT 976, and on erythromycin against CECT 976, RN4220 and MJMC001; *P. communis* (4 g l^{-1}) potentiated tetracycline and erythromycin against CECT 976; and *F. carica* (2 g l^{-1}) potentiated ciprofloxacin against SA1199B.

4.3.3. Relevant remarks

This study allowed to assess the potential of 29 different plant species to be used in co-therapies against *S. aureus*, a major cause of HAIs. From the 29 tested plants, four (*E. globulus*, *C. sativa*, *A. eupatoria* and *F. excelsior*) were found to have antibacterial activity, being in agreement with their traditional uses (PFAF, 2016), and ten (*A. dealbata*, *C. striatus*, *P. communis*, *P. avium*, *P. domestica*, *P. persica*, *C. nigra*, *E. cannabinum*, *F. carica*, *B. sempervirens*) were able to potentiate antibiotic activity, especially ciprofloxacin, tetracycline and erythromycin. Further studies with these plants would be necessary in order to conclude about the responsible metabolites promoting these antibiotic-potentiating activities.

Resistance to the antibiotics tested can be easily achieved with the expression of efflux pumps from pathogens (Fernández and Hancock, 2012). So, most of these plant extracts may be causing efflux pump inhibition on *S. aureus*, thus explaining the potentiation of ciprofloxacin, tetracycline and erythromycin. Indeed, the number of plant extracts producing efflux pump inhibitors seems to be considerable, as it is being extensively reported (Gibbons et al., 2003a; Smith et al., 2007b; Stavri et al., 2007). *C. striatus* was highlighted in this initial screening and further studies focusing on the isolation and characterization of the main metabolites responsible for this antibiotic-potentiating activity were performed and will be further described in *Sections 4.4* and *4.5*.

No plant extract significantly potentiated β-lactam antibiotics, but additive effects were found with *B. sempervirens*. Thus, *B. sempervirens* seems to act as a general potentiator for the several antibiotics, not being dependent of the antibiotic-class. Thus, other interesting possibilities to explain this potentiation that not an efflux pump activity emerge. Antibiotic potentiation can be reached by compounds that are interfering with other mechanisms of the bacterial cell that not involve drug resistance mechanisms, such as quorum-sensing, virulence activation, biofilm formation, adherence to the host tissues (Chatterjee et al., 2016), etc. For this reason, *B. sempervirens* was also selected for further investigation and phytochemical elucidation, which results will be further presented and discussed in *Section 4.6*.

4.4. TASK 4. THE POTENTIAL OF *CYTISUS STRIATUS* FOR ANTIBIOTIC-POTENTIATING ACTIVITY: ISOLATION AND IDENTIFICATION OF ANTIBIOTIC ADJUVANTS

Abstract

Antibacterial therapy is perhaps the only field of medicine in which therapeutic options have declined over time. The spread of multidrug resistant *S. aureus* strains, including MRSA, has shortened the useful life of anti-staphylococcal drugs extremely. Disabling resistance mechanisms may restore the activity of old-generation antibiotics. Herein, the use of ^{1}H NMR-based metabolomics to identify the antimicrobial and antibiotic potentiating compounds of *C. striatus* is described. Luteolin was isolated from the plant and showed antibacterial activity (MIC = 30-120 mg l^{-1}). Genistein, 2'-hydroxygenistein and apigenin are potentially implicated in the potentiation of ciprofloxacin and erythromycin against MRSA strains belonging to CC8 (ST239 and USA300). Interesting activities were also shown by the isolated compounds from *C. striatus*, namely EPI activity and *S. aureus* biofilm control.

4.4.1. Antibacterial and antibiotic-synergistic activities of *Cytisus striatus* leaf, flower and twig methanolic extracts

Previously, the methanolic extract of *C. striatus* leaves were assessed for antibacterial and antibiotic-potentiating activities. To determine if other parts of the plant have the same activity, methanolic extracts of flowers and twigs of this plant were assessed as well. The MICs of the EtOAc fractions of the methanolic extracts of *C. striatus* leaves, flowers and twigs were determined by microdilution techniques according to CLSI (2015a) guidelines against seven *S. aureus* strains including USA300 and ST239 MRSA strains. Characterization of these strains concerning the origin was detailed previously in TABLE 3.1 in *Section 3.1.4*. None of the extracts exhibited a MIC below 1 g l^{-1} against the *S. aureus* strains. In TABLE 4.7, MICs of ciprofloxacin and erythromycin are presented for each strain.

TABLE 4.7 | Minimal inhibitory concentrations (MIC, mg l^{-1}) were determined for each strain according to CLSI (2015a) guidelines and classified as resistant (R), intermediate (I) or susceptible (S) to ciprofloxacin (CIP) and erythromycin (ERY)

S. aureus	**CIP**	**ERY**
M116	32 (R)	32 (R)
RWW337	16 (R)	> 10 000 (R)
RWW50	2 (I)	64 (R)
M82	0.25 (S)	0.5 (S)
RN6390	0.25 (S)	0.5 (S)
CECT 976	1.0 (S)	0.25 (S)
SA1199B	4 (R)	n.p.

n.p. not performed

The EtOAc fractions of methanolic extracts of leaves, flowers and twigs of *C. striatus* (0.5 g l^{-1}) were assessed for their potentiating effect of ciprofloxacin and erythromycin activity against the seven *S. aureus* strains, using the disk-diffusion method (TABLE 4.8).

TABLE 4.8 | Antibiotic-potentiating activity of the methanolic extracts of *Cytisus striatus* leaf, flower and twig (0.5 g l^{-1}). The activity is expressed as the increase on inhibition zone diameters (IZDs, mm) promoted by ciprofloxacin (CIP) or erythromycin (ERY) in the presence of the plant extracts

	Increased IZD ($IZD_c - IZD_a$) (mm)					
	Leaf		**Flower**		**Twig**	
S. aureus	**CIP**	**ERY**	**CIP**	**ERY**	**CIP**	**ERY**
M116	**7.0 ± 1.0 (P)**	**10.0 ± 0.0 (P)**	-	-	-	-
RWW337	**8.3 ± 0.6 (P)**	-	-	-	-	-
RWW50	**7.0 ± 0.0 (P)**	5.0 ± 1.0 (A)	-	-	-	-
M82	4.0 ± 0.0 (A)	4.5 ± 0.0 (A)	-	-	-	-
RN6390	-	5.0 ± 0.3 (A)	-	-	-	-
CECT 976	**6.3 ± 1.0 (P)**	**9.1 ± 0.6 (P)**	5.4 ± 1.2 (A)	4.5 ± 1.0 (A)	-	-
SA1199B	**14.0 ± 2.5 (P)**	n.p.	**17.3 ± 0.3 (P)**	n.p.	-	n.p.

Inhibition zones obtained with the combinations (IZD_c) over antibiotic-single activity (IZD_a) are given and classified as: (-) indifferent ($IZD_c - IZD_a < 4$ mm); (A) additive ($4 \leq IZD_c - IZD_a < 6$ mm); and (P) potentiation ($IZD_c - IZD_a \geq 6$ mm, in bold). Data are means and SD from at least three independent experiments; n.p. not performed.

The different parts of the plant showed different antibiotic-potentiating activities. The leaf extract showed the best potentiating effect on both ciprofloxacin and erythromycin against several strains. The flower extract showed additive effects with the antibiotics only against *S. aureus* strains CECT 976 and SA1199B. The twig extract showed no antibiotic-potentiating activity at all against the seven *S. aureus* strains tested. The IZD of each combination was never lower than that produced by each antibiotic alone ($P > 0.05$), which proves there was no antagonism detected in the combinations.

Antibiotic potentiation caused by the methanolic extract of *C. striatus* leaf was also confirmed by checkerboard method (TABLE 4.9).

TABLE 4.9 | Minimal inhibitory concentrations (MIC, g l^{-1}) of *Cytisus striatus* leaf methanolic extract against *Staphylococcus aureus*, when applied alone (MIC_a) and in combination (MIC_b) with ciprofloxacin (CIP) or erythromycin (ERY). Fold-reductions of antibiotic MICs (R) and fractional inhibitory concentration index (FICI) values are shown

		CIP			ERY		
S. aureus	**MIC_a**	**MIC_b**	**R**	**FICI**	**MIC_b**	**R**	**FICI**
M116	> 1.0	0.25	4	**≤ 0.38 (P)**	0.5	4	**≤ 0.5 (P)**
RWW337	> 1.0	0.25	4	**≤ 0.38 (P)**	-	-	I
RWW50	> 1.0	0.125	4	**≤ 0.31 (P)**	0.5	2	> 0.5 (I)
M82	> 1.0	0.5	2	> 0.5 (I)	-	-	I
RN6390	> 1.0	-	-	I	-	-	I
CECT 976	> 1.0	0.25	4	**≤ 0.38 (P)**	0.25	4	**≤ 0.38 (P)**
SA1199B	> 1.0	0.125	4	**≤ 0.31 (P)**	n.p.		

- = no decrease or increase in the MIC was observed; n.p. not performed; plant extracts (EtOAc fractions) were tested in a range of concentrations from 0.06 to 1.0 g l^{-1}. When FICI ≤ 0.5 (in bold), the effect is considered potentiation (P); if FICI > 0.5, the interaction is considered indifferent (I). ERY was not tested against SA1199B. The experiments were repeated three times, and the values presented are the averages of three independent assays.

4.4.2. ^{1}H NMR measurement of the extracts of different parts of *Cytisus striatus*

^{1}H NMR was applied to obtain metabolic profiles of all samples (Ali et al., 2012). ^{1}H NMR is widely used in combination with different multivariate data analyses methods to obtain metabolic profiles of all types of samples (Ali et al., 2012). An ^{1}H NMR spectrum can be roughly divided into three distinct regions: δ 0.80–4.00 corresponds to signals from amino acids and organic acids,

δ 4.00-5.50 is known as the carbohydrates region and δ 5.50–8.60 is considered to be the phenolic region. Visual inspection of the ^{1}H NMR spectra of the EtOAc fractions of the methanolic extracts of leaf, flower and twig revealed some differences in their chemical profiles (FIGURE 4.8), especially in the phenolic region, which could be responsible for differences in antibiotic-potentiating activity: leaves (most active) > flower > twig (non-active).

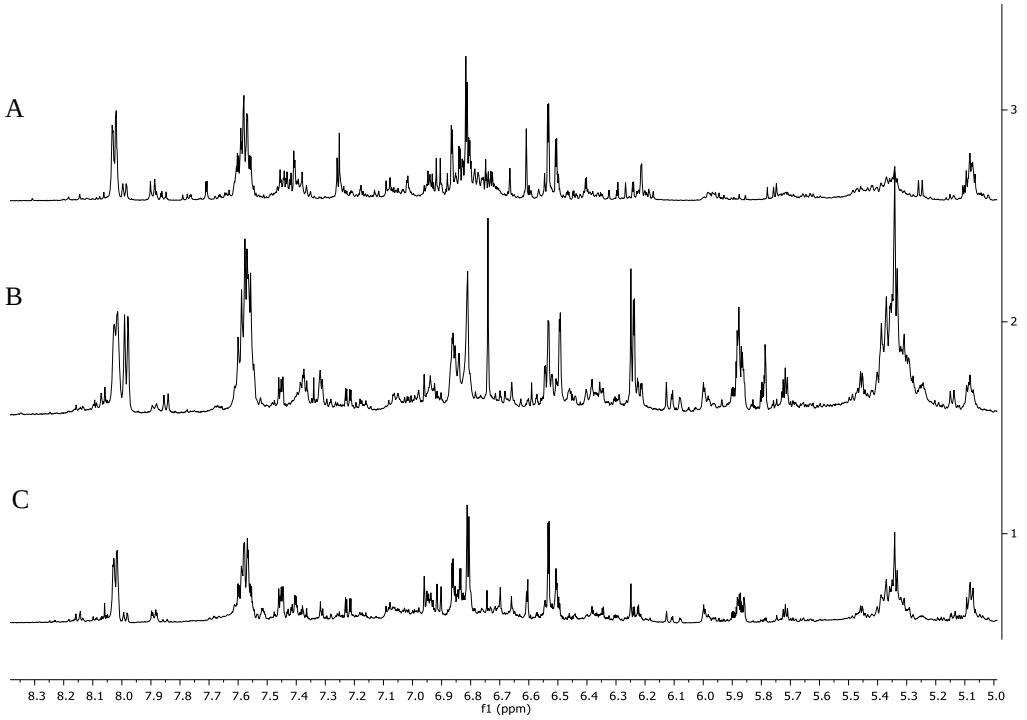


FIGURE 4.8 | Representative ^{1}H NMR spectra of *Cytisus striatus* extracts. ^{1}H NMR spectra (600 MHz, in CD_3OD, phenolic region) of the ethyl acetate fractions of the methanolic extracts of (A) leaf, (B) flower and (C) twig.

4.4.3. Multivariate data analysis for identification of the biomarkers

A number of different extracts was made to enable the use of multivariate data analysis to identify the markers of activity. The experimental diagram performed to find the biomarkers can be found in FIGURE 4.9. For the correlation between activity and chemical data, high chemical variation is essential. Therefore, leaves or flowers (active parts) were mixed with twig (non-active parts) by various % ratio in order to give more variation. Then, mixed parts of *C. striatus* plants were extracted using different solvents, temperature and pressure conditions. TABLE 3.7 in *Section 3.2.4.1* showed the rationale of the extractions performed.

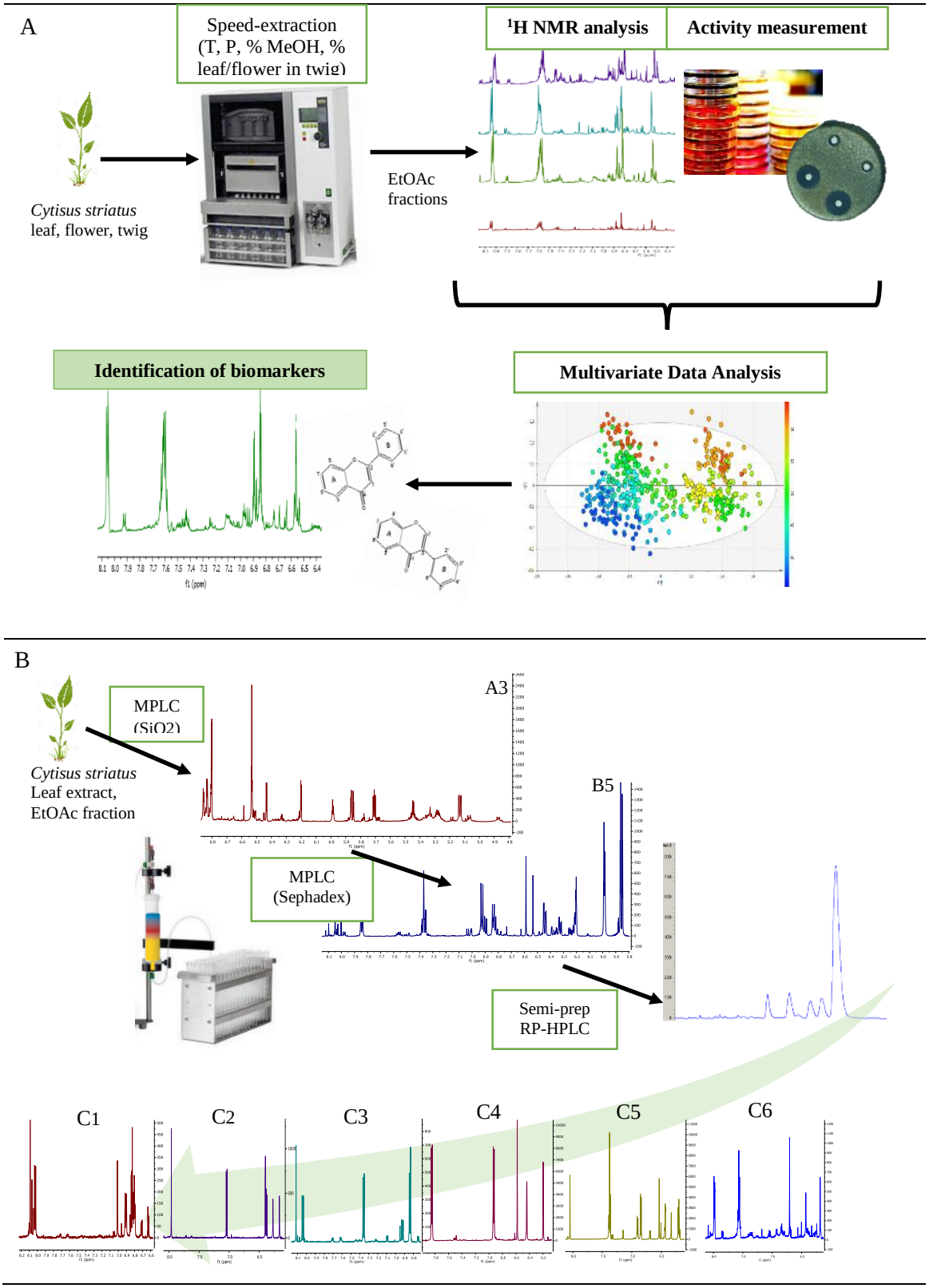


FIGURE 4.9 | Elucidating diagram describing the steps performed during this study. 1st step (A) - identification of the biomarkers correlated with antibiotic-potentiating activity through a multivariate data analysis applied to ^{1}H NMR data; 2nd step (B) - isolation and identification of active compounds.

The resulting 54 EtOAc fractions of the methanolic extracts were assayed for their potentiation of the activity of ciprofloxacin and erythromycin against *S. aureus* CECT 976 using the disk diffusion method (FIGURE 4.10). This strain was chosen for this screening since a clearly distinct response was obtained with the different parts of *C. striatus* (previsouly shown in TABLE 4.8). In general, extracts obtained with 100% methanol showed a higher potentiating activity than those prepared with 75- and 50% MeOH in water ($P < 0.05$); temperature and pressure did not significantly affect the activity ($P < 0.05$).

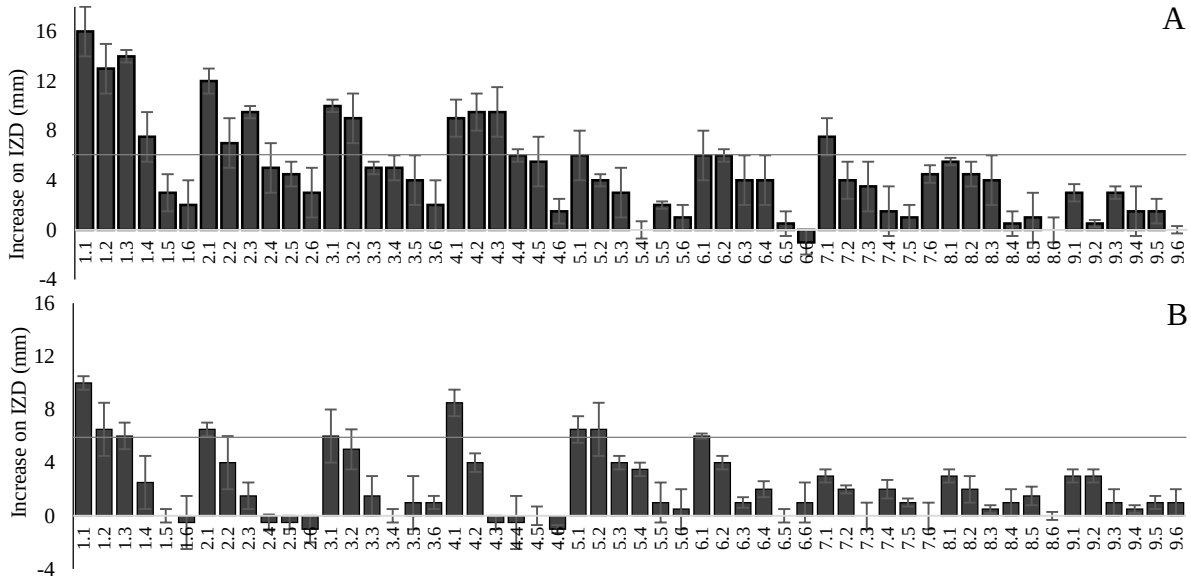


FIGURE 4.10 | Potentiating results obtained for the 54 methanolic extracts of *Cytisus striatus* when combined with antibiotics against *Staphylococcus aureus* CECT 976. The activity is expressed as the increase in the inhibition zone diameters (IZDs, mm) promoted by (A) ciprofloxacin (CIP) or (B) erythromycin (ERY) in the presence of the extracts dissolved in Mueller-Hinton agar medium, compared with reference conditions (without the extracts). Bars above the line y = 6 mm show potentiation of antibiotic. Bars represent means and SD from at least three independent experiments. Classification of the samples was performed as (*x*,*y*) and shown in TABLE 3.7.

The ^{1}H NMR data obtained for the 54 samples were reduced by OPLS modeling to investigate the grouping of the samples according to the potentiating activity of ciprofloxacin (FIGURE 4.11-A) and erythromycin (FIGURE 4.11-B). The aromatic region (δ 6.0 - 8.6) was considered since phenolic compounds were expected to have better therapeutic potential.

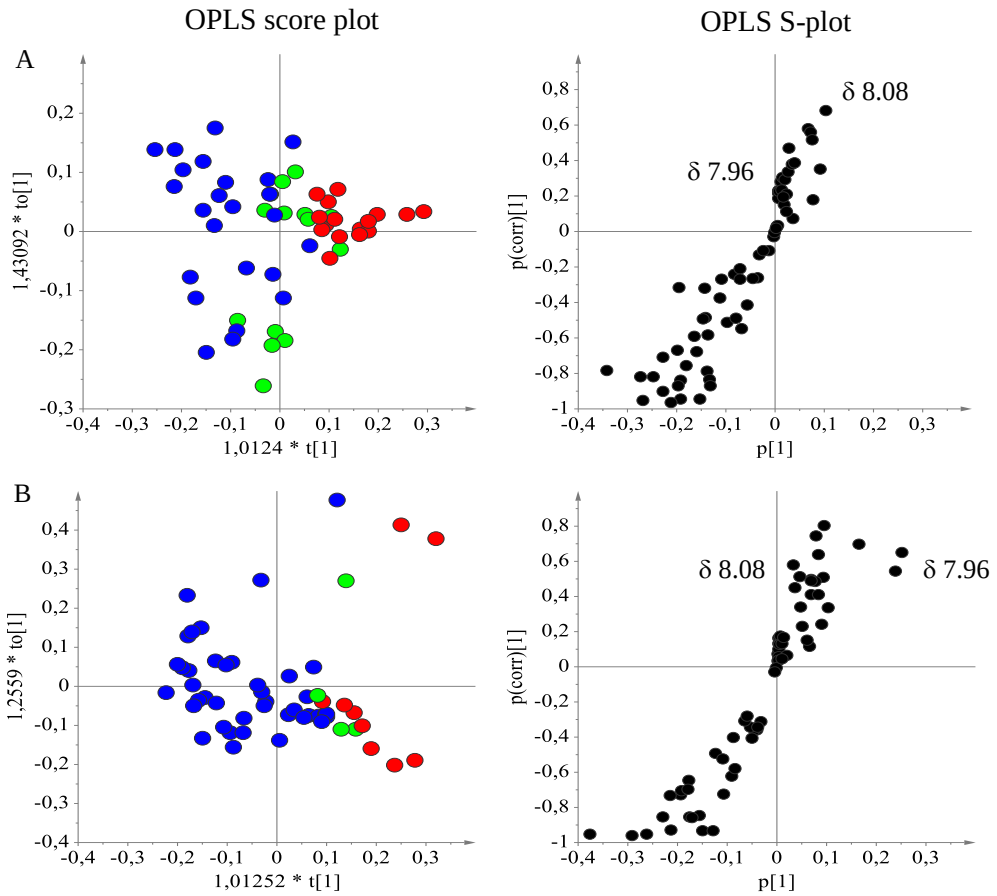


FIGURE 4.11 | Orthogonal partial least squaring model applied to *Cytisus striatus* ^{1}H NMR data. OPLS score- and S-plots obtained from the potentiating activity (1: no activity •, 2: additive activity •, 3: potentiating activity •) of ciprofloxacin (A) and erythromycin (B) and ^{1}H NMR data in the range of the region between δ 6.00 – 8.60 of the different extracts of *C. striatus*. The sample preparation and extraction conditions were performed as shown in TABLE 3.7.

Both OPLS score plots of ciprofloxacin and erythromycin showed that the phenolic ingredients correlated well with the potentiating activity, which was confirmed by permutation test. For identification of the contributing metabolites to the activity, S-plot was employed. The signals in the range of δ 7.9 - 8.2, e.g. H-2 of isoflavonoids or H-2' and H-6' of flavonoids having a 4'-monohydroxy group in B-ring were found to strongly correlate with the potentiating activity.

4.4.4. Identification of compounds correlated with the antibiotic-potentiation

To confirm the chemical structures associated with the activities, the *C. striatus* leaf methanolic extract was separated by column chromatography using silica gel and followed by a Sephadex LH-20 column yielding two fractions, B5 and B6 that exhibited distinct activities: B5

potentiated the activity of ciprofloxacin and erythromycin (similarly to *C. striatus* extract) and B6 showed antibacterial activity against *S. aureus* CECT 976. The B6 fraction was identified as luteolin. Semi-preparative RP-HPLC of fraction B5 allowed the separation of a number of compounds that were identified by ^{1}H NMR as apigenin, chrysin, daidzein, genistein, 2'-hydroxygenistein and 3'-hydroxydaidzein (TABLE 4.10). The identification was carried out using the in-house library of NMR data of common metabolites. When necessary, 2D-NMR techniques were used to confirm the identity (*J*-resolved, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMBC, ^{1}H-^{13}C HSQC).

TABLE 4.10 | Main metabolites detected in active fraction of *Cytisus striatus* leaves

	Chemical formula	**^{1}H NMR [CD_3OD-d4; δ (ppm)]**
Apigenin	$C_{15}H_9O_5$	6.19 (H-6, d, *J* = 1.9), 6.43 (H-8, d, *J* = 1.9), 6.57 (H-3, s), 6.92 (H-3'/H-5', d, *J* = 8.8), 7.84 (H-2'/H-6', d, *J* = 8.8)
Chrysin	$C_{15}H_{10}O_4$	6.18 (H-6, d, *J* = 1.9), 6.42 (H-8, d, *J* = 1.9), 6.70 (H-3, s), 7.56 (H-3'/H-4'/H-5', m); 7.97 (H-2'/H-6', d, *J* = 7.8)
Luteolin	$C_{15}H_9O_6$	6.19 (H-6, d, *J* = 1.9), 6.42 (H-8, d, *J* = 1.9), 6.53 (H-3, s), 6.89 (H-5', d, *J* = 8.4), 7.37 (H-6', dd, *J* = 8.4, 1.9), 7.37 (H-2', d, *J* = 1.9)
Daidzein	$C_{15}H_{10}O_4$	6.83 (H-3'/H-5', d, *J* = 8.4), 6.84 (H-8, d, *J* = 1.8), 6.92 (H-6, dd, *J* = 9.0, 2.4), 7.36 (H-2'/H-6', d, *J* = 8.4), 8.04 (H-5, d, *J* = 8.4), 8.12 (H-2, s)
3'-Hydroxydaidzein	$C_{15}H_9O_5$	6.79 (H-8, d, *J* = 1.8), 6.82 (H-5'/H-6', m), 6.90 (H-6, dd, *J* = 9.0, 2.4),7.00 (H-2', d, *J* = 1.8), 8.02 (H-5, d, *J* = 8.4), 8.08 (H-2, s),
Genistein	$C_{15}H_9O_5$	6.20 (H-6, d, *J* = 2.1), 6.32 (H-8, d, *J* = 2.1), 6.84 (H-3'/H-5', d, *J* = 8.4), 7.36 (H-2'/H-6', d, *J* = 8.4), 8.04 (H-2, s)
2'-Hydroxygenistein	$C_{15}H_{10}O_6$	6.15 (H-6, d, *J* = 1.8), 6.26 (H-8, d, *J* = 1.8), 6.37 (H-5', dd, *J* = 7.8, 2.4), 6.39 (H-3', d, *J* = 1.8), 7.03 (H-6', d, *J* = 8.4), 7.95 (H-2, s)

^{1}H NMR data are measured in ppm and coupling constants (*J*) in Hertz.

4.4.5. Antibacterial evaluation of the compounds isolated from *Cytisus striatus*

The MICs of luteolin, apigenin, chrysin, genistein and daidzein against *S. aureus* strains were determined (TABLE 4.11). Unfortunately, it was not possible to isolate enough 2'-hydroxygenistein and 3'-hydroxydaidzein for biological assays. Luteolin was the only compound that showed antibacterial activity against *S. aureus* with mild MIC between 30 and 120 mg l^{-1}. The potentiating effect of the five isolated compounds on the activity of ciprofloxacin and erythromycin was tested by checkerboard (TABLE 4.11) and the FICIs were calculated. The minimal concentration of compounds that exhibited antibiotic potentiation is shown for each

strain. Synergy (for luteolin) or potentiation (for the non-antibacterial phytochemicals) was considered for FICI ≤ 0.5.

TABLE 4.11 | Minimal inhibitory concentrations (MIC, mg l^{-1}) of the isolated compounds from *Cytisus striatus* against *Staphylococcus aureus* strains applied alone (MIC_a) and in combination (MIC_b) with ciprofloxacin (CIP) or erythromycin (ERY). Antibiotic MICs fold-reductions (R) and fractional inhibitory concentration index (FICI) values are shown

		CIP					ERY				
		Lut	**Apig**	**Chry**	**Gen**	**Daid**	**Lut**	**Apig**	**Chry**	**Gen**	**Daid**
M116	MIC_a	30	>120	>120	>120	>120	30	>120	>120	>120	>120
	MIC_b	-	30	-	60	15	3.5	30	-	30	-
	R	-	4	-	4	2	2	4	-	4	-
	FICI	I	**≤0.38 (P)**	I	**≤0.50 (P)**	>0.5 (I)	0.63 (I)	**≤0.38 (P)**	I	**≤0.38 (P)**	I
RWW337	MIC_a	30	>120	>120	>120	>120	30	>120	>120	>120	>120
	MIC_b	-	60	-	60	-	-	30	-	60	-
	R	-	8	-	8	-	-	≥2	-	≥4	-
	FICI	I	**≤0.38 (P)**	I	**≤0.38 (P)**	I	I*	(*)	I*	**≤0.50 (P)***	I*
RWW50	MIC_a	60	>120	>120	>120	>120	30	>120	>120	>120	>120
	MIC_b	-	15	-	30	-	-	-	-	60	-
	R	-	2	-	2	-	-	-	-	4	-
	FICI	I	>0.5 (I)	I	>0.5 (I)	I	I	I	I	**≤0.50 (P)**	I
M82	MIC_a	120	>120	>120	>120	>120	60	>120	>120	>120	>120
	MIC_b	60	30	-	30	-	60	-	-	30	-
	R	4	2	-	2	-	4	-	-	2	-
	FICI	0.75 (I)	>0.5 (I)	I	>0.5 (I)	I	0.75 (I)	I	I	>0.5 (I)	I
RN6390	MIC_a	120	>120	>120	>120	>120	120	>120	>120	>120	>120
	MIC_b	-	-	-	-	-	-	-	-	-	-
	R	-	-	-	-	-	-	-	-	-	-
	FICI	I	I	I	I	I	I	I	I	I	I
CECT 976	MIC_a	120	>120	>120	>120	>120	120	>120	>120	>120	>120
	MIC_b	-	-	-	30	30	-	-	-	30	-
	R	-	-	-	4	2	-	-	-	4	-
	FICI	I	I	I	**≤0.38 (P)**	>0.5 (I)	I	I	I	**≤0.38 (P)**	I
SA1199B	MIC_a	120	>120	>120	>120	>120					
	MIC_b	-	10	-	60	60			n.p.		
	R	-	2	-	8	4					
	FICI	I	>0.5 (I)	I	**≤0.38 (P)**	**≤0.25 (P)**					

- = no decrease or increase in the MIC was observed; n.p. not performed; *no MIC was detected for erythromycin alone against RWW337, but when combined with apigenin and genistein. MIC for erythromycin was found to be at least ½ (not conclusive) and ¼ of the maximal concentration tested, respectively. When FICI ≤ 0.5 (in bold), if the phytochemical has a determinable MIC value the effect is considered as synergy (S); if the phytochemical has no detectable MIC the effect is called potentiation (P). If FICI > 0.5, the interaction is indifferent (I). Erythromycin was not tested against SA1199B strain. The experiments were repeated three times and the values presented are the averages of at least three independent assays. Lut: luteolin; Apig: apigenin; Chry: chrysin; Gen: genistein.

No synergistic effects were obtained with luteolin when combined with ciprofloxacin or erythromycin (FICI > 0.5). The MIC of ciprofloxacin against ST239 was reduced 4- and 8-fold by apigenin (15 – 60 mg l^{-1}) and its isoflavone analogue genistein (30 – 60 mg l^{-1}), respectively. Genistein also reduced by 8-fold the MIC of ciprofloxacin against SA1199B (reversing the resistance of this strain) but not apigenin. Daidzein (60 mg l^{-1}) only potentiated ciprofloxacin against SA1199B (4-fold reduction of the MIC of this antibiotic). Genistein produced a 4-fold reduction of the MIC of erythromycin against ST239 and USA300 strains. No potentiating effect was obtained with chrysin. No potentiation effect was observed with any of the compounds against the susceptible strains CECT 976, M82 and RN6390, excepting for genistein against CECT 976 (4-fold reduction of the MIC of ciprofloxacin and erythromycin). Ciprofloxacin-potentiation promoted by the compounds isolated from *C. striatus* was confirmed by disk diffusion method (TABLE 4.12), but erythromycin potentiation was more difficult to detect for MRSA strains, maybe because no inhibition halo was observed for erythromycin against some of these strains.

TABLE 4.12 | Antibiotic-potentiating activity promoted by four phytochemicals found in *Cytisus striatus* against *Staphylococcus aureus* detected by disk diffusion method. The activity is expressed as the increase in the diameter of growth inhibition zones (mm) promoted by ciprofloxacin (CIP) and erythromycin (ERY) in the presence of the isoflavonoids (60 mg l^{-1}) dissolved in Mueller-Hinton agar medium

		Increased IZD (IZD_c – IZD_a) (mm)			
		Apigenin	**Chrysin**	**Genistein**	**Daidzein**
CECT 976	CIP	-	-	**10.0 ± 0.0 (P)**	4.0 ± 0.0 (A)
	ERY	-	-	**6.0 ± 0.0 (P)**	-
M116	CIP	**8.0 ± 1.0 (P)**	-	**9.0 ± 0.0 (P)**	-
	ERY	-	-	4.3 ± 0.6 (A)	-
RWW337	CIP	**8.0 ± 1.0 (P)**	4.5 ± 1.0 (A)	**7.0 ± 1.0 (P)**	-
	ERY	-	-	-	-
RWW50	CIP	**10.0 ± 0.0 (P)**	5.0 ± 0.0 (A)	**8.3 ± 0.6 (P)**	-
	ERY	-	-	-	-
M82	CIP	5.3 ± 0.6 (A)	-	4.0 ± 1.0 (A)	-
	ERY	-	-	4.0 ± 0.0 (A)	-
RN6390	CIP	-	-	-	-
	ERY	-	-	-	-
SA1199B	CIP	4.6 ± 0.3 (A)	4.0 ± 0.0 (A)	**10.0 ± 0.0 (P)**	**13.0 ± 1.0 (P)**
	ERY	n.p.	n.p.	n.p.	n.p.

n.p.: not performed; - no significant increase over antibiotic activity ($P > 0.05$). Inhibition zones obtained with the combinations (IZD_c) over antibiotic-single activity (IZD_a) are given and classified as: (-) indifferent (IZD_c – IZD_a < 4 mm); (A) additive (4 ≤ IZD_c – IZD_a < 6 mm); and (P) potentiation (IZD_c – IZD_a ≥ 6 mm, in bold). Data are means and SD from at least three independent experiments.

Dual combinations between luteolin and the other flavonoids/isoflavonoids found in *C. striatus* were performed to understand if luteolin, the main antibacterial compound produced by *C. striatus*, would benefit from the presence of the other compounds. Luteolin MIC was not reduced by the presence of any compound applied alone (120 mg l^{-1}). It would be interesting to test multiple combinations of the compounds. However, detecting the high number of possible dual and multiple interactions of the compounds as well as their optimal doses is extremely challenging. To assess and predict real interactions of the compounds, it would be valuable to test the effects of such combinations against plant pathogens. This would allow a better perception of the defense system of this plant.

Apigenin and genistein presented the best potentiating results, and this in agreement with the analysis of the S-plot in FIGURE 4.11. However, genistein and daidzein were able to potentiate ciprofloxacin (4-8 fold) against the NorA overexpressing SA1199B, indicating that these isoflavonoids may be efflux pump inhibitors. To confirm this hypothesis, EtBr accumulation in SA1199B and on MRSA strains was assessed in the presence of these compounds by fluorometry.

4.4.6. Effect of the compounds isolated from *Cytisus striatus* on EtBr accumulation

Fluoroquinolone resistance in *S. aureus* has been mainly attributed to mutations occurring in the cellular targets GrlA/GrlB (topoisomerase IV, encoded by genes *grlA*/*grlB*) and GyrA/GyrB (DNA gyrase, encoded by genes *gyrA*/*gyrB*). However, reports of antibiotic resistance mediated by efflux pumps in *S. aureus* is also greatly observed for fluoroquinolones, as well as for tetracyclines and macrolides (Costa et al., 2013). *S. aureus* encodes several MDR efflux pumps among which NorA has been extensively examined.

The potential of the non-antibacterial compounds isolated from *C. striatus* to inhibit efflux pumps was assessed on *S. aureus* SA1199B. The MIC of EtBr for SA1199B cells was previously determined according to CLSI (2015a) guidelines as described in *Section 3.2.1.2*. Accumulation of EtBr applied at ½ MIC, to avoid compromising the cellular viability, was measured fluorometrically. The real-time setup and sensitivity of the fluorometric method allowed the observation of the effect of the phytochemicals (60 mg l^{-1}) on the overtime accumulation of EtBr (20 mg l^{-1}) in *S. aureus* SA1199B (FIGURE 4.12).

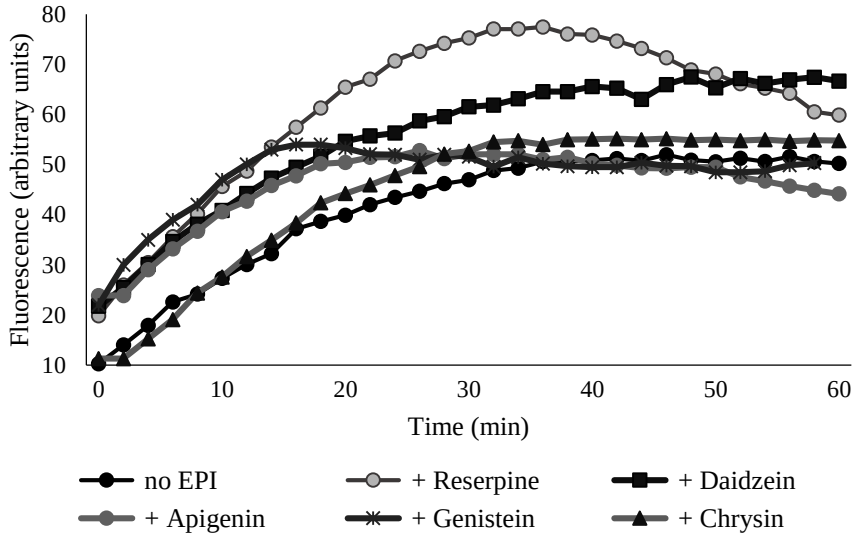


FIGURE 4.12 | Detection of the effect of the compounds isolated from the active fraction of *Cytisus striatus* on ethidium bromide accumulation in *Staphylococcus aureus* SA1199B. The bacteria were loaded with EtBr at 20 mg l^{-1} in the presence of the phytochemicals (60 mg l^{-1}) for 60 min at 37°C and fluorescence measured. Mean values of at least three independent experiments are shown. Reserpine at 20 mg l^{-1} was used as a positive control.

Reserpine control (20 mg l^{-1}) showed the best EtBr accumulation, confirming the EPI activity. Genistein and, in less extension, apigenin increased the accumulation of EtBr in SA1199B until approximately 20 min, after which either the signal levels slightly reduced (for apigenin, to values lower than the control, $P < 0.05$). Daidzein also showed high EtBr accumulation over time ($P < 0.05$). This increased EtBr accumulation obtained with daidzein and genistein may be due to a NorA inhibition, which could explain the ciprofloxacin-potentiation by 4- and 8-fold, respectively, verified against SA1199B (TABLE 4.13). In line with these observations, apigenin only achieved a 2-fold potentiation of ciprofloxacin against SA1199B and no effect was observed with chrysin ($P < 0.05$).

The effect of the phytochemicals on the accumulation of EtBr was also tested by fluorometry for strains M116, RWW337 and RWW50. The MIC of EtBr was determined for each strain using the microdilution technique, resulting in 5 mg l^{-1} for M116 and RWW50 and 40 mg l^{-1} for RWW337. The three MRSA strains also showed a poor accumulation of EtBr, suggesting that, contrarily to the other strains, there is overexpression of NorA or other related MDR efflux pumps in these strains. Relative fluorescence (RF) values were calculated using the maximal fluorescence intensities (MFI) of each assay (FIGURE 4.13).

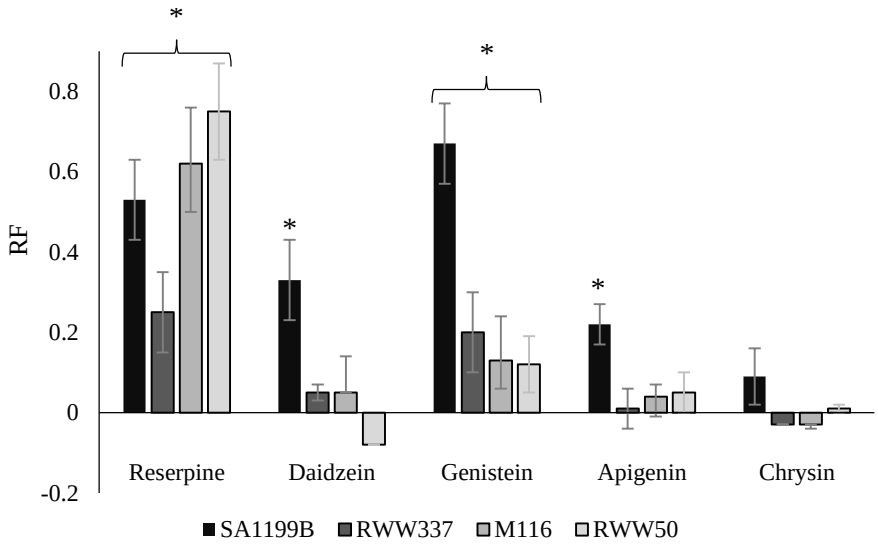


FIGURE 4.13 | Detection of the effect of the compounds isolated from the active fraction of *Cytisus striatus* on ethidium bromide accumulation in ST239 and USA300 strains. Relative fluorescence (RF) values were calculated for the isolated compounds from *C. striatus* by fluorometry in SA1199B and MRSA strains: RF_{assay} = (MFI_{assay} – $FI_{control}$)/ $FI_{control}$, where MFI_{assay} was the Maximal Fluorescence Intensity obtained in each 60 min assay and $FI_{control}$ was the correspondent Fluorescence Intensity obtained with the DMSO control at the same time. Reserpine (20 mg l^{-1}) was used as positive EPI control. Bars with (*) accumulated more EtBr than the control ($P < 0.05$). RFs are averages and standard deviations of three independent assays.

High RF values indicated that cells accumulated more EtBr under the tested conditions than the control and vice-versa for negative values. Reserpine showed high RF values for all the MRSA strains, confirming that this compound is inhibiting MDR efflux pumps. Despite being an EPI universally used for Gram-positive bacteria in predicting pump gene overexpression, it seems that the use of reserpine to predict the contribution of efflux to the reduced susceptibility in clinical *S. aureus* isolates is not reliable and reserpine may fail to identify overexpression of staphylococcal MDR pump genes using microdilution susceptibility testing (Frempong-Manso et al., 2008).

Genistein also showed significant RF values for all MRSA strains ($P < 0.05$). The fact that genistein potentiated ciprofloxacin against these MRSA strains is in line with these observations. Daidzein only showed significant RF values ($P < 0.05$) for SA1199B and, likewise, this compound only caused ciprofloxacin potentiation on SA1199B strain. Apigenin achieved inferior RF values for MRSA strain even though this flavonoid was able to potentiate ciprofloxacin against MRSA strains. Other mechanisms may be involved in the potentiation observed with this compound. Chrysin, as expected, showed inferior RF values than the other compounds.

4.4.7. Effect of the compounds isolated from *Cytisus striatus* on biofilm control

As mentioned before, eradication of biofilms has been a challenging task due to the biological complexity of the biofilm structure (Kishen et al., 2010). Combination of one antibiotic with a biofilm inhibitor could eventually improve the treatment of biofilm-related infections.

The effect of the five compounds isolated from *C. striatus* was evaluated for their activity on biofilms. The exposure of the compounds for 1 and 24 h was first evaluated against biofilms of *S. aureus* CECT 976, which is the reference strain for biofilm assays. The number of CFU cm^{-2} of the biofilms obtained is shown in FIGURE 4.14, as well as the percentages of biofilm reduction. Ciprofloxacin and erythromycin were also applied at MIC, for comparison. Previously (in *Section 4.2.1*), it was found that no differences were obtained among application of these antibiotics at MIC, 10 × MIC or 50 × MIC.

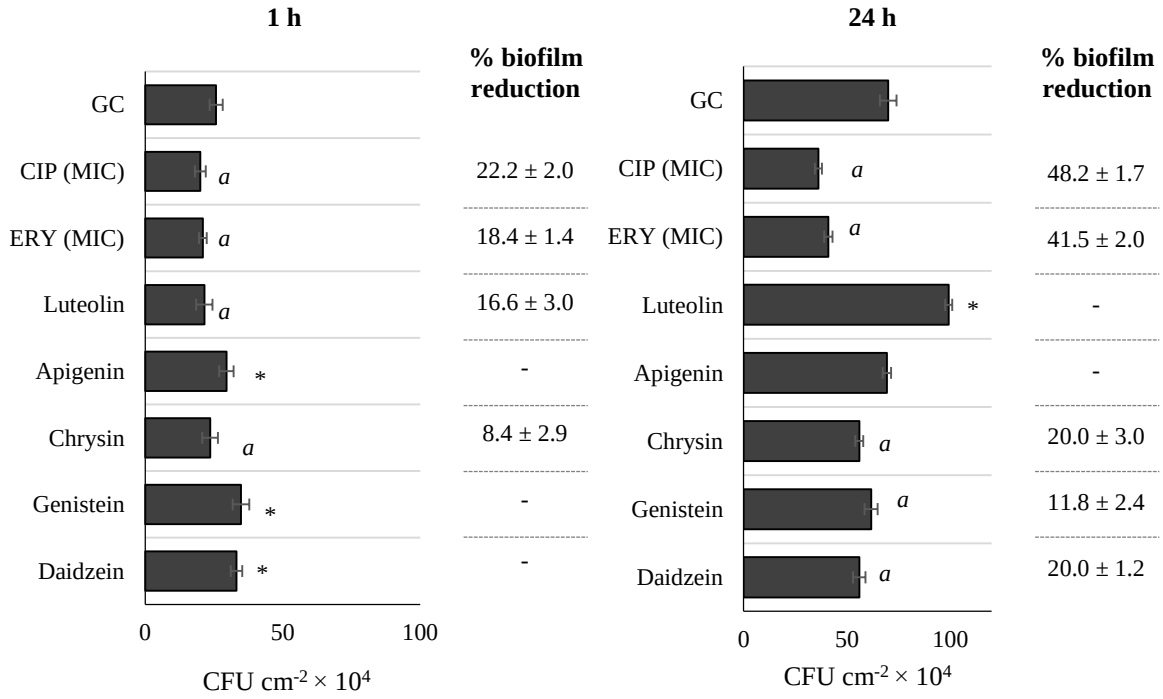


FIGURE 4.14 | CFU cm^{-2} of biofilms of *Staphylococcus aureus* CECT 976 after exposure to the five phytochemicals isolated from *Cytisus striatus* at 60 mg l^{-1} for 1 and 24 h. Biofilm reductions (%) are shown. (*) when statistically higher than GC ($P < 0.05$), (a) when statistically lower than GC ($P < 0.05$). GC – growth control (5% v v^{-1} DMSO).

Despite having antibacterial activity, luteolin at 60 mg l^{-1} did not cause any significant reduction of CECT 976 biofilms when applied for 1 h (only 17%), and even increased the amount

of biofilm formed comparing to the control, when applied for 24 h ($P > 0.05$). Similarly, apigenin and chrysin applied at 60 mg l^{-1}, either for 1 h or 24 h, did not promote any significant reduction of CECT 976 biofilms. The isoflavonoids genistein and daidzein only significantly removed CECT 976 biofilms when applied for 24 h, but only by 12 and 20%, respectively.

The effects of the compounds were also assessed on SA1199B biofilms after 1 and 24 h exposure. The interest of testing this strain was to understand if the phytochemicals acting as EPIs, mostly daidzein and also genistein, could promote better biofilm control activities. The number of CFU cm^{-2} of biofilms obtained is presented in FIGURE 4.15 along with the percentages of biofilm reduction.

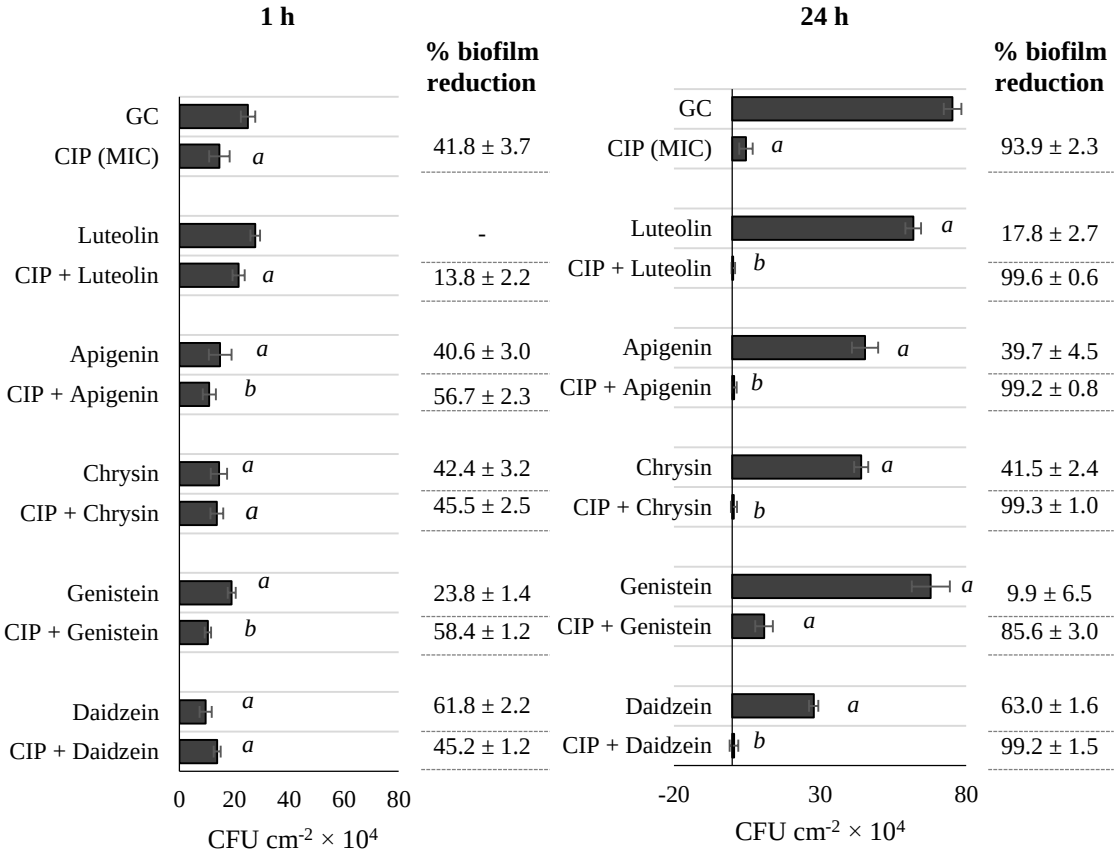


FIGURE 4.15 | CFU cm^{-2} of biofilms of *Staphylococcus aureus* SA1199B after exposure to the five phytochemicals isolated from *Cytisus striatus* at 60 mg l^{-1} for 1 and 24 h. Biofilm reductions (%) are shown. (*a*) when statistically lower than GC ($P < 0.05$), and (*b*) than both GC and ciprofloxacin applied alone ($P < 0.05$). GC – growth control (5% v v^{-1} DMSO).

Similarly, luteolin applied alone at 60 mg l^{-1} caused no considerable reduction on SA1199B biofilms (only of 18% when applied for 24 h, $P < 0.05$) and neither caused genistein (24 and 10% for 1 and 24 of exposure, respectively). Apigenin and chrysin caused approximately 40% of biofilm reduction for both 1 and 24 h of exposure. Daidzein showed the best removal activity when applied alone, achieving 62% of biofilm reduction when exposed for 1 h and 63% for 24 h. This activity may be related to its EPI activity on SA1199B strain.

Combinations between the phytochemicals with ciprofloxacin were not always advantageous for exposures of 1 h, excepting for combination with apigenin and genistein ($P < 0.05$). For exposures of 24 h, combinations ciprofloxacin-phytochemicals achieved better results (around 99% of biofilm reduction) than when the compounds were applied alone ($P < 0.05$), excepting for the combination with genistein that caused worst results comparing to when ciprofloxacin was applied individually ($P < 0.05$). Still, these combinations are not considered synergic according to Monzón et al. (2001).

4.4.8. Relevant remarks

The chemical novelty and diversity of natural products, especially those derived from plants, is higher than that of synthetic sources. Moreover, through evolution they have been selected as effective compounds in various survival strategies of different organisms. In plants this has resulted for example, in resistance against most microorganisms. The antimicrobial properties displayed by a number of plants can be attributed, generally, to mixtures of active constituents (Mukne et al., 2011). It is also known that plants can produce inhibitors as a protection against multidrug resistant pathogens ensuring the efficient delivery of the antimicrobial compounds. A good example of this is provided by Tegos et al. (2002). The role of phytochemistry in the search for *S. aureus* NorA inhibitors is significant, having led to the discovery of several chemically diverse plant-derived EPIs, including flavones, isoflavones, acylated glycosides, porphyrin phaeophorbide A or kaempferol rhamnoside (Handzlik et al., 2013), among others.

As a bioassay-guided fractionation does not generally detect synergy, metabolomics was used as an alternative strategy. Whether using LC-MS or NMR as an analytical platform, it is necessary to have a larger number of samples with different levels of the different plant metabolites to be able to detect signals or peaks related to activity. These could be samples of different plants, or different extracts from the same plant and/or their fractions. An example of the use of fractions was reported by Yuliana et al. (2011). When fractionation is used there is, of course, the risk that interacting molecules have been separated. In this strategy, this could be circumvented by using different extracts for fractionation. With this approach, it was proven that even using one single

plant, it was possible to obtain a variety of different extracts by mixing the different plant parts in different rations and extracting them under different conditions. Applying multivariate data analysis to the resulting NMR spectra then allowed the identification of signals that were correlated to activity.

The present study resulted in the identification of one main antibacterial compound in *C. striatus* leaf extract - luteolin - with MICs between 30 and 120 mg l^{-1} against various *S. aureus* strains. Surprisingly, luteolin had lower MICs for ST239-MRSA strains when compared to antibiotic-susceptible strains. The multivariate data analysis also revealed correlations of the activity with a number of other phenolics. These were isolated and identified as apigenin, daidzein, genistein, 2'-hydroxygenistein and 3'-hydroxydaidzein. Among these, apigenin and genistein clearly showed potentiation of the antibiotics against ST239 and USA300-MRSA strains but not against the susceptible strains M82 and RN6390. Daidzein showed some weaker potentiating activity against cell line M116 and against the resistant line SA1199B (4-fold increase).

Sato et al. (2000) also reported an antibacterial activity for luteolin with MICs between 62.5 – 125 mg l^{-1} against MRSA and MSSA strains. Apigenin showed no MIC for concentrations below 120 mg l^{-1}, in agreement with other studies that reported values of up to 128 mg l^{-1} (Basile et al., 2000). Other authors, however, report a MIC in the range of 3.9 to 15.6 mg l^{-1} for 15 MRSA and 5 MSSA strains (Sato et al., 2000). Genistein also showed no detectable MIC until 120 mg l^{-1}. In agreement, Albert Dhayakaran et al. (2015) reported that MRSA strains and their biofilms were not inhibited by soy isoflavones, including genistein and daidzein (until 100 mg l^{-1}), and Morán et al. (2014) reported no MIC for genistein until 2 g l^{-1} against *S. aureus* CECT 59. Contrarily, other group found growth inhibition of an enteroisolate of *S. aureus* of more than 90% by either genistein or daidzein at 125 mg l^{-1} (Parkar et al., 2008). Hong et al. (2006) revealed that CFU determination results at 8 h of incubation showed the inhibitory effects of genistein (270 mg l^{-1}) on *S. aureus* and MRSA strains but not at 15 and 24 hours, probably due to the degradation of genistein in culture or outgrowth of bacteria. It is known and it was mentioned before that results of antibacterial activity of phenolic compounds can be widely inconsistent as reported by Cushnie and Lamb (2005), probably owing to variations in their susceptibility testing and differences in genetic determinants of the strains, culture conditions, antimicrobial tests, etc. Wang et al. (2014) reported that genistein reduced the MIC of norfloxacin against SA1199B by four times and had a moderate efflux pump inhibitory effect against the same strain.

Flavonoids have been reported to exert a number of biological activities. These activities have been attributed to many molecular mechanisms, including the modulation of the activities of phase I and II detoxification enzymes, direct and indirect antioxidant activities, inhibition of

protein kinases, effects on cell cycle, modulation of gene transcription, and epigenetic activities (Busch et al., 2015). The B ring of flavonoids can intercalate or form hydrogen bonds with the stacking of nucleic acid bases and further lead to inhibition of DNA and RNA synthesis in bacteria and influence DNA gyrase activity (Mierziak et al., 2014). Genistein has been described as a tyrosine kinase and topoisomerase II inhibitor (Verdrengh et al., 2004) and for its antioxidant, estrogenic and antiangiogenic properties. Other study also refers that genistein, apigenin and other isoflavone, biochanin A, inhibited topoisomerase II to the same extent as etoposide, a specific inhibitor of the enzyme (Azuma et al., 1995). This inhibition could explain the potentiation observed with genistein and apigenin with ciprofloxacin. Since ciprofloxacin also functions by inhibiting DNA gyrase, the presence of a second compound with the same target could inhibit or potentiate its activity. It was also observed the same effect with quercetin (in *Sections 4.1* and *4.2*). However, more conclusive studies are necessary to verify such effect.

Ethidium bromide accumulation assays allowed to conclude that genistein and daidzein increased EtBr accumulation in SA1199B cells, thus presenting a EPI activity on NorA efflux pump. This result could explain the potentiation of ciprofloxacin against the same strain. Daidzein failed to show the same effect for the other MRSA strains, while genistein showed significant results, being in line with the fact that genistein potentiated ciprofloxacin also against the MRSA strains.

When studying efflux activity in clinical isolates instead of well-defined collection strains, the myriad of bacterial responses can complicate the clear interpretation of data. The role played by each individual efflux system is difficult to determine. Each efflux system is regulated by several specific and/or global regulators that act in an intricate network of regulatory/sensory pathways. Bacterial response to ciprofloxacin is mediated, in most of the cases, not by one single efflux pump but by several (such as SdrM, MdeA, MepA, NorB or NorC) (Costa et al., 2013) hampering the perception of the role played by each individual pump on the overall efflux activity/resistance phenotype. For example, even if the isoflavonoids are successful in inhibiting NorA, which could be detected by an increase EtBr accumulation in SA1199B strain, when testing other clinical isolates, efflux pumps others than NorA can be overexpressed and can still extrude ciprofloxacin out of the cell. Frempong-Manso et al. (2008) revealed that nearly one-half of clinical isolates with an efflux phenotype were overexpressing *norA-B-C*, *mepA* or *mdeA*. Costa et al. (2011) described the predominance of *norB* overexpression among a collection of *S. aureus* bloodstream isolates, while *norA* was the only for which no overexpression was detected. Thus, it is suggested that other efflux pumps than NorA can have a more relevant role in the resistance to fluoroquinolones and EtBr in *S. aureus*. The same substrate can also promote the expression of different efflux pump genes depending on its concentration and/or time of exposure (Costa et al.,

2011). Even isolates belonging to the same clonal type can have distinct levels of efflux activity and respond to the same agent through the activation of different efflux pumps (Costa et al., 2011). More studies are necessary to clearly define the molecular, functional and genetic bases of the efflux pumps being expressed by these *S. aureus* strains. Detection of MDR efflux pumps gene expression analysis using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) would be valuable for a deeper study of this phenomenon.

Among the five phytochemicals tested, daidzein showed the best eradication of SA1199B biofilms: 62 and 63 % of biofilm reduction when exposed for 1 and 24 h, respectively. However, against CECT 976 biofilms, the maximal biofilm reduction was only 20%, obtained after 24 h exposure. The apparently opposite effect observed with the same metabolite on biofilms formed by different strains can be a consequence of the multiple molecules and factors that characterize a biofilm (adhesion factors, quorum sensing, extracellular polymers in the matrix, etc.) and that indeed can differ between bacterial species (Morán et al., 2014). Also, it is suggestive that the anti-biofilm effect of daidzein may be related to its EPI activity. Kvist et al. (2008) refers that some EPIs reduced biofilm formation and, when in combination, they could abolish biofilm formation completely. Any relevant study was found about the activity of daidzein on biofilms. Nevertheless, an anti-biofilm activity against *S. aureus* was demonstrated for genistein at 500 mg l^{-1}, which inhibited *S. aureus* biofilm formation by 45% (Morán et al., 2014).

This study aimed to identify active antibiotic adjuvants from a promising plant, *Cytisus striatus*, by a metabolomics approach, which proved to be very efficient on the detection of synergistic activities among plant metabolites. These results reveal very clear potentiation effects between plant secondary metabolites (belonging to flavonoid and isoflavonoid class) and two antibiotics, showing a promising potential for the application of these systems in the treatment of infectious diseases caused by MRSA in clinical settings.

4.5. TASK 5. EVALUATION OF THE STRUCTURE-ACTIVITY RELATIONSHIP OF ISOFLAVONOIDS AS ANTIBIOTIC ADJUVANTS IN CLINICAL THERAPY

Abstract

For SAR evaluation purposes, 22 isoflavonoids were assessed as antibiotic adjuvants against *S. aureus* and MRSA strains. Genistein, tectorigenin and biochanin A were highlighted being able to potentiate ciprofloxacin and erythromycin by 2-8 fold, especially against ST239 and USA300 MRSA strains. The NorA efflux pump inhibitory activity of the isoflavonoids was also tested as a possible mechanism for this coadjuvant activity and, besides genistein and daidzein, biochanin A, calycosin, irigenin, irisflorentin and tectorigenin were found to be active. The possible inhibition of Erm methyltransferases was tested but no effect was found. The effect of the isoflavonoids on the eradication of biofilms was also assessed, being highlighted daidzein and irisflorentin. This study reveals the great potential for the application of isoflavonoids in clinical therapy of MRSA infection.

4.5.1. Antibacterial evaluation of isoflavonoids

The antimicrobial activity of isoflavonoids (low or moderate activity in most cases) has been reported (Chacha et al., 2005; Dastidar et al., 2004; Osawa et al., 1992), and they are seemingly involved in the inducible defense response of Leguminosae plants. However, their mechanism of action remains largely unexplained. Similarly, the potentiating activity of ciprofloxacin against *S. aureus* described for some isoflavonoids (Belofsky et al., 2006; Morel et al., 2003; Tanaka et al., 2002; Wang et al., 2014), has yet to be understood.

For SAR purposes, twenty-two structurally different isoflavonoids were analysed for their antibacterial and antibiotic-potentiating properties. The chemical structures of the compounds tested were previously shown in TABLE 3.4, *Section 3.1.6*. TABLE 4.13 shows the MICs obtained for the active isoflavonoids alone and combined with ciprofloxacin and erythromycin.

From the 22 isoflavonoids tested, only three showed antibacterial activity below 120 mg l^{-1}: neobavaisoflavone, corylifol A and orobol. Synergy was obtained with neobavaisoflavone combined with ciprofloxacin against SA1199B (FICI of 0.5) and corylifol A combined with both ciprofloxacin and erythromycin against RWW337 (FICIs of 0.38).

TABLE 4.13 | Minimal inhibitory concentrations (MICs) of the isoflavonoids against *Staphylococcus aureus* when applied alone (MIC_a) and in combination (MIC_b) with ciprofloxacin (CIP) or erythromycin (ERY). Fold-reductions of antibiotic MICs (R) and fractional inhibitory concentration index (FICIs) values are shown

		CIP										
		Neob	**CorA**	**Orob**	**Gen**	**Tect**	**BiochA**	**Calyc**	**Irig**	**Gly**	**Daid**	**Irisfl**
M116	MIC_a	20	2.5	120	>120	>120	>120	>120	>120	>120	>120	>120
	MIC_b	5	-	60	60	60	30	-	-	-	15	-
	R	2	-	8	4	2	4	-	-	-	2	-
	FICI	0.75 (I)	I	0.63 (I)	**≤0.50 (P)**	>0.5 (I)	**≤0.38 (P)**	I	I	I	>0.5 (I)	I
RWW337	MIC_a	20	20	120	>120	>120	>120	>120	>120	>120	>120	>120
	MIC_b	5	5	60	60	60	30	30	60	-	-	-
	R	2	8	4	8	4	4	2	2	-	-	-
	FICI	0.75 (I)	**0.38 (S)**	0.63 (I)	**≤0.38 (P)**	**≤0.50 (P)**	**≤0.38 (P)**	>0.5 (I)	>0.5 (I)	I	I	I
RWW50	MIC_a	20	2.5	120	>120	>120	>120	>120	>120	>120	>120	>120
	MIC_b	10	-	-	30	60	15	30	30	-	-	-
	R	4	-	-	2	2	2	2	2	-	-	-
	FICI	0.75 (I)	I	I	>0.5 (I)	>0.5 (I)	>0.5 (I)	>0.5 (I)	>0.5 (I)	I	I	I
M82	MIC_a	20	2.5	60	>120	>120	>120	>120	>120	>120	>120	>120
	MIC_b	10	1.25	-	30	30	30	-	60	-	-	-
	R	4	2	-	2	2	2	-	2	-	-	-
	FICI	0.75 (I)	1 (I)	I	>0.5 (I)	>0.5 (I)	>0.5 (I)	I	I	I	I	I
RN6390	MIC_a	20	2.5	60	>120	>120	>120	>120	>120	>120	>120	>120
	MIC_b	10	-	-	-	-	-	-	-	-	-	-
	R	4	-	-	-	-	-	-	-	-	-	-
	FICI	0.75 (I)	I	I	I	I	I	I	I	I	I	I
CECT 976	MIC_a	20	2.5	60	>120	>120	>120	>120	>120	>120	>120	>120
	MIC_b	10	-	-	30	60	60	60	60	-	30	60
	R	2	-	-	4	2	2	2	2	-	2	2
	FICI	1.0 (I)	I	I	**≤0.38 (P)**	>0.5 (I)	>0.5 (I)	>0.5 (I)	>0.5 (I)	I	>0.5 (I)	>0.5 (I)
SA1199B	MIC_a	20	0.06	60	>120	>120	>120	>120	>120	>120	>120	>120
	MIC_b	5	-	15	60	60	30	60	60	60	60	60
	R	4	-	2	8	4	8	4	4	2	4	4
	FICI	**0.50 (S)**	I	>0.5 (I)	**≤0.38 (P)**	**≤0.50 (P)**	**≤0.38 (P)**	**≤0.50 (P)**	**≤0.50 (P)**	>0.5 (I)	**≤0.25 (P)**	**≤0.50 (P)**

		ERY										
		Neob.	**Cor.A**	**Orob**	**Gen**	**Tect**	**Bioch.A**	**Calyc**	**Irig**	**Gly**	**Daid**	**Irisfl**
M116	MIC_a	20	2.5	120	>120	>120	>120	>120	>120	>120	>120	>120
	MIC_b	-	1.25	-	30	60	15	30	60	-	-	-
	R	-	2	-	4	4	4	2	2	-	-	-
	FICI	I	1 (I)	I	**≤0.38 (P)**	**≤0.38 (P)**	**≤0.28 (P)**	>0.5 (I)	>0.5 (I)	I	I	I
RWW337	MIC_a	20	20	120	240	>240	>240	>240	>240	>120	>120	>120
	MIC_b	-	5	60	60	120	60	-	-	-	-	-
	R	-	≥8	≥4	**≥4**	≥2	**≥4**	-	-	-	-	-
	FICI	I*	**≤0.38 (S)***	>0.50 (I)*	**≤0.50 (P)***	(*)	**≤0.50 (P)***	I*	I*	I	I	I
RWW50	MIC_a	20	2.5	120	>120	>120	>120	>120	>120	>120	>120	>120
	MIC_b	10	-	-	60	60	60	30	60	-	-	-
	R	8	-	-	4	4	4	2	2	-	-	-
	FICI	0.63 (I)	I*	I*	**≤0.50 (P)**	**≤0.50 (P)**	**≤0.50 (P)**	>0.5 (I)	>0.5 (I)	I	I	I
M82	MIC_a	20	2.5	60	>120	>120	>120	>120	>120	>120	>120	>120
	MIC_b	5	1.25	-	30	60	30	120	60	-	-	-
	R	2	2	-	2	2	2	2	2	-	-	-
	FICI	0.75 (I)	1 (I)	I	≥0.56 (I)	≥0.63 (I)	≥0.56 (I)	>0.5 (I)	>0.5 (I)	I	I	I
RN6390	MIC_a	20	2.5	60	>120	>120	>120	>120	>120	>120	>120	>120
	MIC_b	10	-	-	-	-	-	-	-	-	-	-
	R	4	-	-	-	-	-	-	-	-	-	-
	FICI	0.75 (I)	I	I	I	I	I	I	I	I	I	I
CECT 976	MIC_a	20	2.5	60	>120	>120	>120	>120	>120	>120	>120	>120
	MIC_b	-	1.25	-	30	60	-	60	60	-	-	-
	R	-	2	-	4	2	-	2	2	-	-	-
	FICI	I	1 (I)	I	**≤0.38 (P)**	>0.5 (I)	I	>0.5 (I)	>0.5 (I)	I	I	I

- = no decrease or increase in the MIC was observed; n.p. not performed; *no MIC was detected for erythromycin alone against RWW337, but when combined with genistein and biochanin A, erythromycin MIC was found to be at least ¼ of the maximal concentration tested, and at least ½ for tectorigenin. When FICI ≤ 0.5 (in bold), if the isoflavonoid has a determinable MIC value, the effect is considered as synergy (S); if the isoflavonoid has no detectable MIC, the effect is called potentiation (P). If FICI > 0.5, the interaction is considered indifferent (I). Isoflavonoids showing only indifferent effects were not included. ERY was not tested against SA1199B. The experiments were repeated three times, and the values presented are the averages of three independent assays. Neob: neobavaisoflavone; CoryA: corylifol A; Orob: orobol; Gen: genistein; Tect: tectorigenin; BiochA: biochanin A; Calyc: calycosin; Irig: irigenin; Gly: glycitein; Daid: daidzein; Irisfl: irisflorentin.

Concerning the non-antibacterial isoflavonoids, biochanin A (15 – 60 mg l^{-1}) and tectorigenin (60 – 120 mg l^{-1}) have a similar activity to genistein (30 – 60 mg l^{-1}) when combined with ciprofloxacin and erythromycin against all cell lines except for the susceptible strains CECT 976, M82 and RN6390, for which no activity was found. Additionally, calycosin, irisflorentin, irigenin

and daidzein (60 mg l^{-1}) were also able to reduce the MIC of ciprofloxacin by 4-fold against the NorA overexpressing strain SA1199B.

The disk diffusion method was also performed to confirm these antibiotic-potentiating results promoted by the non-antibacterial isoflavonoids, which were dissolved in MH agar at 60 mg l^{-1}. The increase on IZDs in the presence of isoflavonoids over antibiotic single activity is shown in TABLE 4.14.

TABLE 4.14 | Antibiotic-potentiating activity of isoflavonoids by disk diffusion method. The activity is expressed as the increase in the diameter of growth inhibition zones (mm) promoted by the antibiotics with ciprofloxacin (CIP) and erythromycin (ERY) in the presence of the isoflavonoids in MH agar medium and applied at 60 mg l^{-1}

		Increased IZD ($IZD_c - IZD_a$) (mm)							
S. aureus		**Gen**	**Bioch A**	**Tecto**	**Irig**	**Calyc**	**Daid**	**Glyc**	**Irisfl**
M116	CIP	**9.0 ± 0.0 (P)**	**7.0 ± 0.0 (P)**	5.0 ± 0.0 (A)	**4.3 ± 0.6 (P)**	**4.0 ± 0.0 (P)**	-	-	-
	ERY	4.3 ± 0.6 (A)	-	0	-	-	-	-	-
RWW337	CIP	**7.0 ± 1.0 (P)**	**6.5 ± 1.0 (P)**	**6.0 ± 0.0 (P)**	-	-	-	-	-
	ERY	-	-	-	-	-	-	-	-
RWW50	CIP	**8.3 ± 0.6 (P)**	**6.0 ± 1.0 (P)**	**6.5 ± 1.0 (P)**	4.6 ± 0.3 (A)	5.0 ± 1.0 (A)	-	-	-
	ERY	-	-	-	-	-	-	-	-
M82	CIP	4.0 ± 1.0 (A)	5.0 ± 0.0 (A)	4.0 ± 0.0 (A)	-	-	-	-	-
	ERY	4.0 ± 0.0 (A)	-	5.0 ± 0.0 (A)	-	-	-	-	-
RN6390	CIP	-	-	-	-	-	-	-	-
	ERY	-	-	-	-	-	-	-	-
CECT 976	CIP	**10.0 ± 0.0 (P)**	-	4.0 ± 0.0 (A)	5.0 ± 0.0 (A)	5.0 ± 0.0 (A)	4.0 ± 0.0 (A)	-	5.0 ± 0.0 (A)
	ERY	**6.0 ± 0.0 (P)**	-	5.0 ± 0.0 (A)	5.0 ± 0.0 (A)	4.0 ± 0.0 (A)	-	-	-
SA1199B	CIP	**10.0 ± 0.0 (P)**	**12.0 ± 2.0 (P)**	**10.0 ± 0.0 (P)**	**11.0 ± 1.0 (P)**	**8.0 ± 0.0 (P)**	**13.0 ± 1.0 (P)**	5.0 ± 1.0 (A)	**6.0 ± 0.0 (P)**
	ERY	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.

n.p.: not performed; - no significant increased over antibiotic activity ($P > 0.05$); Inhibition zones obtained with the combinations (IZD_c) over antibiotic-single activity (IZDa) are given and classified as: (-) indifferent ($IZD_c - IZD_a < 4$ mm); (A) additive ($4 \leq IZD_c - IZD_a < 6$ mm); and (P) potentiation ($IZD_c - IZD_a \geq 6$ mm, in bold). Data are means and SD from at least three independent experiments.

As previously observed, potentiation of erythromycin by any isoflavonoid was poorly detected for MRSA strains using the disk diffusion method. Despite that, several potentiation effects of ciprofloxacin obtained by this method were in agreement with the checkerboard results. Besides genistein and daidzein, as previously reported, many isoflavonoids showed to potentiate ciprofloxacin against SA1199B, which is resistant to ciprofloxacin due to the overexpression of NorA. Thus, the possibility of inhibition of this efflux pump was also investigated for all the isoflavonoids.

4.5.2. Effect of isoflavonoids on EtBr accumulation

The potential of the isoflavonoids to inhibit efflux pumps was assessed fluorometrically with an efflux accumulation assay of EtBr at ½ MIC to avoid compromising the cellular viability. The fluorometric method allowed the observation of the effect of the isoflavonoids on the overtime accumulation of EtBr (20 mg l^{-1}) in *S. aureus* SA1199B (FIGURE 4.16), using reserpine at 20 mg l^{-1} as positive control.

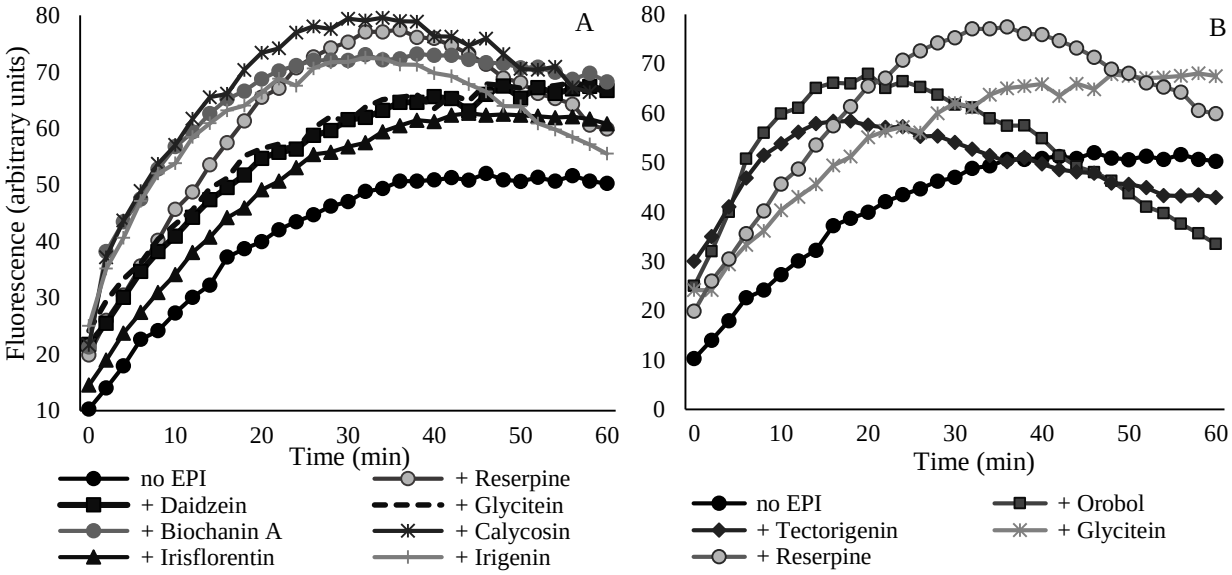


FIGURE 4.16 | Detection of the effect of the isoflavonoids on ethidium bromide accumulation for 60 min in *Staphylococcus aureus* SA1199B cells by fluorometry. Bacteria were loaded with EtBr at 20 mg l^{-1} in the presence of isoflavonoids (60 mg l^{-1}, except orobol at 30 mg l^{-1}) for 60 min at 37°C and fluorescence was measured. Mean values of least three independent experiments are shown. Reserpine at 20 mg l^{-1} was used as a positive control. (A) the isoflavonoids increasing the accumulation over control over time ($P < 0.05$) are presented; (B) Genistein, tectorigenin and orobol increased EtBr accumulation in the first 20-30 min, but then this accumulation was reverted for values similar or lower than the control.

As can be observed in FIGURE 4.16-A, isoflavonoids increased the accumulation of EtBr in SA1199B until approximately 40 min, after which either the signal levels stabilized or were slightly reduced again. As previously observed with genistein, tectorigenin and orobol showed a different pattern (FIGURE 4.16-B), producing a significant increase on the accumulation of EtBr in the first 20-30 min ($P < 0.05$) after which values reverted to levels that were similar (for genistein, $P > 0.05$) or lower (for orobol and tectorigenin, $P < 0.05$) than the control. If these results are related to the ciprofloxacin-potentiating activity data shown in TABLE 4.13, it can be observed that the isoflavonoids that most increased the accumulation of EtBr in SA1199B were also those that potentiated ciprofloxacin against the same strain.

There is a lack of consensual methodological approaches that guarantee appropriate address of all the issues related to efflux pump expression and inhibition. So, the positive effect of some isoflavonoids on the accumulation of EtBr in SA1199B cells was also shown and confirmed by flow cytometry by the acquisition of mean fluorescence intensities (FL3-A) after incubation of SA1199B cells with EtBr (1/2 MIC), reserpine (20 mg l^{-1}) and each isoflavonoid. Mean fluorescence intensities are shown in FIGURE 4.17.

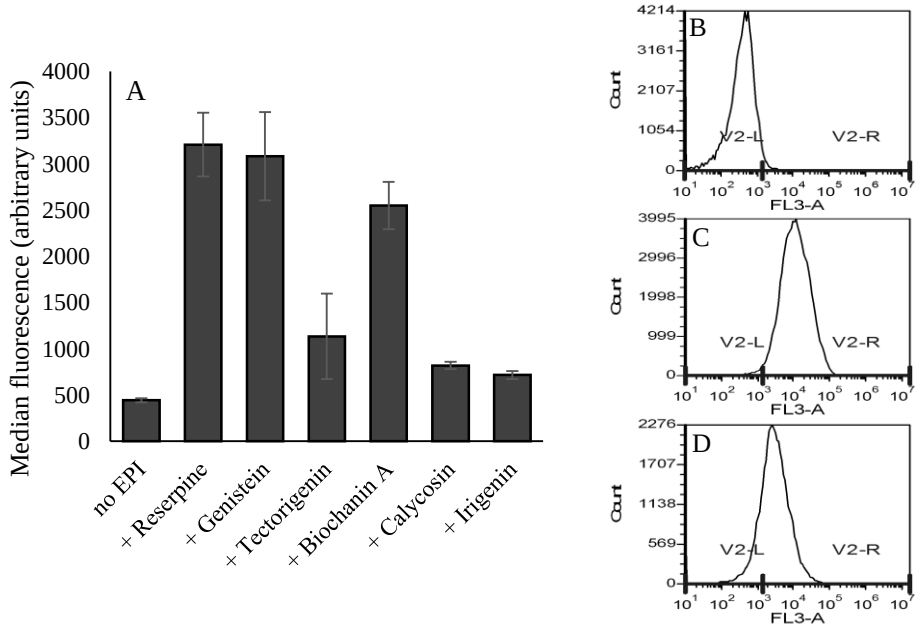


FIGURE 4.17 | Detection of the effect of the isoflavonoids on ethidium bromide accumulation for 60 min in *Staphylococcus aureus* SA1199B cells by flow cytometry. (A) Median fluorescence values obtained by flow cytometry for SA1199B after 60 min of exposure to EtBr (20 mg l^{-1}) at 37°C with reserpine (20 mg l^{-1}) and isoflavonoids (60 mg l^{-1}, except orobol at 30 mg l^{-1}); only the efflux pump inhibitors (EPI) which fluorescence values statistically differ from the control (no EPI, $P < 0.05$) are presented. Bars represent means and SD from at least three independent experiments. The effect of EPIs on the accumulation of EtBr in SA1199B is observed with a histogram shift: (B) without EPI, (C) with genistein and (D) biochanin A.

FIGURE 4.17-A shows that the highest accumulation of EtBr was obtained in the presence of genistein (similar to reserpine, $P > 0.05$) and biochanin A. Tectorigenin, calycosin and irigenin also promoted higher EtBr accumulation than control ($P < 0.05$) but the results were not so satisfactory. In this assay, the fluorescence values were only recorded after 60 min of incubation, and since it was observed that the final fluorescence values are not necessarily the ones that best reflect EtBr accumulation for all isoflavonoids, the results could be unreliable. Usually, the distribution of fluorescence was well defined by the population and the intensity distributed around one peak. FIGURE 4.17 (B-D) show the histogram peak shifts promoted by genistein and biochanin A.

The effect of the isoflavonoids on the accumulation of EtBr was also tested by fluorometry for strains M116, RWW337 and RWW50. RF values were calculated using the MFI values of each assay (FIGURE 4.18) as previously described. Reserpine showed high values of RF for all the MRSA strains. Among the isoflavonoids, biochanin A and tectorigenin caused the highest EtBr accumulation in MRSA strains. Calycosin and irigenin also showed high RF values ($P < 0.05$) for all MRSA strains, followed by genistein.

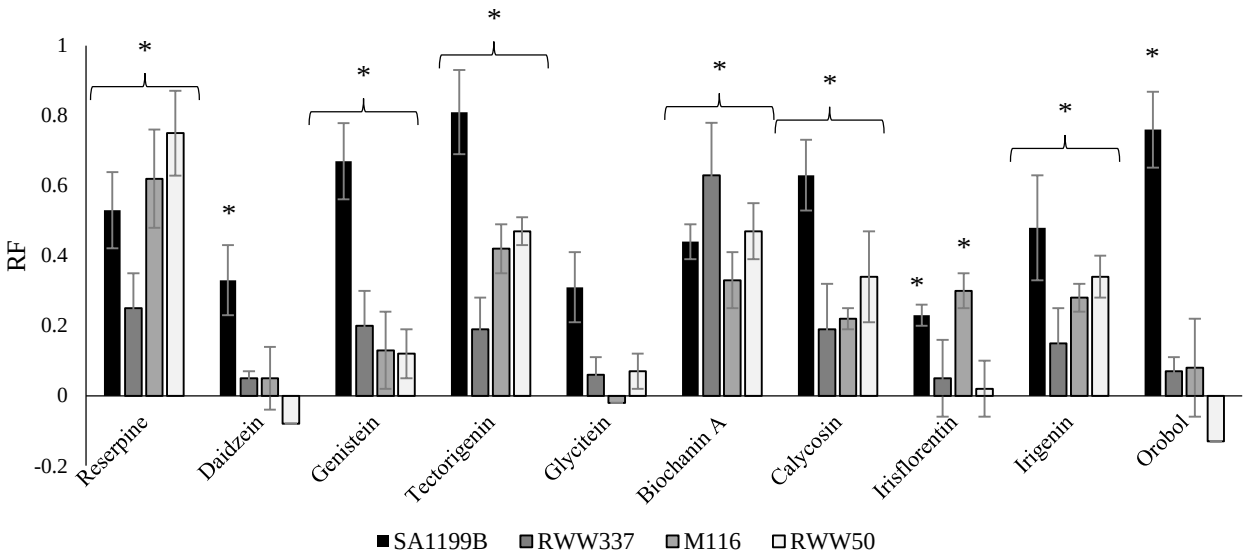


FIGURE 4.18 | Detection of the effect of isoflavonoids on ethidium bromide accumulation for 60 min in SA1199B, ST239 and USA300 strains by fluorometry. Relative fluorescence (RF) values were calculated for the isoflavonoids by fluorometry in SA1199B and MRSA strains: $RF_{assay} = (MFI_{assay} - FI_{control})/ FI_{control}$, where MFI_{assay} was the Maximal Fluorescence Intensity obtained in each 60 min assay and $FI_{control}$ was the correspondent Fluorescence Intensity obtained with the DMSO control at the same time. Reserpine (20 mg l^{-1}) was used as positive EPI control. Bars with (*) accumulated more EtBr than the DMSO control ($P < 0.05$). RFs are averages and standard deviations of three independent assays.

4.5.3. Effect of isoflavonoids on preventing inducible resistance to clindamycin

The inhibition of NorA or other related MDR efflux pumps may explain the potentiation of ciprofloxacin by some isoflavonoids against MRSA and SA1199B strains. However, the potentiation of erythromycin, which was mainly observed against MRSA strains, is still not understood. Staphylococci resist macrolide and lincosamide antibiotics in 2 ways: (1) ribosomal target modification - Erm family of methyltransferases confers resistance to the macrolide-lincosamide-streptogramin type B (MLS_B) antibiotics through the methylation of 23S ribosomal RNA (Fiebelkorn et al., 2003); (2) an active efflux mechanism encoded by *msrA* (Leclercq, 2002). Modification of the ribosomal target confers broad-spectrum resistance to macrolides and lincosamides, whereas efflux or inactivation affect only some of these molecules.

MLS_B resistance is either constitutive or inducible following exposure to a macrolide and can be detected by induction tests ("D-test") using erythromycin and clindamycin disks as shown in FIGURE 4.19. The MRSA strain M116 expressed an erythromycin-inducible methylase ($iMLS_B$ phenotype), as it can be concluded by a positive D-test (FIGURE 4.19-A), RWW50 was resistant to erythromycin but susceptible to clindamycin (MS phenotype, FIGURE 4.19-B); and RWW337 was constitutively resistant to clindamycin ($cMLS_B$ phenotype, FIGURE 4.19-C).

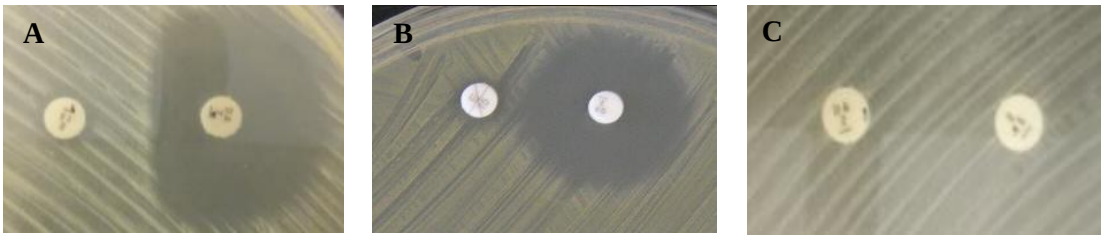


FIGURE 4.19 | D-test for detecting if resistance to the macrolide-lincosamide-streptogramin type B (MLS_B) antibiotics in staphylocci is constitutive or inducible. (A) D-test positive: inducible resistance to clindamycin ($iMLS_B$ phenotype); D-test negative: isolate resistant to erythromycin and susceptible to clindamycin (MS phenotype); (C) constitutive resistance to clindamycin ($cMLS_B$ phenotype).

Thus, macrolide resistance in M116 and RWW337 (ST239) strains is caused by *erm* genes, while in RWW50 it is caused most probably by ABC transporters encoded by *msrA* genes. However, it was previously observed that genistein, biochanin A and tectorigenin can potentiate erythromycin in the three strains; thus one would expect that they are affecting a common trait in the three strains. Nevertheless, the D-test was also performed in MH plates containing the isoflavonoids at 60 mg l^{-1}. The aim was to check if isoflavonoids would be able to inhibit Erm methyltransferases, thus avoiding the methylation of the 23S rRNA, and preventing bacteria to

become resistant to macrolides. Genistein and other dietary polyphenols, such as (-)-epigallocatechin 3-gallate, have been demonstrated to inhibit DNA methyltransferases *in vitro* (Fang et al., 2007). No phenotype changes were observed from control to isoflavonoids plates.

To understand if the isoflavonoids are inhibiting MsrA efflux pump, the activity of erythromycin in the presence of the isoflavonoids was evaluated on the overexpressing MsrA efflux pump *S. aureus* RN4220 strain. Interestingly, genistein and tectorigenin effectively potentiated erythromycin (causing 4-fold reduction of the MIC), while biochanin A promoted only a 2-fold reduction. Additional genetic tests are necessary to characterize the ST239 strains regarding the presence of *msrA* genes.

4.5.4. Effect of isoflavonoids on biofilm control

In this section, it is aimed to assess the effect of isoflavonoids for their activity on biofilm control. Besides genistein and daidzein that were previously tested in *Section 4.4.7*, only the isoflavonoids showing antibiotic potentiation and/or EPI activity were selected (tectorigenin, biochanin A, calycosin, irigenin and irisflorentin). The exposure of biofilms of *S. aureus* CECT 976 to isoflavonoids and antibiotics for 1 and 24 h was evaluated (FIGURE 4.20).

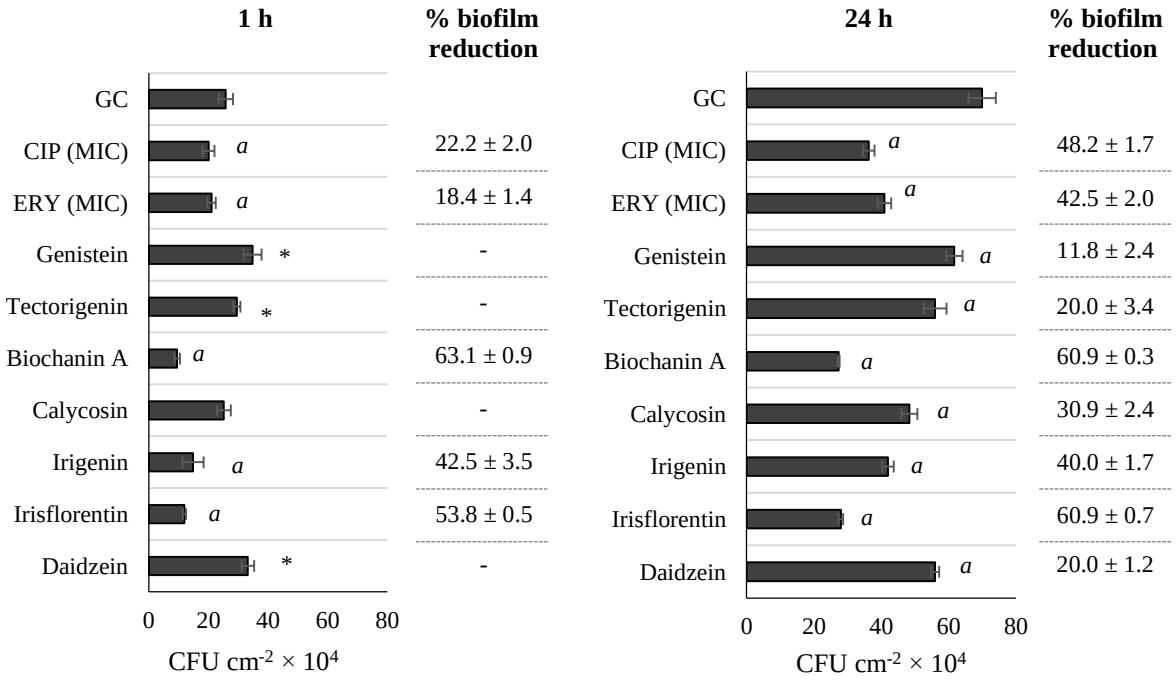


FIGURE 4.20 | CFU cm^{-2} of biofilms of *Staphylococcus aureus* CECT 976 after 1 or 24 h exposure to isoflavonoids (60 mg l^{-1}), ciprofloxacin (CIP) and erythromycin (ERY) at MIC. Percentages of biofilm reduction are shown. (*) when statistically higher than GC, or (a) lower than GC ($P < 0.05$); GC – growth control (5% v v^{-1} DMSO).

Among the seven isoflavonoids tested, biochanin A promoted the highest reductions of *S. aureus* CECT 976 biofilms for both exposure times of 1 and 24 h (63 and 61%, respectively). Irisflorentin was also highlighted, being able to reduce biofilms of CECT 976 strain by 54 and 61%, for 1 and 24 h of exposure, respectively. These biofilm reductions were higher than those obtained with the individual exposure of ciprofloxacin (22 and 48%) and erythromycin (18 and 43%), for exposure times of 1 and 24 h, respectively ($P < 0.05$).

The effect of the isoflavonoids was also assessed against biofilms of SA1199B strain for 1 and 24 h exposure. Additionally, ciprofloxacin at MIC was applied either alone or combined with the isoflavonoids at 60 mg l^{-1} searching for potentiation effects, as obtained against planktonic cells. CFU cm^{-2} of biofilms after exposure to isoflavonoids and/or ciprofloxacin for 1 and 24 h are shown in FIGURE 4.21. Percentages of biofilm reduction after drug treatment are also shown.

Within 1 h of exposure, ciprofloxacin caused 42% reduction on SA1199B biofilms. The highest biofilm reduction was obtained with irisflorentin applied alone at 60 mg l^{-1} (83%). Besides daidzein, which as mentioned before promoted reduction of SA1199B biofilm of 62%, interesting activities were also obtained with irigenin, daidzein and biochanin A at 60 mg l^{-1} (83, 70 and 59%, respectively) applied individually.

Generally, the combination of ciprofloxacin with isoflavonoids was unfavorable, disturbing the anti-biofilm activity of isoflavonoids, excepting for combinations between ciprofloxacin with reserpine and calycosin (biofilm reduction increased from 24- to 58% and from 38- to 51%, respectively). Still, these combinations are not considered synergic according to Monzón et al. (2001).

Within 24 h exposure, ciprofloxacin applied at MIC reduced the amount of biofilm by 94% regardless of the presence of isoflavonoids. Some isoflavonoids applied alone promoted relevant biofilm reduction ($P < 0.05$), being highlighted daidzein, irisflorentin and biochanin A (with 63, 54 and 51 % of biofilm reduction, respectively), which were also quite active when exposed for 1 h. Despite irisflorentin achieved lower biofilm reduction when applied for 24 h ($P < 0.05$) than when exposed for 1 h, daidzein still promoted a similar biofilm reduction in both exposure times ($P > 0.05$). On the contrary, the exposure of biochanin A and irigenin to SA1199B biofilms for 24 h did not achieve so interesting as when they were applied for 1 h. The decreased susceptibility to phytochemicals after a prolonged exposure period (24 h) was generally observed in this study. As previously mentioned, longer incubation periods can allow bacteria to adopt more resistant profiles or a dormant behavior, being thus able to survive (Shafahi and Vafai, 2010).

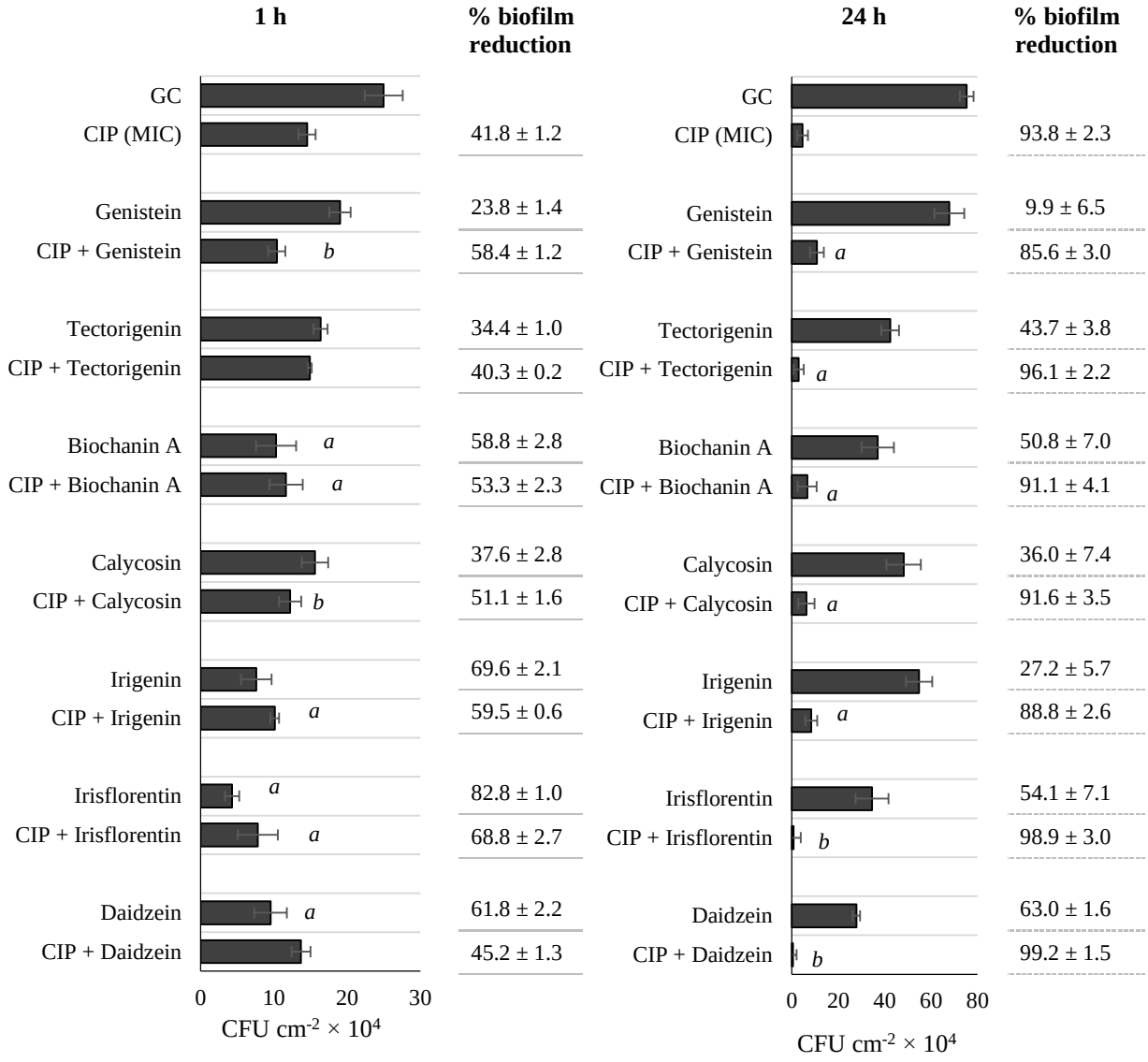


FIGURE 4.21 | CFU cm^{-2} of *Staphylococcus aureus* SA1199B biofilms after 1 h or 24 h exposure to isoflavonoids (60 mg l^{-1}), ciprofloxacin (CIP, MIC), and their combinations. Percentages of biofilm reduction are shown. Only isoflavonoids active as EPIs were tested and were applied at 60 mg l^{-1}. All assays are statistically lower than GC ($P < 0.05$); (*a*) when statistically lower than ciprofloxacin applied alone ($P < 0.05$); and (*b*) than ciprofloxacin and the respective isoflavonoid applied alone ($P < 0.05$). GC – growth control (5% v v^{-1} DMSO).

Additionally, the combinations between ciprofloxacin and daidzein or irisflorentin allowed a higher biofilm reduction than when ciprofloxacin applied alone ($P < 0.05$), causing 99% reduction on CFU cm^{-2}. Likewise, these combinations are not synergic according to Monzón et al. (2001)

4.5.5. Relevant remarks

Isoflavones are well known for their therapeutic properties having anti-inflammatory, estrogenic, anti-estrogenic, anticancer, antibiotic as well as radical scavenging activities (Dixon and Steele, 1999; Hummelova et al., 2015; Pandey and Rizvi, 2009; Russo et al., 2012). They have also long been thought to play a role in plant–microbial interactions as part of the defensive arsenal of the host plant but neither this (Dixon and Steele, 1999) nor the exact relationship between chemical structure and activity are fully understood (Osawa et al., 1992; Pandey and Rizvi, 2009; Wu et al., 2013).

Only three compounds revealed antimicrobial activity against all *S. aureus* strains, neobavaisoflavone (MIC of 20 mg l^{-1}), corylifol A (MIC between 0.06 - 2.5 mg l^{-1}), which both have a lipophilic prenyl group attached to the B-ring, and orobol (MIC between 60 - 120 mg l^{-1}).

Besides genistein, biochanin A (a structurally similar isoflavonoid) and tectorigenin were able to potentiate ciprofloxacin and erythromycin (2 to 8-fold MIC reduction) against MRSA strains. Besides studies previously described about genistein potentiating effects, a range of MIC values between 64-512 mg l^{-1} against 12 *S. aureus* strains have been reported for biochanin A, which also showed synergism with ciprofloxacin (Liu et al., 2011). A MIC of 125 mg l^{-1} against MRSA strains has been reported for tectorigenin (Joung et al., 2014). The possible mode of action may be based either on DNA topoisomerases inhibition, as reported for genistein and biochanin A (Azuma et al., 1995). Considering their structural similarity, it can be assumed that tectorigenin could also have a similar effect. Also, tectorigenin seems to affect membrane permeability and ABC transporters (Joung et al., 2014). As previously mentioned, the inhibition of DNA topoisomerases could explain both potentiation effects with ciprofloxacin (having the same target it could either benefit as detected in this study, or disturb the activity of ciprofloxacin) and erythromycin (by a multi-target effect). However, it does not explain why the potentiation is notorious against MRSA strains and not against susceptible strains. Thus, it is expected that other targets may be involved, also considering the reactivity of these phenolic compounds and their ability to complex with several extracellular and soluble proteins. In line with this idea, it has been suggested that the mechanism of flavonoid antibacterial activity can based on their ability to inactivate cell envelope transport proteins (Mierziak et al., 2014).

In our study, seven of the tested non-antimicrobial active isoflavonoids were able to potentiate ciprofloxacin activity (with 4- to 8-fold reductions of the MIC value) against the resistant SA1199B, in decreasing order of activity these were: genistein, biochanin A, tectorigenin, daidzein, calycosin, irigenin and irisflorentin. Also the active antimicrobial neobavaisoflavone, showed synergism, with a FICI of 0.5 with ciprofloxacin against SA1199B.

Genistein and biochanin A were the most active of all the tested isoflavonoids, potentiating ciprofloxacin and erythromycin against resistant *S. aureus* strains. A comparison of their structures suggests that the two meta-hydroxyl groups in the A-ring present in these compounds, are a key feature for this antibiotic-potentiation. Biochanin A has the same substitution pattern on the A-ring, but the hydroxyl groups in the B-ring is replaced by a methoxy group. Orobol which also has the same A-ring substitution but two *ortho*-hydroxyl groups in the B-ring, shows antibacterial activity. Daidzein which lacks the 5-hydroxyl group but is otherwise identical to genistein is also less active. It seems that synergistic properties might parallel the estrogenic activity (Miyazaki, 2004). Furthermore, all compounds in which the C-7 hydroxyl group was glycosylated were inactive. Hummelova et al. (2015) coincides partially with our observations, concluding that the isoflavonoids with strongest *S. aureus* inhibitory effect were *ortho*-dihydroxyisoflavones. Other studies showed that 5,7-dihydroxylation of the flavonoid A ring was important for anti-MRSA activity (Alcaráz et al., 2000; Hummelova et al., 2015; Tsuchiya et al., 1996) and monomethoxy B-ring derivatives were generally found to be more active (antibiotic synergy) than the disubstituted B-ring derivatives (Wang et al., 2014).

The next question to answer is the possible mode of action of the synergistic activity. The uptake and efflux of the antibiotics are decisive for this activity. To measure the effect on the uptake and efflux, the accumulation of the highly fluorescent EtBr in the bacteria was measured in the presence of the isoflavonoids. The seven isoflavonoids that potentiated ciprofloxacin against SA1199B were the ones that most increased the accumulation of EtBr. Against the MRSA strains, biochanin A, genistein and tectorigenin were the most active EPIs, similarly to reserpine. Calycosin and irigenin had lower RFs, but still improved EtBr accumulation as compared to the control in all MRSA strains. The effects obtained with these five isoflavonoids active as EPIs were successfully confirmed by flow cytometry against SA1199B. There is clear evidence that inhibition of MDR transporters, and in particular NorA, do play a role in resistance.

Accumulation is the net result of uptake and efflux. Transport in cells occurs in diverse ways, including non-selective diffusion, and more or less selective transporters for uptake or efflux. A higher uptake can thus be related to each of the three processes. Only in the case of efflux there is clear evidence of the role of MDR-like transporters, and in particular NorA, in resistance. Cell membrane damage has also been reported in connection with the isoflavonoids (Joung et al., 2014; Mierziak et al., 2014). Considering the results obtained, isoflavonoids seem to induce a fast uptake, but over time the levels in the cell reverted to those of the controls, meaning that an equilibrium was reached after a certain time. This means that the synergistic effect may have other modes of action as well.

Additional experiments were conducted in order to understand the potentiation of erythromycin by genistein, tectorigenin and biochanin A against ST239 and USA300 strains. The resistance to macrolide can be mediated by *msr*A gene coding for efflux mechanism or via *erm* gene encoding for enzymes that confer inducible or constitutive resistance to MLS_B antibiotics. There are reports of some compounds that bind weakly to ErmAM (Hajduk et al., 1999) and ErmC (Clancy et al., 1995) methyltransferases, thus inhibiting the Erm-mediated methylation of rRNA. These studies suggest that Erm inhibitors used in combination with a broad-spectrum macrolide antibiotic could be useful for the treatment of infections caused by MLS-resistant pathogenic bacteria. Despite genistein has been demonstrated to inhibit DNA methyltransferases *in vitro* (Fang et al., 2007), inhibition of Erm methyltransferases by isoflavonoids, which were causing macrolide resistance on ST239 strains, was not detected by D-test. So, inhibition of MsrA efflux pump could be a reasonable explanation if ST239 is also expressing these efflux pumps. By testing the MrsA overexpressing strain RN4220 it was possible to observe a potentiating/additive effect of genistein, tectorigenin and biochanin A with erythromycin. However, it seems that while the prevalence of *erm*-mediated macrolide resistance is notorious among MRSA strains, *msrA* genes are not so predominant: Moosavian et al. (2014) reported that *ermA* and *ermC* are the most common among 124 *S. aureus* isolates, but *ermB* and *msrA* were never detected; Schmitz et al. (2000) showed that the most prevalent resistance gene in 851 *S. aureus* isolates was *ermA* (67%), followed by *ermC* (23%) and *msrA/B* (6%). Yet, further studies would be necessary to fully understand this phenomenon.

The mechanism behind transport inhibition is unknown. Inhibition of several MDR efflux pumps may be the explanation to these effects promoted by the isoflavonoids. In fact, there are reports of compounds that can inhibit more than one efflux pump, such as reserpine. For example, Gibbons et al. (2003a) reported that GG918 (drug specifically developed for overcoming MDR phenotype) and reserpine potentiated norfloxacin and ciprofloxacin in a fluoroquinolone-susceptible *S. aureus* strain and in strains expressing the MsrA and TetK proteins, which mediate efflux-related resistance to macrolides and tetracyclines, respectively. NorA appears to export a surprisingly large variety of structurally unrelated drugs, such as fluoroquinolones, EtBr, cetrimide, benzalkonium chloride, tetraphenylphosphonium bromide and acriflavine (Deng et al., 2012). So, the explanation may lie in the low substrate specificity of these systems (Markham et al., 1999). Nevertheless, it seems that the most active agents belong to the families of compounds possessing conjugated double bonds (Handzlik et al., 2013). Finally, there is still a lack of consensual methodological approaches that guarantee appropriate address of all these issues (Costa et al., 2013). The use of molecular approaches would be necessary to fully understand the function that each individual efflux pump undertakes in the bacterial cell response to antimicrobial compounds and how they are being affected by the presence of EPIs.

Currently, increasing evidence has elucidated that efflux pump systems are not merely pumps involved in the transport of drugs or other toxic substances out of the cells but they have been also implicated in quorum-sensing regulation and the subsequent expression of genes responsible for virulence and biofilm formation (Beceiro et al., 2013; Sun et al., 2014). For example, mutants of *Salmonella typhimurium* that lack TolC or AcrB were compromised in their ability to form biofilms, but not in the presence of efflux pump inhibitors (Baugh et al., 2014).

From this study, irisflorentin is highlighted for its anti-biofilm activity being able to reduce biofilms of *S. aureus* CECT 976 and SA1199B by 54 and 83% when applied for 1 h, and by 61 and 54%, when applied for 24 h, respectively. Daidzein was able to cause biofilm reductions of 62 and 63% when applied for 1 and 24 h, respectively against *S. aureus* SA1199B, but no significant effects were obtained against CECT 976 strain. Biochanin A also promoted interesting effects on biofilms, being able to reduce CECT 976 biofilms by 63 and 61%, and SA1199B biofilms by 59 and 51%, when applied for 1 and 24 h, respectively.

Testing twenty-two structurally different isoflavonoids allowed to detect a relationship between the potentiation of ciprofloxacin and inhibition of NorA efflux pump. Also, it was shown that antimicrobial and antibiotic-potentiation properties of isoflavonoids are highly structure-dependent. Further questions arise, namely whether the refereed isoflavonoids could also inhibit other similar efflux pumps, also possibly in other organisms. Additionally, inhibition of NorA does not explain the potentiation observed for erythromycin; thus interference with other cellular efflux pumps, mechanisms and/or targets is most probably occurring and should be subject of further research. The concept of restoring and enhancing the therapeutic value of antimicrobials by employing EPIs is a formidable challenge. The development of combinations of fluoroquinolone antibiotics with a transporter inhibitor appears to be a feasible alternative to the discovery of “rare” compounds that are poorly recognized by multidrug transporters. However, this is not yet at a therapeutic stage and a number of methodological gaps should be overcome for the clinical implementation of EPIs.

4.6. TASK 6. THE POTENTIAL OF *BUXUS SEMPERVIRENS* FOR ANTIBIOTIC-POTENTIATING ACTIVITY: ISOLATION AND IDENTIFICATION OF ANTIBIOTIC ADJUVANTS

Abstract

Among the tested plants, *Buxus sempervirens* showed to potentiate mostly ciprofloxacin, erythromycin and tetracycline but also showed an additive effect when combined with β-lactams. In this study, *B. sempervirens* (1 g l^{-1}) was able to cause 88% reduction of 24 h-old biofilms of *S. aureus* CECT 976 within 1 h of exposure. Further phytochemical investigation of *B. sempervirens* allowed to identify one candidate, betulinic acid. This and other similar terpernoids, lupeol, betulin, hederagenin, ursolic acid and oleanolic acid, were tested for antibacterial and antibiotic-potentiating activities. Among the compounds tested, oleanolic acid and ursolic acid were highlighted, showing MIC of 62.5 and 15.6 mg l^{-1}, respectively, against *S. aureus*. Additionally, oleanolic acid showed synergism when combined with tetracycline and erythromycin and biofilm reductions of 70, 81 and 85% when applied at ½ MIC, MIC and 2 × MIC, respectively.

4.6.1. Effect of *Buxus sempervirens* methanolic extract on biofilm control

Any effective strategy able to impair biofilm formation or disturb, weaken or disperse its structure is urgently needed and for long desired. In this study, the MeOH extract of *Buxus sempervirens* was analysed for its ability to control biofilms within 1 h of contact of the susceptible strain *S. aureus* CECT 976, against which the combinations with *B. sempervirens* were generally more effective (as previously shown in *Section 4.3.2*, TABLE 4.6).

The MeOH extract of *B. sempervirens* was evaluated against CECT 976 24 h-old biofilms at several concentrations (FIGURE 4.22). It was possible to observe an overall concentration-dependent effect, and increasing concentrations of *B. sempervirens* extract caused higher biofilm removal percentages ($P < 0.05$), except for applications at 0.25 g l^{-1} and 5 g l^{-1}, which did not show improvement over the preceding concentrations of 0.1 and 1 g l^{-1}, respectively ($P > 0.05$). The minimal concentration causing potentiation with planktonic cells (1 g l^{-1}) was the one causing the highest biofilm reduction (88%).

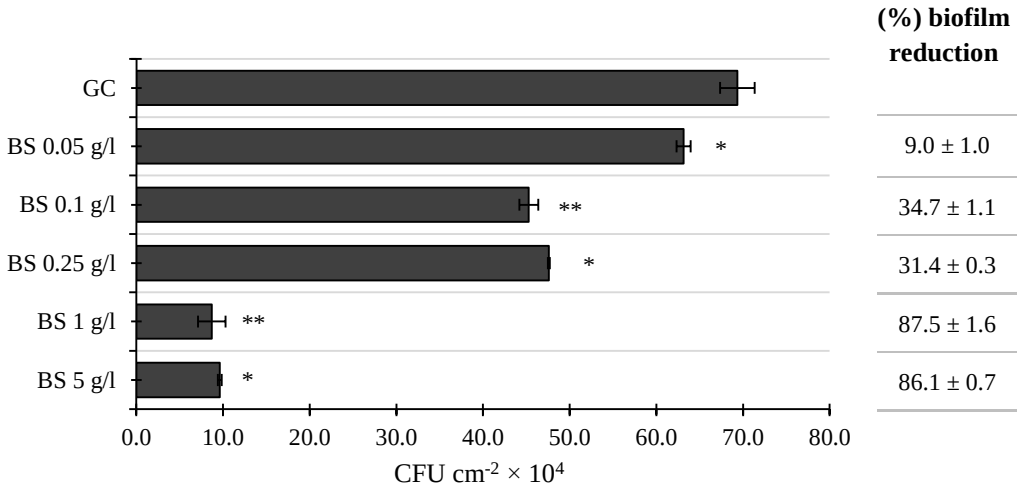


FIGURE 4.22 | CFU cm^{-2} of *Staphylococcus aureus* CECT 976 biofilms after 1 h exposure to methanolic extract of *Buxus sempervirens* (0.05, 0.1, 0.25, 1 and 5 g l^{-1}). Biofilm reductions (%) are shown for each assay. (*) when statistically lower than GC and (**) than the preceding concentration ($P < 0.05$). GC – growth control (5% v v^{-1} DMSO); BS – *B. sempervirens*.

Additionally, combinations between *B. sempervirens* MeOH extract with ciprofloxacin, tetracycline and erythromycin against 24 h-old biofilms were assessed (FIGURE 4.23). Antibiotics were applied at MIC and 50 × MIC and the extract of *B. sempervirens* was applied at the concentration causing highest biofilm reduction (1 g l^{-1}).

Concerning the single activity of the antibiotics at MIC, ciprofloxacin, tetracycline and erythromycin promoted biofilm reductions of 38, 31 and 21%, respectively. Both ciprofloxacin and erythromycin applied at 50 × MIC did not show any improvement over application at MIC ($P > 0.05$). Tetracycline at 50 × MIC did not cause ant biofilm reduction at all (similar CFU cm^{-2} values to the growth control, $P > 0.05$). This supports the concept of how bacteria are much more protected within a biofilm.

Comparing individual activities, *B. sempervirens* MeOH extract surprisingly achieved the best ability to control *S. aureus* CECT 976 biofilms within 1 h of application, even not showing antibacterial activity at this concentration. According to Monzón et al. (2001), it is possible to classify a combination between a plant extract/phytochemical and an antibiotic as synergic if the $\log_{10}$ reduction CFU cm^{-2} caused by the combination is significantly higher ($P < 0.05$) than the sum of reductions of individual treatments. In this case, the application of antibiotics at MIC did not promote any significant improvement over the activity of the plant extract alone ($P > 0.05$).

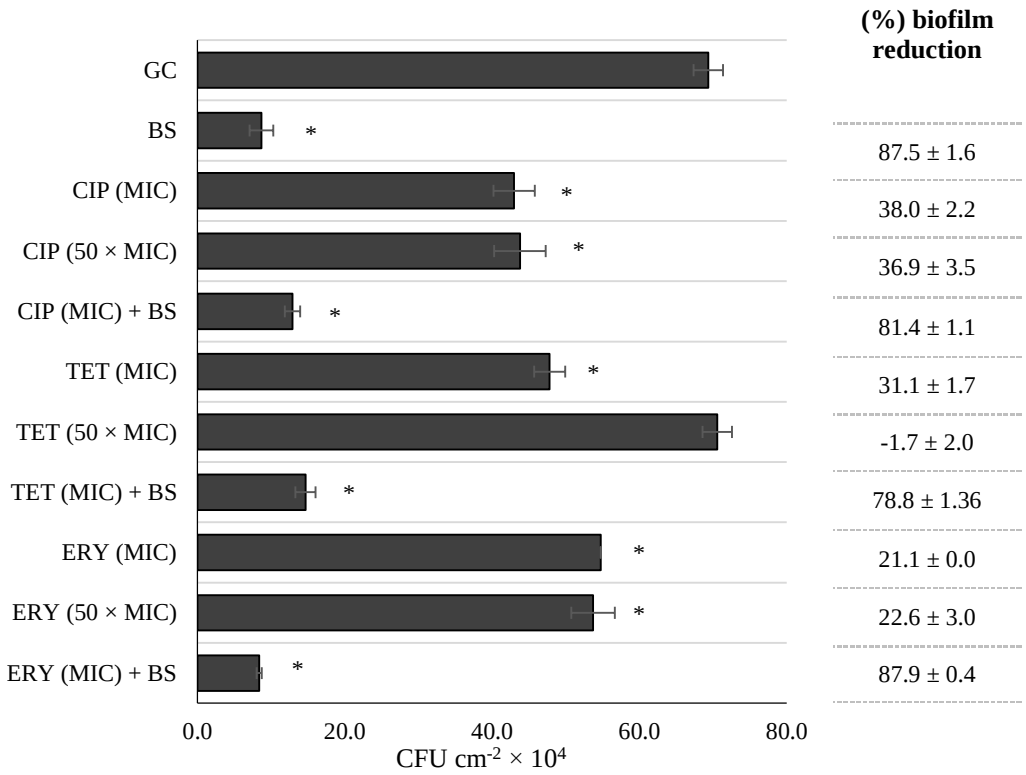


FIGURE 4.23 | CFU cm^{-2} of *Staphylococcus aureus* CECT 976 biofilms after 1 h exposure to ciprofloxacin (CIP), tetracycline (TET) and erythromycin (ERY) at MIC and 50 × MIC, and combined with the methanolic extract of *Buxus sempervirens* (BS) at 1 g l^{-1}. Biofilm reductions (%) are shown. (*) when statistically different from GC ($P < 0.05$). GC – growth control (5% v v^{-1} DMSO).

4.6.2. Isolation of bioactive compounds from Buxus *sempervirens*

Afterwards, *B. sempervirens* MeOH extract was submitted to fractionation for the identification of the bioactive compounds. Among the subfractions obtained from the *n*-BuOH fraction of *B. sempervirens*, through silica gel column, F1 and F2 were differentiated, showing antibiotic-potentiating activity (0.5 g l^{-1}). Analysing the ^{1}H NMR for all the eight fractions obtained, it was possible to compare the spectra of the active fractions F1 and F2 with the non-active ones. Bearing in mind the similar activity, some signals were found in both fractions (FIGURE 4.24-A), which were not found in the non-active ones. The identification was carried out using an in-house library of NMR data of common metabolites and based on characteristic methyl and olefinic signals it was possible to identify betulinic acid (FIGURE 4.24-B) as a major component together with oleanane and ursane type terpenoids.

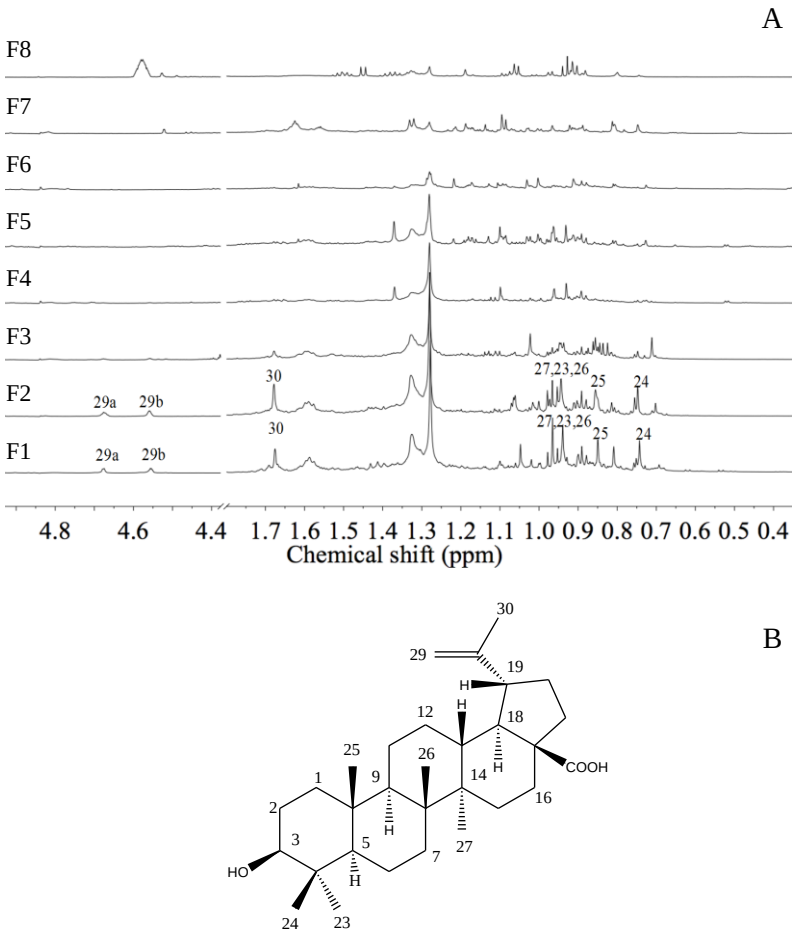


FIGURE 4.24 | ^{1}H NMR spectra (0.4 – 5.0 ppm) of the subfractions obtained from n-BuOH fraction of *B. sempervirens* (A); the numbering in the fractions is related to the ^{1}H assignments of betulinic acid (B), which is only observed in the active fractions F1 and F2.

4.6.3. Evaluation of triterpenoids as antibiotic potentiators

Betulinic acid and other similar terpenoids, – lupeol, betulin, hederagenin, ursolic acid and oleanolic acid – were tested for their antibacterial activity by microdilution technique as previously explained. Pentacyclic triterpenoids α-amyrin, betulinic acid and betulinaldehyde, and other related triterpenes such as imberbic acid, oleanolic acid, ursolic acid, ulsolic acid, rotundic acid and zeylasteral have been reported to possess antimicrobial activity against many bacterial species, especially Gram-positive, but also against Gram-negative (Chung et al., 2011; Kurek et al., 2009). In this study, only oleanolic acid and ursolic acid showed a MIC up to 120 mg l^{-1}, which was 62.5 and 15.6 mg l^{-1}, respectively, against *S. aureus* CECT976.

After MIC determination, these terpenoids were evaluated in combination with antibiotics searching for a synergistic activity through checkerboard method (TABLE 4.15).

TABLE 4.15 | Minimal inhibitory concentrations (MIC)-fold reductions obtained with the combination between oleanolic acid and ursolic acid and the antibiotics. Fractional inhibitory concentration index (FICI) values were determined for each combination. Classification of the combinations is given as synergy (S) or indifference (I)

	Antibiotic - Oleanolic acid			**Antibiotic - Ursolic acid**		
	MIC fold reduction	**MIC fold reduction**	**FICI***	**MIC fold reduction**	**MIC fold reduction**	**FICI***
AMP	2	2	1.0 (I)	2	2	1.0 (I)
CIP	4	2	0.75 (I)	8	2	0.63 (I)
TET	4	4	**0.5 (S)**	4	16	**0.31 (S)**
ERY	8	4	**0.38 (S)**	2	2	1.0 (I)

A FICI value of ≤ 0.5 (in bold) was interpreted as synergy, > 4 as antagonism and > 0.5 – 4 as indifferent. FIC: fractional inhibitory concentration; FICI: FIC index. AMP: ampicillin; CIP: ciprofloxacin, TET: tetracycline; ERY: erythromycin

Analysing the FICI values, it is possible to detect synergism only between oleanolic acid with erythromycin and tetracycline (FICI ≤ 0.5) and between ursolic acid and tetracycline (FICI = 0.31) against *S. aureus* CECT 976. Similarly, Fontanay et al. (2008) found a MIC for ursolic acid and oleanolic acid of 8 and 32-64 mg l^{-1} against *S. aureus* ATCC25923 and ATCC29213 but not for betulinic acid. No MIC was found against one MRSA strain (Fontanay et al., 2008). Contrarily, in other study, oleanolic acid was reported to inhibit MRSA with a MIC between 16 and 128 mg l^{-1} (Kim et al., 2012) and to synergize with ampicillin against *S. aureus* (Kurek et al., 2012). Chung et al. (2011) showed that betulinic acid and similar compounds, α-amyrin and betulinaldehyde, inhibited MSSA and MRSA (MIC between 64 and 512 mg l^{-1}), and synergized with methicillin and vancomycin against the same strains. Therefore, it seems that the antibacterial and synergistic activities of triterpenoids vary widely, not only between susceptibility methods, but also between strains belonging to the same species. Considering the low activity displayed by betulinic acid, which was found in the active fractions of *B. sempervirens*, other triterpenoids also existing in this plant, as oleanane and ursane type terpenoids may contribute to the antibiotic-potentiation and anti-biofilm effects displayed by this plant.

4.6.4. Evaluation of triterpenoids on biofilm control

Plenty studies were initiated to identify the cellular targets and molecular mechanisms of triterpenoids. Besides their influence on bacterial gene expression (Shin and Park, 2015), cell autolysis and peptidoglycan turnover (Kurek et al., 2009), oleanolic acid and related compounds also seem to affect the formation and maintenance of biofilms (Wolska et al., 2010). Indeed,

terpenes are believed to influence the fatty acid composition of the cell membrane, and thus cell hydrophobicity, which can lead to biofilm eradication (Perumal and Mahmud, 2013). To confirm this, oleanolic acid, which caused the best antibiotic-potentiation in this study, was evaluated for its anti-biofilm activity against *S. aureus* CECT 976 biofilms. FIGURE 4.25 shows the number of CFU cm^{-2} of biofilms obtained after 1 h exposure to oleanolic acid at ½ MIC, MIC and 2 × MIC as well as with antibiotics at MIC, individually and in combination.

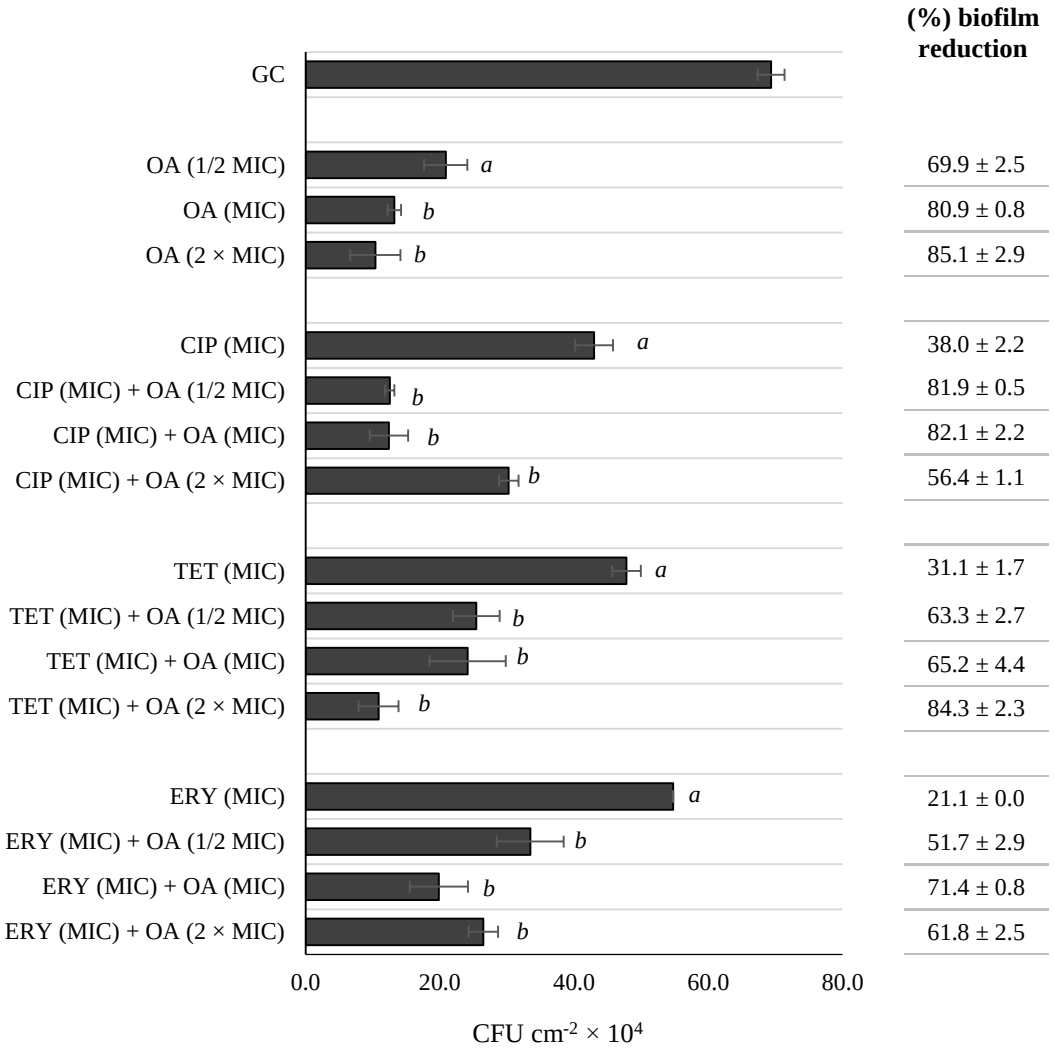


FIGURE 4.25 | CFU cm^{-2} of *Staphylococcus aureus* CECT 976 biofilms after 1 h exposure to oleanolic acid (OA) applied at ½ MIC, MIC or 2 × MIC, individually and combined with ciprofloxacin (CIP), tetracycline (TET) and erythromycin (ERY) at their MICs. Biofilm reductions (%) are shown. (*a*) when statistically different from GC ($P < 0.05$); (*b*) when statistically different from the respective antibiotic applied individually. GC – growth control (5% v v^{-1} DMSO).

Oleanolic acid applied at ½ MIC, MIC and 2 × MIC caused high biofilm CFU reduction, 70, 81 and 85%, respectively. The combination between the antibiotics and oleanolic acid never promoted higher biofilm reductions than those obtained with oleanolic acid alone. The exception was the combination of ciprofloxacin (MIC) with oleanolic acid (½ MIC), that showed to be significantly than oleanolic acid alone at the same concentration (82%, $P < 0.05$). However, diverse combinations achieved worst biofilm reductions than those obtained with oleanolic acid alone ($P < 0.05$): between oleanolic acid and erythromycin, between oleanolic acid (MIC) and tetracycline (MIC) and between oleanolic acid (2 × MIC) and ciprofloxacin (MIC).

One could expect that by combining antibiotics with a possible biofilm inhibitor, the outcome would be an improved therapeutic benefit. Nevertheless, probably by applying the combination in a preliminary stage of bacteria adhesion/biofilm formation, the combinations would be more effective, which would explain the potentiation observed.

4.6.5. Relevant remarks

Although it has been used medicinally in the past as a sedative and to treat syphilis, box (*B. sempervirens*) is very rarely used in modern herbalism. Still, the leaves possess a serious of therapeutic properties, and were inclusive used as a quinine substitute in the treatment of malaria (PFAF, 2016). In this study, it was observed that the methanolic extract of *B. sempervirens* (1 g l^{-1}) showed an 88% of *S. aureus* CECT 976 biofilm reduction when applied for 1 h. This effect on *S. aureus* biofilms may be the reason for the antibiotic-potentiation previously observed for this plant extract (*Section 4.3.2*, TABLE 4.6).

Further fractionation of *B. sempervirens* allowed to suggest that betulinic acid together with oleanane and ursane type terpenoids may be responsible for the activity of the plant. Triterpenoids are widely distributed in the plant kingdom and their therapeutic activities (such as antibacterial, antiviral, antiulcer, anti-inflammatory and anticancer) have been described in numerous reports. Betulinic acid showed weak antibacterial and antibiotic-potentiation activities. Considering this, other triterpenoids also existing in this plant, as reported for example by Abramson et al. (1973), or other sterols, alkaloids and anthocyanins that are typical of *Buxus* spp. (Orhan et al., 2012), might synergistically contribute to the antibiotic-potentiation and anti-biofilm effects displayed by this plant. Further isolation of the active fractions of the plant towards the identification of all the involved metabolites is apparently necessary.

Meanwhile, six structurally similar triterpenoids were studied for antibacterial and antibiotic-potentiation activities. Among them, oleanolic acid and ursolic acid were highlighted for showing a MIC of 62.5 and 15.6 mg l^{-1}, respectively against *S. aureus* CECT976 and synergism when

combined with erythromycin (for both) and tetracycline (only for oleanolic acid) against the same strain. Additionally, oleanolic acid was found to remove biofilm CFU by 70, 81 and 85%, when applied at ½ MIC, MIC and 2 × MIC, respectively. Kurek et al. (2012) also found synergistic antibacterial effects of oleanolic acid in combination with ampicillin against biofilms of *S. aureus* and *S. epidermidis*, and with oxacillin against biofilms of *L. monocytogenes*, *S. epidermis* and *S. aureus*. Ursolic acid was found to inhibit biofilm formation of MRSA by reducing amino-acids metabolism and expression of adhesins (Qin et al., 2014), to induce genes related to chemotaxis, mobility and heat shock response, and to repress genes that have functions in cysteine synthesis and sulfur metabolism (Ren et al., 2005).

Restoring the activity of antibiotics that were already accepted and approved for clinical safety aspects, minimal toxicity and side effects, could thereby potentially reduce the costs associated to drug preclinical and clinical development. This strategy is possible by combining antibiotic with adjuvants that are able to inhibit drug-resistance mechanisms expressed by the pathogens. This study highlights the potential of *B. sempervirens* extract, and particularly of triterpenoids for their relevant ability to act against *S. aureus* biofilms and opens new perspectives for further research.

Chapter

5

CONCLUDING REMARKS AND PERSPECTIVES FOR FURTHER RESEARCH

In this section, the main conclusions obtained with this thesis are highlighted and summarized. An overview of all the work developed is discussed. Some main difficulties and challenges that arised during this project are described. Perspectives and suggestions for further research are also identified and provided.

5.1. GENERAL CONCLUSIONS

Over the last decade there has been a resurgence of interest in the search for products that will restore the activity of licensed antimicrobial agents. It is known by now that plants do not produce individual compounds as their defense mechanisms, but they generate complex defense systems involving a myriad of metabolites. Indeed, plants have been fighting against the resistance mechanisms developed by their attackers for millions of years ago and one could make use of their potential.

The present thesis showed that the prospects of finding useful plant-derived products are enormous. Products that interfere with efflux of antibiotics from the cell or other resistance mechanisms may be easily found in nature. Within this project, 29 plants and 41 phytochemicals were assessed for their antibiotic-potentiating effects. Some were highlighted and selected for further investigation and open new perspectives for an antibiotic-combined therapy. Such phytochemicals can serve as scaffolds for the design of new and more attractive compounds that could be applied in clinical setting towards the treatment of *S. aureus* and MRSA. Some main achievements reached within this thesis will be highlighted in this section.

- The use of two or more methods to assess antibiotic-potentiation allowed to overcome and circumvent the limitations of each method and to characterize drug potentiation with more confidence. Disk-diffusion method raised as an excellent strategy and showed good agreement with checkerboard and Etest methods in detecting potentiation for ciprofloxacin, tetracycline and erythromycin. This method is suitable especially when dealing with plant extracts and non-purified fractions. Checkerboard can be easily performed and allow to test several concentrations at the same time. Furthermore, characterization of synergy by this method is well established among the scientific community.

- From the initial screening of several plant alkaloids and flavonoids, reserpine, pyrrolidine, quinine, morin and quercetin were highlighted as adjuvants of antimicrobial therapy against planktonic *S. aureus*. Reserpine is already known as an EPI for the NorA and TetK efflux pumps. This study supports the results already obtained by other authors. Pyrrolidine showed a capacity for potentiating erythromycin and ciprofloxacin against some *S. aureus* strains. Quinine would be a viable option if not for the high toxicity of this compound. Nevertheless, it had a significant effect on ciprofloxacin against SA1199B and MSSA strains. Morin and quercetin increased the activity of ciprofloxacin against *S. aureus* CECT 976 and SA1199B and tetracycline against *S. aureus* CECT 976 and all MRSA and MSSA strains.

- Regarding the effects of these phytochemicals against sessile *S. aureus*, morin caused the highest biofilm reduction when applied for 1 h and also inhibited biofilm formation. Pyrrolidine had a significant effect against biofilms for 1 h exposure, while quercetin was highlighted for preventing biofilm formation. Several significant synergic combinations of antibiotics with the phytochemicals were obtained being highlighted the combinations between ciprofloxacin and quinine to control SA1199B biofilms (within 24 h exposure with $\log_{10}$ CFU cm^{-2} reduction of 2.1) and between ciprofloxacin with reserpine to prevent SA1199B biofilm formation ($\log_{10}$ CFU cm^{-2} reduction of 3.2). These effects depend on the incubation time. Additionally, quinine and morin were successful in reducing antibiotic tolerance of SA1199B strain to ciprofloxacin within 15 days of exposure, the first probably due to an EPI effect (it increased accumulation of EtBr) and the second for acting as a biofilm inhibitor. In this study, it was also showed that quercetin had EPI activity on strain SA1199B.

- Among twenty-nine different plant species, four were found to have antibacterial activity, (*E. globulus, C. sativa, A. eupatoria* and *F. excelsior* with MICs of 0.125-0.5, 0.5-1.0, 1.0-2.0, and 2.0-4.0 g l^{-1}, respectively) being in agreement with their traditional uses, and nine (*A. dealbata, P. communis, P. avium, P. domestica, P. persica, C. nigra, E. cannabinum, F. carica, B. sempervirens*) were able to potentiate antibiotic activity, especially ciprofloxacin, tetracycline and erythromycin. *C. striatus* was highlighted in this initial screening for promoting potentiation of ciprofloxacin and erythromycin even against one MRSA strain (MJMC001). *B. sempervirens* seems to act as a general potentiator for several antibiotics. For these reasons, both plants were selected for further study focusing on the isolation and characterization of the main metabolites responsible for these antibiotic-potentiating activities.

- NMR-based metabolomics was proven to be a powerful tool to identify compounds that are related to an activity. With this approach, it was proven that even using one single plant, it was possible to obtain a variety of different extracts and activities. Submitting all this data to multivariate data analysis allowed the identification of all signals related to the activity. Three non-antibacterial compounds (up to 120 mg l^{-1}) were positively correlated with an antibacterial-potentiating activity - apigenin, genistein and 2'-hydroxygenistein. Both apigenin and its analogue genistein caused 2-8 fold reductions of ciprofloxacin and erythromycin MICs against ST239 and USA300-MRSA strains. Suggestions were made that this activity could be associated to inhibition of DNA and RNA synthesis in bacteria and influence DNA gyrase activity (Mierziak et al., 2014), inhibition of tyrosine kinase (Verdrengh et al., 2004) or topoisomerase II (Azuma et al., 1995). However, further studies

would be necessary to demonstrate such inhibition. One antibacterial compound was also found from *C. striatus* leaf extract - luteolin - with MICs between 30 and 120 mg l^{-1} against 7 *S. aureus* strains, including the ST239 and USA300 MRSA strains. Other isoflavonoid, daidzein, was found to potentiate ciprofloxacin against SA1199B possibly due to EPI activity. The same EPI effect was also confirmed for genistein, including for the other MRSA strains. Additionally, daidzein showed to reduce SA1199B biofilms by 62 and 63 % when exposed for 1 and 24 h, respectively, probably due to its EPI activity.

- Testing twenty-two structurally showed that antimicrobial and antibiotic-potentiation properties of isoflavonoids are highly structure-dependent. Besides genistein, tectorigenin and biochanin A were highlighted being able to potentiate ciprofloxacin and erythromycin by 2-8 fold, especially against ST239 and USA300 MRSA strains. The hydroxyl group in position 5` seems to be correlated with the activity. A relationship between the potentiation of ciprofloxacin and inhibition of NorA efflux pump was also detected. Further experiments are required to understand the possible interactions of isoflavonoids namely whether these compounds can also interact with other efflux pumps, even in other organisms. Additionally, irisflorentin and biochanin A showed significant effects on *S. aureus* biofilm control.

- *Buxus sempervirens* leaf methanolic extract showed to potentiate mostly ciprofloxacin, erythromycin, but also tetracycline, and caused an additive effect on β-lactam antibiotics activity. *B. sempervirens* (1 g l^{-1}) was able to cause an 88% reduction of 24 h-old biofilms of *S. aureus* CECT 976 within 1 h of exposure. Further phytochemical investigation of *B. sempervirens* active fractions allowed to identify one major compound, betulinic acid. Among this and other five similar triterpenoids, only oleanolic acid and ursolic acid showed antibacterial activity (MIC of 62.5 and 15.6 mg l^{-1}, respectively) against *S. aureus* CECT 976 and synergy when combined with tetracycline (for both) and erythromycin (only for oleanolic acid). Additionally, oleanolic acid promoted biofilm reductions of 70, 81 and 85% when applied at ½ MIC, MIC and 2 × MIC, respectively.

The continued evolution and spread of multiple antibiotic resistance in human pathogens is an alarming clinical challenge. It is clear from the past two decades of effort that antibiotics that are highly effective, safe and broad spectrum are incredibly difficult to find. Consequently, one must ask whether the current medical practices and ideas about infection control are appropriate in the resistance era. It is important to highlight that researchers should continue investigating the synergistic capacity of plant extracts or other natural products, independently of the antimicrobial activity they might have alone. Thus, a more integrated approach is necessary to fully and deeply

evaluate the defense features of each plant. A NMR-based metabolomics strategy, as it was applied in this study, proved to be a valuable and strong tool to reveal synergistic activities between phytochemicals from the same plant and allowed the identification of interesting metabolites to be applied in the flight of MRSA strains.

The presence of MDR pumps in bacteria is certainly not just for drug resistance. However, understanding the physiological roles of the MDR pumps may continue to be rather difficult as the functions of these pumps are often involved in a complex, and overlapping network of reactions in the bacterial cell (Li and Nikaido, 2009). Understanding how EPIs block the transport of antibacterials is critical for designing and optimizing EPIs. The effect of efflux pumps needs to be considered in the design of future antibiotics and the role of inhibitors assessed in order to maximize the efficacy of current and future antibiotics. Also, efflux inhibition can be a promising anti-biofilm strategy.

This study reveals the great potential for the application of several phytochemical, especially of isoflavonoids, in clinical therapy of MRSA infection. The results of this thesis might contribute to pave the way for new treatments for *S. aureus*, MRSA and biofilm-related infections. Additionally, they may open new perspectives for future work on efflux pumps, which may include a new therapeutic concept on the usefulness of EPI as adjuvants of the conventional antibiotic therapy to overcome the scarcity of therapeutic approaches for these MDR infections.

5.2. SUGGESTIONS FOR FUTURE WORK

Although different assays were performed to study the mode of action of the several phytochemicals tested along this project, more tests are needed to understand the interaction of phytochemicals with bacterial cells and their possible targets. A lot of mechanistic details still remains to be explained. A confirmation of the achieved conclusions on the mode of action of these phytochemicals on all bacteria tested should be taken through the study of the expression of membrane proteins by proteomic analysis and morphological changes of the bacterial cells by transmission electron microscopy (TEM). The use of molecular approaches would be necessary to fully understand the role of each efflux pump and how the bacterial cell responds to the presence of isoflavonoids.

All strains used in studies designed to assess MDR efflux pump gene expression in clinical isolates should also be evaluated by a method independent of *in vitro* susceptibility testing.

Among the 29 plants screened, only two were subject to further research. However, it would be interesting to study in more detail other promising plants displaying antibiotic-potentiation effect and proceed with the identification of the bioactive compounds.

It would also be desirable to test the ability of antibiotic-potentiators found in this study in a different cell culture system (e.g., a different strain of the same bacterial species or in different bacterial species). Additionally, it would be interesting to evaluate the specificity of the candidate compound by testing its ability to potentiate the activity of other members of the same class of antibiotics. Moreover, the potential for the emergence of resistance in long term, unexpected drug–drug interactions, side effects and toxicity issues that might arise from drug combination must also be examined thoroughly.

The apparently opposite effect observed with the same metabolite on biofilm formed by different strains can be a consequence of the multiple molecules and factors that characterize a biofilm (adhesion factors, quorum sensing, capsular polymers, among others) and that indeed can differ between bacterial species (Morán et al., 2014). So, it would be important to evaluate the interference of phytochemicals with certain components of the extracellular polymeric matrix of the biofilms, which could provide relevant information on the biofilm aspects that confer resistance. Additionally, it would be interesting to test these effects on MRSA biofilms, which were not included in the biofilm experiments. Efflux pumps seem to be highly active in bacterial biofilms, so the use of EPIs could be an attractive measure to control biofilm formation and/or reduce biofilm tolerance to antibiotics (Borges et al., 2016). Also, instead of applying the antibiotic and the potentiating compound concurrently, it would be interesting to test their

administration sequentially, by applying first the RMA/EPI compound, and only after, the antibiotic. These questions need to be properly addressed.

The clinical benefits and the effective synergy of these combinations have to be determined *in vivo* through carefully designed pharmacokinetic studies in animals. Determination of synergy or potentiation *in vitro* might not be reflected *in vivo* because of the potential failure to achieve synergistic levels of drugs in the desired tissue, for the differences in plasma protein binding and the drug metabolism (Kalan and Wright, 2011). Suitable animal models and models of bacterial infection (e.g. implant/pulmonary infection mouse model) could provide new important data. The data obtained from cell culture assays and animal studies could be used to formulate a range of dosage to ascertain the potential clinical success of the most promising compounds. Additionally, the application of such compounds on implants and other medical surfaces would be interesting to test. Their possible use in combination for surface coating could also be a potential immediate application.

Moreover, due to their extensive functional group chemistry and chirality those products are potentially interesting scaffolds for the discovery and development of antibacterial therapeutic approaches. Knowing the core structure that characterizes a compound shown to display potentiating activity for a particular antibiotic agent, subsequent libraries may be generated with the features shown to have potentiating activity, until a group of compounds with high potentiating activity and, optionally, one or more additional desirable properties (e.g., cell permeability) can be found. The modification and optimization of the compounds based on SAR, in a way that the intrinsic pharmacological effect of each one is completely suppressed and only the potentiating effect with the antibiotics is present, is also a further step of research.

REFERENCES

Abascal, K. and Yarnell, E. (2002) Herbs and drug resistance: Part 1 - herbs and microbial resistance to antibiotics. *Alternat Complement Ther*; **8**: 237-241.

Abimanyu, N., Murugesan, S. and Krishnan, P. (2012) Emergence of methicillin-resistant *Staphylococcus aureus* ST239 with high-level mupirocin and inducible clindamycin resistance in a tertiary care center in Chennai, South India. *J Clin Microbiol*; **50**: 3412-3413.

Abramson, D., Goad, L. J. and Goodwin, T. W. (1973) Triterpenes and sterols of *Buxus sempervirens* and local variations in their levels. *Phytochemistry*; **12**: 2211-2216.

Adonizio, A., Kong, K.-F. and Mathee, K. (2008) Inhibition of quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa* by south Florida plant extracts. *Antimicrob Agents Chemother*; **52**: 198-203.

Adwan, G., Abu-Shanab, B. and Adwan, K. (2010) Antibacterial activities of some plant extracts alone and in combination with different antimicrobials against multidrug-resistant *Pseudomonas aeruginosa* strains. *Asian Pac J Trop Med*: 266-269.

Adwan, G. and Mhanna, M. (2008) Synergistic effects of plant extracts and antibiotics on *Staphylococcus aureus* strains isolated from clinical specimens. *Middle-East J Sci Res*; **3**: 134-139.

Aeschlimann, J. R., Dresser, L. D., Kaatz, G. W. and Rybak, M. J. (1999) Effects of NorA inhibitors on *in vitro* antibacterial activities and postantibiotic effects of levofloxacin, ciprofloxacin, and norfloxacin in genetically related strains of *Staphylococcus aureus*. *Antimicrob Agents Chemother*; **43**: 335-340.

Ahmad, I. and Aqil, F. (2007) *In vitro* efficacy of bioactive extracts of 15 medicinal plants against ESβL-producing multidrug-resistant enteric bacteria. *Microbiol Res*; **162**: 264-275.

Aiyegoro, O. A., Afolayan, A. J. and Okoh, A. I. (2009a) *In vitro* antibacterial activities of crude extracts of the leaves of *Helichrysum longifolium* in combination with selected antibiotics. *Afr J Pharm Pharmaco*; **36**: 293-300.

Aiyegoro, O. A., Afolayan, A. J. and Okoh, A. I. (2009b) Synergistic interaction of *Helichrysum pedunculatum* leaf extracts with antibiotics against wound infection associated bacteria. *Biol Res*; **42**: 327-338.

Aiyegoro, O. A. and Okoh, A. I. (2009) Use of bioactive plant products in combination with standard antibiotics: Implications in antimicrobial chemotherapy. *J Med Plants Res*; **3**: 1147-1152.

Akram, M., Hamid, A., Khalil, A., Ghaffar, A., Tayyaba, N., Saeed, A., Ali, M. and Naveed, A. (2014) Review on medicinal uses, pharmacological, phytochemistry and immunomodulatory activity of plants. *Int J Immunopathol Pharmacol*; **27**: 313-319.

Al-hebshi, N., Al-haroni, M. and Skaug, N. (2006) *In vitro* antimicrobial and resistance-modifying activities of aqueous crude khat extracts against oral microorganisms. *Arch Oral Biol*; **51**: 183-188.

Albert Dhayakaran, R. P., Neethirajan, S., Xue, J. and Shi, J. (2015) Characterization of antimicrobial efficacy of soy isoflavones against pathogenic biofilms. *LWT - Food Sci Technol*; **63**: 859-865.

Alcaráz, L. E., Blanco, S. E., Puig, O. N., Tomás, F. and Ferretti, F. H. (2000) Antibacterial activity of flavonoids against methicillin-resistant *Staphylococcus aureus* strains. *J Theor Biol*; **205**: 231-240.

Alekshun, M. N. and Levy, S. B. (2007) Molecular mechanisms of antibacterial multidrug resistance. *Cell*; **128**: 1037-1050.

Ali, K., Iqbal, M., Korthout, H. A. A. J., Maltese, F., Fortes, A. M., Pais, M. S., Verpoorte, R. and Choi, Y. H. (2012) NMR spectroscopy and chemometrics as a tool for anti-TNFα activity screening in crude extracts of grapes and other berries. *Metabolomics*; **8**: 1148-1161.

Allen, H. K., Donato, J., Wang, H. H., Cloud-Hansen, K. A., Davies, J. and Handelsman, J. (2010) Call of the wild: antibiotic resistance genes in natural environments. *Nat Rev Microbiol*; **8**: 251-259.

Alves, M. J., Ferreira, I. C. F. R., Lourenço, I., Castro, A., Pereira, L., Martins, A. and Pintado, M. (2014) Wild mushroom extracts potentiate the action of standard antibiotics against multiresistant bacteria. *J Appl Microbiol*; **116**: 32-38.

Alzoreky, N. S. and Nakahara, K. (2003) Antibacterial activity of extracts from some edible plants commonly consumed in Asia. *Int J Food Microbiol*; **80**: 223-230.

Andersson, D. I. and Hughes, D. (2010) Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Microbiol*; **8**: 260 - 271.

Andlauer, W., Stumpf, C. and Fürst, P. (2001) Intestinal absorption of rutin in free and conjugated forms. *Biochem Pharmacol*; **62**: 369-374.

Ankri, S. and Mirelman, D. (1999) Antimicrobial properties of allicin from garlic. *Microbes Infect*; **1**: 125-129.

Aqil, F., Ahmad, I. and Owais, M. (2006) Evaluation of anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activity and synergy of some bioactive plant extracts. *Biotechnol J*; **1**: 1093-1102.

Aqil, F., Khan, M. S. A., Owais, M. and Ahmad, I. (2005) Effect of certain bioactive plant extracts on clinical isolates of β-lactamase producing methicillin resistant *Staphylococcus aureus*. *J Basic Microbiol*; **45**: 106-114.

Aumeeruddy-Elalfi, Z., Gurib-Fakim, A. and Mahomoodally, F. (2015) Antimicrobial, antibiotic potentiating activity and phytochemical profile of essential oils from exotic and endemic medicinal plants of Mauritius. *Ind Crop Prod*; **71**: 197-204.

Azmir, J., Zaidul, I. S. M., Rahman, M. M., Sharif, K. M., Mohamed, A., Sahena, F., Jahurul, M. H. A., Ghafoor, K., Norulaini, N. A. N. and Omar, A. K. M. (2013) Techniques for extraction of bioactive compounds from plant materials: A review. *J Food Eng*; **117**: 426-436.

Azuma, Y., Onishi, Y., Sato, Y. and Kizaki, H. (1995) Effects of protein tyrosine kinase inhibitors with different modes of action on topoisomerase activity and death of IL-2-dependent CTLL-2 cells. *J Biochem*; **118**: 312-318.

Bachir, R. G. and Benali, M. (2012) Antibacterial activity of the essential oils from the leaves of *Eucalyptus globulus* against *Escherichia coli* and *Staphylococcus aureus*. *Asian Pac J Trop Biomed*; **2**: 739-742.

Baran, E. T., Tuzlakoglu, K., Salgado, A. J. and Reis, R. L. (2004) Multichannel mould processing of 3D structures from microporous coralline hydroxyapatite granules and chitosan support materials for guided tissue regeneration/engineering. *J Mater Sci - Mater Med*; **15**: 161-165.

Barker, K. F. (1999) Antibiotic resistance: a current perspective. *Br J Clin Pharmacol*; **48**: 109-124.

Barreto, H. M., Coelho, K. M. R. N., Ferreira, J. H. L., dos Santos, B. H. C., de Abreu, A. P. L., Coutinho, H. D. M., da Silva, R. A. C., de Sousa, T. O., Citó, A. M. d. G. L. and Lopes, J. A. D. (2015) Enhancement of the antibiotic activity of aminoglycosides by extracts from *Anadenanthera colubrine* (Vell.) Brenan var. cebil against multi-drug resistant bacteria. *Nat Prod Res*: 1-4.

Barreto, H. M., de Lima, I. S., Coelho, K. M. R. N., Osório, L. R., de Almeida Mourão, R., Santos, B. H. C. d., Coutinho, H. D. M., de Abreu, A. P. L., de Medeiros, M. d. G. F., Citó, A. M. d. G. L. and Lopes, J. A. D. (2014) Effect of Lippia origanoides H.B.K. essential oil in the resistance to aminoglycosides in methicillin resistant *Staphylococcus aureus*. *Eur J Integr Med*; **6**: 560-564.

Bartel, J., Krumsiek, J. and Theis, F. J. (2013) Statistical methods for the analysis of high-throughput metabolomics data. *Comput Struct Biotechnol J*; **4**: Article ID e201301009.

Basile, A., Sorbo, S., Giordano, S., Ricciardi, L., Ferrara, S., Montesano, D., Castaldo Cobianchi, R., Vuotto, M. L. and Ferrara, L. (2000) Antibacterial and allelopathic activity of extract from *Castanea sativa* leaves. *Fitoterapia*; **71**, Supplement 1: 110-116.

Basri, D., Zin, N. M., Bakar, N. S., Rahmat, F. and Mohtar, M. (2008) Synergistic effects of phytochemicals and oxacillin on laboratory passage-derived vancomycin-intermediate *Staphylococcus aureus* strain. *J Med Sci*; **8**: 131-136.

Baugh, S., Phillips, C. R., Ekanayaka, A. S., Piddock, L. J. V. and Webber, M. A. (2014) Inhibition of multidrug efflux as a strategy to prevent biofilm formation. *J Antimicrob Chemother*; **69**: 673-681.

Bax, R., Mullan, N. and Verhoef, J. (2000) The millennium bugs - the need for and development of new antibacterials. *Int J Antimicrob Agents*; **16**: 51-59.

Beceiro, A., Tomás, M. and Bou, G. (2013) Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? *Clin Microbiol Rev*; **26**: 185-230.

Belofsky, G., Carreno, R., Lewis, K., Ball, A., Casadei, G. and Tegos, G. P. (2006) Metabolites of the "smoke tree", *Dalea spinosa*, potentiate antibiotic activity against multidrug-resistant *Staphylococcus aureus*. *J Nat Prod*; **69**: 261-264.

Belofsky, G., Percivill, D., Lewis, K., Tegos, G. P. and Ekart, J. (2004) Phenolic metabolites of *Dalea versicolor* that enhance antibiotic activity against model pathogenic bacteria. *J Nat Prod*; **67**: 481-484.

Berdy, J. (2012) Thoughts and facts about antibiotics: Where we are now and where we are heading. *J Antibiot*; **65**: 385-395.

Bernier, S. P. and Surette, M. G. (2013) Concentration-dependent activity of antibiotics in natural environments. *Front Microbiol*; **4**: Article ID 20.

Berrueta, L. A., Alonso-Salces, R. M. and Héberger, K. (2007) Supervised pattern recognition in food analysis. *J Chromatogr A*; **1158**: 196-214.

Betoni, J. E. C., Mantovani, R. P., Barbosa, L. N., Di Stasi, L. C. and Fernandes Junior, A. (2006) Synergism between plant extract and antimicrobial drugs used on *Staphylococcus aureus* diseases. *Mem Inst Oswaldo Cruz*; **101**: 387-390.

Bhardwaj, A. K. and Mohanty, P. (2012) Bacterial efflux pumps involved in multidrug resistance and their inhibitors: rejuvinating the antimicrobial chemotherapy. *Recent Pat Antiinfect Drug Discov*; **7**: 73-89.

Bischoff, S. C. (2008) Quercetin: potentials in the prevention and therapy of disease. *Curr Opin Clin Nutr Metab Care*; **11**: 733-740.

Bohm, B. A. (1998) *Introduction to flavonoids*. Australia: Harwood Academic Publishers.

Bolla, J.-M., Alibert-Franco, S., Handzlik, J., Chevalier, J., Mahamoud, A., Boyer, G., Kieć-Kononowicz, K. and Pagès, J.-M. (2011) Strategies for bypassing the membrane barrier in multidrug resistant Gram-negative bacteria. *FEBS Letters*; **585**: 1682-1690.

Bonapace, C. R., Bosso, J. A., Friedrich, L. V. and White, R. L. (2002) Comparison of methods of interpretation of checkerboard synergy testing. *Diagn Microbiol Infect Dis*; **44**: 363-366.

Bonapace, C. R., White, R. L., Friedrich, L. V. and Bosso, J. A. (2000) Evaluation of antibiotic synergy against *Acinetobacter baumannii*: a comparison with Etest, time-kill, and checkerboard methods. *Diagn Microbiol Infect Dis*; **38**: 43-50.

Borges-Walmsley, M. I., McKeegan, K. S. and Walmsley, A. R. (2003) Structure and function of efflux pumps that confer resistance to drugs. *Biochem J*; **376**: 313-338.

Borges, A., Abreu, A., Dias, C., Saavedra, M., Borges, F. and Simões, M. (2016) New perspectives on the use of phytochemicals as an emergent strategy to control bacterial infections including biofilms. *Molecules*; **21**: Article ID 877.

Borges, A., Saavedra, M. J. and Simões, M. (2012) The activity of ferulic and gallic acids in biofilm prevention and control of pathogenic bacteria. *Biofouling*; **28**: 755-767.

Braga, L. C., Leite, A. A. M., Xavier, K. G. S., Takahashi, J. A., Bemquerer, M. P., Chartone-Souza, E. and Nascimento, A. M. A. (2005) Synergic interaction between pomegranate extract and antibiotics against *Staphylococcus aureus*. *Can J Microbiol*; **51**: 541-547.

Brehm-Stecher, B. F. and Johnson, E. A. (2003) Sensitization of *Staphylococcus aureus* and *Escherichia coli* to antibiotics by the sesquiterpenoids nerolidol, farnesol, bisabolol, and apritone. *Antimicrob Agents Chemother*; **47**: 3357-3360.

Brennan, L. (2013) Metabolomics in nutrition research: current status and perspectives. *Biochem Soc Trans*; **41**: 670-673.

Bridier, A., Briandet, R., Thomas, V. and Dubois-Brissonnet, F. (2011) Resistance of bacterial biofilms to disinfectants: a review. *Biofouling*; **27**: 1017-1032.

Brooun, A., Liu, S. and Lewis, K. (2000) A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother*; **44**: 640-646.

Brown, E. D. and Wright, G. D. (2016) Antibacterial drug discovery in the resistance era. *Nature*; **529**: 336-343.

Brown, M. G. and Balkwill, D. L. (2008) Antibiotic resistance in bacteria isolated from the deep terrestrial subsurface. *Microb Ecol*; **57**: 484-493.

Burts, M. L., Alexeff, I., Meek, E. T. and McCullers, J. A. (2009) Use of atmospheric non-thermal plasma as a disinfectant for objects contaminated with methicillin-resistant *Staphylococcus aureus. Am J Infect Control*; **37**: 729-733.

Busch, C., Burkard, M., Leischner, C., Lauer, U. M., Frank, J. and Venturelli, S. (2015) Epigenetic activities of flavonoids in the prevention and treatment of cancer. *Clin Epigenetics*; **7**: Article ID 64.

Bylka, W., Matlawska, I. and Pilewski, N. A. (2004) Natural flavonoids as antimicrobial agents. *JAMA*; **7**: 24-31.

Cai, Y., Wang, R., Pei, F. and Liang, B.-B (2007) Antibacterial activity of allicin alone and in combination with β-lactams against *Staphylococcus spp*. and *Pseudomonas aeruginosa*. *J Antibiot*; **60**: 335-338.

Caiazza, N. C. and O'Toole, G. A. (2003) Alpha-Toxin is required for biofilm formation by *Staphylococcus aureus*. *J Bacteriol*; **185**: 3214-3217.

Cantón, E., Pemán, J., Gobernado, M., Viudes, A. and Espinel-Ingroff, A. (2005) Synergistic activities of fluconazole and voriconazole with terbinafine against four Candida species determined by checkerboard, time-Kill, and Etest methods. *Antimicrob Agents Chemother*; **49**: 1593-1596.

Cappelletty, D. M. and Rybak, M. J. (1996) Comparison of methodologies for synergism testing of drug combinations against resistant strains of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*; **40**: 677-683.

Chacha, M., Bojase-Moleta, G. and Majinda, R. R. T. (2005) Antimicrobial and radical scavenging flavonoids from the stem wood of *Erythrina latissima*. *Phytochemistry*; **66**: 99-104.

Chambers, H. F. and DeLeo, F. R. (2010) Waves of resistance: *Staphylococcus aureus* in the antibiotic era. In: Threats IoMUFoM, ed. *Antibiotic Resistance: Implications for Global Health and Novel Intervention Strategies: Workshop Summary*. Washington (DC): National Academies Press (US).

Chan, B. C. L., Ip, M., Lau, C. B. S., Lui, S. L., Jolivalt, C., Ganem-Elbaz, C., Litaudon, M., Reiner, N. E., Gong, H., See, R. H., Fung, K. P. and Leung, P. C. (2011) Synergistic effects of baicalein with ciprofloxacin against NorA over-expressed methicillin-resistant *Staphylococcus aureus* (MRSA) and inhibition of MRSA pyruvate kinase. *J Ethnopharmacol*; **137**: 767-773.

Chang, P.-C., Li, H.-Y., Tang, H.-J., Liu, J.-W., Wang, J.-J. and Chuang, Y.-C. (2007) *In vitro* synergy of baicalein and gentamicin against vancomycin-resistant Enterococcus. *J Microbiol Immunol Infect*; **40**: 56-61.

Chatterjee, M., Anju, C. P., Biswas, L., Anil Kumar, V., Gopi Mohan, C. and Biswas, R. (2016) Antibiotic resistance in *Pseudomonas aeruginosa* and alternative therapeutic options. *Int J Med Microbiol*; **306**: 48-58.

Chen, F., Di, H., Wang, Y., Cao, Q., Xu, B., Zhang, X., Yang, N., Liu, G., Yang, C.-G., Xu, Y., Jiang, H., Lian, F., Zhang, N., Li, J. and Lan, L. (2016) Small-molecule targeting of a diapophytoene desaturase inhibits *S. aureus* virulence. *Nat Chem Biol*; **12**: 174-179.

Chopra, I., Hodgson, J., Metcalf, B. and Poste, G. (1997) The search for antimicrobial agents effective against bacteria resistant to multiple antibiotics. *Antimicrob Agents Chemother*; **41**: 497-503.

Chowdhury, A. K., Ahsan, M., Islam, S. N. and Ahmed, Z. U. (1991) Efficacy of aqueous extract of garlic & allicin in experimental shigellosis in rabbits. *Indian J Med Res*; **93**: 33-36.

Chung, P. Y., Navaratnam, P. and Chung, L. Y. (2011) Synergistic antimicrobial activity between pentacyclic triterpenoids and antibiotics against *Staphylococcus aureus* strains. *Ann Clin Microbiol Antimicrob*; **10**: 1-6.

Čižman, M. (2003) The use and resistance to antibiotics in the community. *Int J Antimicrob Agents*; **21**: 297-307.

Clancy, J., Schmeider, B. J., Petitpas, J. W., Manousos, M., Williams, J. A., Faiella, J. A., Girard, A. E. and McGuirk, P. R. (1995) Assays to detect and characterize synthetic agents that inhibit the ErmC methyltransferase. *J Antibiot*; **48**: 1273-1279.

Clardy, J., Fischbach, M. A. and Walsh, C. T. (2006) New antibiotics from bacterial natural products. *Nat Biotechnol*; **24**: 1541-1550.

CLSI. (2015a) Clinical and Laboratory Standards Institute: Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically. Approved standard M7-A10. Wayne, Pa, USA.

CLSI. (2015b) Clinical and Laboratory Standards Institute: Performance Standards for Antimicrobial Susceptibility Testing - Twenty-Fifth Informational Supplement. Approved standard M100-S25. Wayne, Pa, USA.

Conly, J. M. (2004) Antimicrobial resistance – Judicious use is the key. *Can J Infect Dis Med Microbiol*; **15**: 249-51.

Copland, A., Nahar, L., Tomlinson, C. T. M., Hamilton, V., Middleton, M., Kumarasamy, Y. and Sarker, S. D. (2003) Antibacterial and free radical scavenging activity of the seeds of *Agrimonia eupatoria*. *Fitoterapia*; **74**: 133-135.

Costa, S. S., Falcão, C., Viveiros, M., Machado, D., Martins, M., Melo-Cristino, J., Amaral, L. and Couto, I. (2011) Exploring the contribution of efflux on the resistance to fluoroquinolones in clinical isolates of *Staphylococcus aureus*. *BMC Microbiol*; **11**: 1-12.

Costa, S. S., Viveiros, M., Amaral, L. and Couto, I. (2013) Multidrug efflux pumps in *Staphylococcus aureus*: an update. *Open Microbiol J*; **7**: 59-71.

Costerton, J. W., Montanaro, L. and Arciola, C. R. (2005) Biofilms in implant infections: its production and regulation. *Int J Artif Organs*; **28**: 1062-1068.

Costerton, J. W., Stewart, P. S. and Greenberg, E. P. (1999) Bacterial biofilms: a common cause of persistent infections. *Science*; **284**: 1318-1322.

Cottarel, G., Gardner, T. S., Lei, X., Porco, J., Schaus, S. E., Wierzbowski, J. and Pal, K. (2010) Compositions and methods for potentiating antibiotic activity. Google Patents US20100234348 A1, Trustees Of Boston University.

Cotter, J. J., Maguire, P., Soberon, F., Daniels, S., O'Gara, J. P. and Casey, E. (2011) Disinfection of meticillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms using a remote non-thermal gas plasma. *J Hosp Infect*; **78**: 204-207.

Coutinho, H. D. M., Costa, J. G. M., Falcão-Silva, V. S., Siqueira-Júnior, J. P. and Lima, E. O. (2010a) Effect of *Momordica charantia* L. in the resistance to aminoglycosides in methicilin-resistant *Staphylococcus aureus*. *Comp Immunol Microbiol*; **33**: 467-471.

Coutinho, H. D. M., Costa, J. G. M., Falcão-Silva, V. S., Siqueira-Júnior, J. P. and Lima, E. O (2010b) Potentiation of antibiotic activity by *Eugenia uniflora* and *Eugenia jambolanum*. *J Med Food*; **13**: 1024-1026.

Coutinho, H. D. M., Costa, J. G. M., Lima, E. O., Falcao-Silva, V. S. and Siqueira, J. P. (2009) Herbal therapy associated with antibiotic therapy: potentiation of the antibiotic activity against methicillin – resistant *Staphylococcus aureus* by *Turnera ulmifolia* L. *BMC Complement Altern Med*; **9**: 9-13.

Couto, I., Costa, S. S., Viveiros, M., Martins, M. and Amaral, L. (2008) Efflux-mediated response of *Staphylococcus aureus* exposed to ethidium bromide. *J Antimicrob Chemother*; **62**: 504-513.

Cowan, M. M. (1999) Plant products as antimicrobial agents. *Clin Microbiol Rev*; **12**: 564-582.

Cozad, A. and Jones, R. D. (2003) Disinfection and the prevention of infectious disease. *Am J Infect Control*; **31**: 243-254.

Cragg, G. M.and Newman, D. J. (2013) Natural products: A continuing source of novel drug leads. *Biochim Biophys Acta*; **1830**: 3670-3695.

Cruz, F. and Davies J. (2000) Horizontal gene transfer and the origin of species: lessons from bacteria. *Trends Microbiol*; **8**: 128-133.

Cushnie, T. P. T. and Lamb, A. J. (2005) Antimicrobial activity of flavonoids. *Int J Antimicrob Agents*; **26**: 343-356.

Cvitkovitch, D. G., Li, Y.-H. and Ellen, R. P. (2003) Quorum sensing and biofilm formation in *Streptococcal* infections. *J Clin Invest*; **112**: 1626-1632.

D'Costa, V. M., McGrann, K. M., Hughes, D. W. and Wright, G. D. (2006) Sampling the antibiotic resistome. *Science*; **311**: 374-377.

da Silva, J. A. T., Yonekura, L., Kaganda, J., Mookdasanit, J., Nhut, D. T. and Afach, G (2005) Important secondary metabolites and essential oils of species within the *Anthemideae* (*Asteraceae*). *J Herbs Spices Med Plants*; **11**: 1-46.

Dangl, J. L. and Jones, J. D. G. (2001) Plant pathogens and integrated defence responses to infection. *Nature*; **411**: 826-833.

Dantas, G., Sommer, M. O. A., Oluwasegun, R. D. and Church, G. M. (2008) Bacteria subsisting on antibiotics. *Science*; **320**: 100-103.

Darwish, R. M., Aburjai, T., Al-Khalil, S. and Mahafzah, A. (2002) Screening of antibiotic resistant inhibitors from local plant materials against two different strains of *Staphylococcus aureus*. *J Ethnopharmacol*; **79**: 359-364.

Dastidar, S. G., Manna, A., Kumar, K. A., Mazumdar, K., Dutta, N. K., Chakrabarty, A. N., Motohashi, N. and Shirataki, Y. (2004) Studies on the antibacterial potentiality of isoflavones. *Int J Antimicrob Agents*; **23**: 99-102.

David, M. Z., Taylor, A., Lynfield, R., Boxrud, D. J., Short, G., Zychowski, D., Boyle-Vavra, S. and Daum, R. S. (2013) Comparing pulsed-field gel electrophoresis with multilocus sequence typing, *spa* typing, Staphylococcal Cassette Chromosome *mec* (SCC*mec*) typing, and PCR for Panton-Valentine Leukocidin, *arcA*, and *opp3* in methicillin-resistant *Staphylococcus aureus* isolates at a U.S. Medical Center. *J Clin Microbiol*; **51**: 814-819.

Davies, J. and Davies, D. (2010) Origins and evolution of antibiotic resistance. *Microb Mol Biol Rev*; **74**: 417-433.

Dehghan, H., Sarrafi, Y. and Salehi, P. (2016) Antioxidant and antidiabetic activities of 11 herbal plants from Hyrcania region, Iran. *J Food Drug Anal*; **24**: 179-188.

Demanèche, S., Sanguin, H., Poté, J., Navarro, E., Bernillon, D., Mavingui, P., Wildi, W., Vogel, T. M. and Simonet, P. (2008) Antibiotic-resistant soil bacteria in transgenic plant fields. *Proc Natl Acad Sci*; **105**: 3957-3962.

Deng, X., Sun, F., Ji, Q., Liang, H., Missiakas, D., Lan, L. and He, C. (2012) Expression of multidrug resistance efflux pump gene NorA is iron responsive in *Staphylococcus aureus*. *J Bacteriol*; **194**: 1753-1762.

Dias, D. A., Urban, S. and Roessner, U. (2012) A Historical Overview of Natural Products in Drug Discovery. *Metabolites*; **2**: 303-336.

Dickson, R. A., Houghton, P. J., Hylands, P. J. and Gibbons, S. (2006) Antimicrobial, resistance-modifying effects, antioxidant and free radical scavenging activities of *Mezoneuron benthamianum* Baill., *Securinega virosa* Roxb. & Wlld. and *Microglossa pyrifolia* Lam. *Phytother Res*; **20**: 41-45.

Dixon, R. A. and Steele, C. L. (1999) Flavonoids and isoflavonoids – a gold mine for metabolic engineering. *Trends Plant Sci*; **4**: 394-400.

Donato, J. J., Moe, L. A., Converse, B. J., Smart, K. D., Berklein, F. C., McManus, P. S. and Handelsman, J. (2010) Metagenomic analysis of apple orchard soil reveals antibiotic resistance genes encoding predicted bifunctional proteins. *Appl Environ Microbiol*; **76**: 4396-4401.

Dötsch, A., Becker, T., Pommerenke, C., Magnowska, Z., Jänsch, L. and Häussler, S. (2009) Genome-wide identification of genetic determinants of antimicrobial drug resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*: 2522-2531.

Doughari, J. H., Human, I. S., Bennade, S. and Ndakidemi, P. A. (2009) Phytochemicals as chemotherapeutic agents and antioxidants: Possible solution to the control of antibiotic resistant verocytotoxin producing bacteria. *J Med Plant Res*; **3**: 839-848.

Drawz, S. M. and Bonomo, R. A. (2010) Three decades of β-lactamase inhibitors. *Clin Microbiol Rev*; **23**: 160-201.

Dulgerm, B. and Gonuz, A. (2004) Antimirobial activity of some Turkish medicinal plants. *Pak J Biol Sci*; **7**: 1559-1562.

Duthie, S. J. and Dobson, V. L. (1999) Dietary flavonoids protect human colonocyte DNA from oxidative attack *in vitro*. *Eur J Nutr*; **38**: 28-34.

Ekor, M. (2013) The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *Front Pharmacol*; **4**: Article ID 177.

Eliopoulos, G. M. and Eliopoulos, C. T. (1988) Antibiotic combinations: Should they be tested? *Clin Microbiol Rev*; **1**: 149-156.

Ellinger, J. J., Chylla, R. A., Ulrich, E. L. and Markley, J. L (2013) Databases and software for NMR-based metabolomics. *Curr Metabolomics*; **1**: Article ID 1.

Erlund, I., Kosonen, T., Alfthan, G., Mäenpää, J., Perttunen, K., Kenraali, J., Parantainen, J. and Aro, A. (2000) Pharmacokinetics of quercetin from quercetin aglycone and rutin in healthy volunteers. *Eur J Clin Pharmacol*; **56**: 545-553.

Ettefagh, K. A., Burns, J. T., Junio, H. A., Kaatz, G. W. and Cech, N. B. (2011) Goldenseal (*Hydrastis canadensis* L.) extracts synergistically enhance the antibacterial activity of berberine via efflux pump inhibition. *Planta med*; **77**: 835-840.

EUCAST. (2000) Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution - European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID). *Clin Microbiol Infect*; **6**: 509-515.

EUCAST. (2003) Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution - European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID). *Clin Microbiol Infect*; **9**: 1-7.

Eumkeb, G., Sakdarat, S. and Siriwong, S. (2010) Reversing β-lactam antibiotic resistance of *Staphylococcus aureus* with galangin from Alpinia officinarum Hance and synergism with ceftazidime. *Phytomedicine*; **18**: 40-45.

Fang, M., Chen, D. and Yang, C. S. (2007) Dietary polyphenols may affect DNA methylation. *J Nutrition*; **137**: 223S-8S.

Fankam, A. G., Kuiate, J. R. and Kuete, V. (2015) Antibacterial and antibiotic resistance modifying activity of the extracts from A*lanblackia gabonensis*, *Combretum molle* and *Gladiolus quartinianus* against Gram-negative bacteria including multi-drug resistant phenotypes. *BMC Complement Altern Med*; **15**: 1-12.

Fernández, L., Breidenstein, E. B. M. and Hancock, R. E. W. (2011) Creeping baselines and adaptive resistance to antibiotics. *Drug Resist Updat*; **14**: 1-21.

Fernández, L. and Hancock, R. E. W. (2012) Adaptive and Mutational Resistance: Role of Porins and Efflux Pumps in Drug Resistance. *Clin Microbiol Rev*; **25**: 661-681.

Ferry, D. R., Smith, A., Malkhandi, J., Fyfe, D. W., deTakats, P. G., Anderson, D., Baker, J. and Kerr, D. J. (1996) Phase I clinical trial of the flavonoid quercetin: pharmacokinetics and evidence for *in vivo* tyrosine kinase inhibition. *Clin Cancer Res*; **2**: 659-668.

Fiamegos, Y. C., Kastritis, P. L., Exarchou, V., Han, H., Bonvin, A. M. J. J., Vervoort, J., Lewis, K., Hamblin, M. R. and Tegos, G. P. (2011) Antimicrobial and efflux pump inhibitory activity of caffeoylquinic acids from *Artemisia absinthium* against Gram-positive pathogenic bacteria. *PLoS ONE*; **6**: 1-12.

Fiebelkorn, K. R., Crawford, S. A., McElmeel, M. L. and Jorgensen, J. H. (2003) Practical disk diffusion method for detection of inducible clindamycin resistance in *Staphylococcus aureus* and coagulase-negative Staphylococci. *J Clin Micobiol*; **41**: 4740-4744.

Fleming-Dutra, K. E., Hersh, A. L., Shapiro, D. J., Bartoces, M., Enns, E. A., File, T. M. Jr., Finkelstein, J. A., Gerber, J. S., Hyun, D. Y., Linder, J. A., Lynfield, R., Margolis, D. J., May, L. S., Merenstein, D., Metlay, J. P., Newland, J. G., Piccirillo, J. F., Roberts, R. M., Sanchez, G. V., Suda, K. J., Thomas, A., Woo, T. M., Zetts, R. M. and Hicks, L. A. (2016) Prevalence of inappropriate antibiotic prescriptions among us ambulatory care visits, 2010-2011. *JAMA*; **315**: 1864-1873.

Fontanay, S., Grare, M., Mayer, J., Finance, C. and Duval, R. E. (2008) Ursolic, oleanolic and betulinic acids: Antibacterial spectra and selectivity indexes. *J Ethnopharmacol*; **120**: 272-276.

Forsberg, K. J., Reyes, A., Wang, B., Selleck, E. M., Sommer, M. O. A. and Dantas, G. (2012) The shared antibiotic resistome of soil bacteria and human pathogens. *Science*; **337**: 1107-1111.

Frankel, E. N., Huang, S.-W., Aeschbach, R. and Prior, E. (1996) Antioxidant activity of a rosemary extract and its constituents, carnosic acid, carnosol, and rosmarinic acid, in bulk oil and oil-in-water emulsion. *J Agr Food Chem*; **44**: 131-135.

Frempong-Manso, E., Raygada, J. L., DeMarco, C. E., Seo, S. M. and Kaatz, G. W. (2008) Inability of a reserpine-based screen to identify strains overexpressing efflux pump genes in clinical isolates of *Staphylococcus aureus*. *Int J Antimicrob Agents*; **33**: 360-363.

Fujita, M., Shiota, S., Kuroda, T., Hatano, T., Yoshida, T., Mizushima, T. and Tsuchiya, T. (2005) Remarkable synergies between baicalein and tetracycline, and baicalein and β-lactams against methicillin-resistant *Staphylococcus aureus*. *Microbiol Immunol*; **49**: 391-396.

Fürstenberg-Hägg, J., Zagrobelny, M. and Bak, S. (2013) Plant defense against insect herbivores. *Int J Mol Sci*; **14**: 10242-10297.

Fux, C. A., Costerton, J. W., Stewart, P. S. and Stoodley, P. (2005) Survival strategies of infectious biofilms. *Trends Microbiol*; **13**: 34-40.

Galani, I., Kontopidou, F., Souli, M., Rekatsina, P.-D., Koratzanis, E., Deliolanis, J. and Giamarellou, H. (2008) Colistin susceptibility testing by Etest and disk diffusion methods. *Int J Antimicrob Agents*; **31**: 434-439.

Garcia, L. S. and Isenberg, H. D. (2007) *Clinical Microbiology Procedures Handbook*. Washington, D.C.: ASM Press.

Garvey, M. I., Rahman, M. M., Gibbons, S. and Piddock, L. J. V. (2011) Medicinal plant extracts with efflux inhibitory activity against Gram-negative bacteria. *Int J Antimicrob Agents*; **37**: 145-151.

Gaynor, M. and Mankin, A. S. (2003) Macrolide antibiotics: binding site, mechanism of action, resistance. *Curr Top Med Chem*; **3**: 949-960.

Gibbons, S. (2004) Anti-staphylococcal plant natural products. *Nat Prod Rep*; **21**: 263 - 277.

Gibbons, S. (2005) Plants as a source of bacterial resistance modulators and anti-infective agents. *Phytochem Rev*; **4**: 63-78.

Gibbons, S. (2008) Phytochemicals for bacterial resistance – strengths, weaknesses and opportunities. *Planta Med*; **74**: 594–602.

Gibbons, S., Moser, E. and Kaatz, G. W. (2004) Catechin gallates inhibit multidrug resistance (MDR) in *Staphylococcus aureus*. *Planta Med*; **70**: 1240-1242.

Gibbons, S., Oluwatuyi, M. and Kaatz, G. W. (2003a) A novel inhibitor of multidrug efflux pumps in *Staphylococcus aureus*. *J Antimicrob Chemother*; **51**: 13-17.

Gibbons, S., Oluwatuyi, M., Veitch, N. C. and Gray, A. I. (2003b) Bacterial resistance modifying agents from *Lycopus europaeus*. *Phytochemistry*; **62**: 83-87.

Gibbons, S. and Udo, E. E. (2000) The effect of reserpine, a modulator of multidrug efflux pumps, on the *in vitro* activity of tetracycline against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) possessing the Tet(K) determinant. *Phytother Res*; **14**: 139-140.

Gilbert, P., Das, J. and Foley, I. (1997) Biofilm susceptibility to antimicrobials. *Adv Dental Res*; **11**: 160-167.

Gilbert, P., Maira-Litran, T., McBain, A. J. Rickard, A. H. and Whyte, F. W. (2002) The physiology and collective recalcitrance of microbial biofilm communities. *Adv Microb Physiol*; **46**: 203-256.

Gill, E. E., Franco, O. L. and Hancock, R. E. W. (2015) Antibiotic adjuvants: Diverse strategies for controlling drug-resistant pathogens. *Chem Biol Drug Des*; **85**: 56-78.

Gogoi-Tiwari, J., Williams, V., Waryah, C. B., Eto, K. Y., Tau, M., Costantino, P., Tiwari, H. K. and Mukkur, T. (2015) Comparative studies of the immunogenicity and protective potential of biofilm vs planktonic *Staphylococcus aureus* vaccine against bovine mastitis using non-invasive mouse mastitis as a model system. *Biofouling*; **31**: 543-554.

González-Lamothe, R., Mitchell, G., Gattuso, M., Diarra, M. S., Malouin, F. and Bouarab, K. (2009) Plant antimicrobial agents and their effects on plant and human pathogens. *Int J Mol Sci*; **10**: 3400-3419.

Gould, I. M., Reilly, J., Bunyan, D. and Walker, A. (2010) Costs of healthcare-associated methicillin-resistant *Staphylococcus aureus* and its control. *Clin Microbiol Infect*; **16**: 1721-1728.

Griep, M. A., Blood, S., Larson, M. A. Koepsell, S. A. and Hinrichs, S. H. (2007) Myricetin inhibits *Escherichia coli* DnaB helicase but not primase. *Bioorg Med Chem Lett*; **15**: 7203-7208.

Griffith, C. J., Cooper, R. A., Gilmore, J., Davies, C. and Lewis, M. (2000) An evaluation of hospital cleaning regimes and standards. *J Hosp Infect*; **45**: 19-28.

Guardia, T., Rotelli, A. E., Juarez, A. O. and Pelzer, L. E. (2001) Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. *II Farmaco*; **56**: 683-687.

Guay, G. G., Tuckman, M. and Rothstein, D. M. (1994) Mutations in the *tetA*(*B*) gene that cause a change in substrate specificity of the tetracycline efflux pump. *Antimicrob Agents Chemother*; **38**: 857-60.

Guo, Q., Sun, S., Yu, J. and Cao, L. (2008) Synergistic activity of azoles with amiodarone against clinically resistant *Candida albicans* tested by chequerboard and time–kill methods. *J Med Microbiol*; **57**: 457-462.

Guz, N. R., Stermitz, F. R., Johnson, J. B., Beeson, T. D., Willen, S., Hsiang, J.-F. and Lewis, K. (2000) Flavonolignan and flavone inhibitors of a *Staphylococcus aureus* multidrug resistance pump: structure-activity relationships. *J Med Chem*; **44**: 261-268.

Hajduk, P. J., Dinges, J., Schkeryantz, J. M., Janowick, D., Kaminski, M., Tufano, M., Augeri, D. J., Petros, A., Nienaber, V., Zhong, P., Hammond, R., Coen, M., Beutel, B., Katz, L. and Fesik, S. W. (1999) Novel inhibitors of Erm methyltransferases from NMR and parallel synthesis. *J Med Chem*; **42**: 3852-3859.

Hammer, B. K. and Bassler, B. L. (2003) Quorum sensing controls biofilm formation in *Vibrio cholerae*. *Mol Microbiol*; **50**: 101-104.

Handzlik, J., Matys, A. and Kieć-Kononowicz, K. (2013) Recent advances in multi-drug resistance (MDR) efflux pump inhibitors of Gram-positive bacteria *S. aureus*. *Antibiotics*; **2**: 28-45.

Harborne, J. B. and Williams, C. A. (2000) Advances in flavonoid research since 1992. *Phytochemistry*; **55**: 481-504.

Harwood, M., Danielewska-Nikiel, B., Borzelleca, J. F., Flamm, G. W., Williams, G. M. and Lines, T. C. (2007) A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. *Food Chem Toxicol*; **45**: 2179-2205.

Helander, I. M., Alakomi, H.-L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E. J., Gorris, L. G. M. and von Wright, A. (1998) Characterization of the action of selected essential oil components on Gram-negative bacteria. *J Agr Food Chem*; **46**: 3590-3595.

Hemaiswarya, S., Kruthiventi, A. K. and Doble, M. (2008) Synergism between natural products and antibiotics against infectious diseases. *Phytomedicine*; **15**: 639-652.

Hirai, I., Okuno, M., Katsuma, R., Arita, N., Tachibana, M. and Yamamoto, Y. (2010) Characterisation of anti-*Staphylococcus aureus* activity of quercetin. *Int J Food Sci Technol*; **45**: 1250-1254.

Hoffman, S. B. (2001) Mechanisms of antibiotic resistance. *Compendium*; **23**: 464-473.

Holler, J. G., Christensen, S. B., Slotved, H.-C., Rasmussen, H. B., Gúzman, A., Olsen, C.-E., Petersen, B. and Mølgaard, P. (2012) Novel inhibitory activity of the *Staphylococcus aureus* NorA efflux pump by a kaempferol rhamnoside isolated from *Persea lingue* Nees. *J Antimicrob Chemother*; **67**: 1138-1144.

Hong, H., Landauer, M. R., Foriska, M. A. and Ledney, G. D. (2006) Antibacterial activity of the soy isoflavone genistein. *J Basic Microbiol*; **46**: 329-335.

Hu, Z.-Q., Zhao, W.-H., Asano, N., Yoda, Y., Hara, Y. and Shimamura, T. (2002) Epigallocatechin gallate synergistically enhances the activity of carbapenems against methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother*; **46**: 558-560.

Hughes, D. (2003) Expoliting genomics, genetics and chemistry to combat antibiotic resistance. *Nat Rev*; **4**: 432-441.

Huie, C. W. (2002) A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. *Anal Bioanal Chem*; **373**: 23-30.

Hummelova, J., Rondevaldova, J., Balastikova, A., Lapcik, O. and Kokoska, L. (2015) The relationship between structure and *in vitro* antibacterial activity of selected isoflavones and their metabolites with special focus on antistaphylococcal effect of demethyltexasin. *Lett Appl Microbiol*; **60**: 242-247.

Infed, N., Hanekop, N., Driessen, A. J. M., Smits, S. H. J. and Schmitt, L. (2011) Influence of detergents on the activity of the ABC transporter LmrA. *BBA-Biomemebranes*; **1808**: 2313-2321.

Inoue, T., Shingaki, R. and Fukui, K. (2008) Inhibition of swarming motility of *Pseudomonas aeruginosa* by branched-chain fatty acids. *FEMS Microbiol Lett*; **281**: 81-86.

Iriti, M. and Faoro, F. (2009) Chemical diversity and defence metabolism: How plants cope with pathogens and ozone pollution. *Int J Mol Sci*; **10**: 3371-3399.

Isbrucker, R. A., A., E. J., Wolz, E., Davidovich, A. and Bausch, J. (2006) Safety studies on epigallocatechin gallate (EGCG) preparations. Part 2: Dermal, acute and short-term toxicity studies. *Food Chem Toxicol*; **44**: 636–650.

ISO (1992) Internation Organization for Standardization (ISO)'s Report: Biological compatibility of medical devices. 5. Test for cytotoxicity: *In vitro* methods. ISO document 10993.

Isogai, E., Isogai, H., Hirose, K., Hayashi, S. and Oguma, K. (2001) *In vivo* synergy between green tea extract and levofloxacin against enterohemorrhagic *Escherichia coli* O157 infection. *Curr Microbiol*; **42**: 248-251.

Jayaraman, P., Sakharkar, M. K., Lim, C. S., Tang, T. H. and Sakharkar, K. R. (2010) Activity and interactions of antibiotic and phytochemical combinations against *Pseudomonas aeruginosa in vitro*. *Int J Biol Sci*; **6**: 556-568.

Jeandet, P., Clément, C., Courot, E. and Cordelier, S. (2013) Modulation of phytoalexin biosynthesis in engineered plants for disease resistance. *Int J Mol Sci*; **14**: 14136-14170.

Jin, J., Zhang, J., Guo, N., Feng, H., Li, L., Liang, J., Sun, K., Wu, X., Wang, X., Liu, M., Deng, X. and Yu, L. (2011) The plant alkaloid piperine as a potential inhibitor of ethidium bromide efflux in *Mycobacterium smegmatis*. *J Med Microbiol*; **60**: 223-229.

Joshi, S. G., Paff, M., Friedman, G., Fridman, G., Fridman, A. and Brooks, A. D. (2010) Control of methicillin-resistant *Staphylococcus aureus* in planktonic form and biofilms: A biocidal efficacy study of nonthermal dielectric-barrier discharge plasma. *Am J Infect Control*; **38**: 293-301.

Joung, D.-K., Mun, S.-H., Lee, K.-S., Kang, O.-H., Choi, J.-G., Kim, S.-B., Gong, R., Chong, M.-S., Kim, Y.-C., Lee, D.-S., Shin, D.-W. and Kwon, D.-Y. (2014) The antibacterial assay of tectorigenin with detergents or ATPase inhibitors against methicillin-resistant *Staphylococcus aureus*. *Evid Based Complement Alternat Med*; **2014**: Article ID 716509.

Jung, R., Husain, M., Choi, M. K. and Fish, D. N. (2004) Synergistic activities of moxifloxacin combined with piperacillin-tazobactam or cefepime against *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Acinetobacter baumannii* clinical isolates. *Antimicrob Agents Chemother*; **48**: 1055-1057.

Kaatz, G. W., Moudgal, V. V., Seo, S. M., Hansen, J. B. and Kristiansen, J. E. (2003) Phenylpiperidine selective serotonin reuptake inhibitors interfere with multidrug efflux pump activity in *Staphylococcus aureus*. *Int J Antimicrob Agents*; **22**: 254-261.

Kalan, L. and Wright, G. D. (2011) Antibiotic adjuvants: multicomponent anti-infective strategies. *Expert Rev Mol Med*; **13**: Article ID e5.

Kang, H.-K., Kim, H.-Y. and Cha, J.-D. (2011) Synergistic effects between silibinin and antibiotics on methicillin-resistant *Staphylococcus aureus* isolated from clinical specimens. *Biotechnol J*; **6**: 1397-1408.

Kaplan, J. B., Izano, E. A., Gopal, P., Karwacki, M. T., Kim, S., Bose, J. L., Bayles, K. W. and Horswill, A. R. (2012) Low levels of β-lactam antibiotics induce extracellular DNA release and biofilm formation in *Staphylococcus aureus*. *mBio*; **3**: Article ID e00198-12.

Khan, I. A., Mirza, Z. M., Kumar, A., Verma, V. and Qazi, G. N. (2006) Piperine, a phytochemical potentiator of ciprofloxacin against *Staphylococcus aureus*. *Antimicrob Agents Chemother*; **50**: 810-812.

Kim, H.-K., Choi, Y. H. and Verpoorte, R. (2010a) NMR-based metabolomic analysis of plants. *Nature Protocols*; **5**: 536-549.

Kim, H. K., Saifullah, Khan, S., Wilson, E. G., Kricun, S. D. P., Meissner, A., Goraler, S., Deelder, A. M., Choi, Y. H. and Verpoorte, R. (2010b) Metabolic classification of South American *Ilex* species by NMR-based metabolomics. *Phytochemistry*; **71**: 773-784.

Kim, S.-G., Kim, M. J., Jin, D., Park, S.-N., Cho, E., Freire, M. O., Jang, S.-J., Park, Y.-J. and Kook, J.-K. (2012) Antimicrobial effect of ursolic acid and oleanolic acid against methicillin-resistant *Staphylococcus aureus*. *Korean J Microbiol*; **48**: 212-215.

Kiraz, N., Daäÿ, I., Yamac, M., Kiremitci, A., Kaåÿifoäÿlu, N. and Akgun, Y. (2009) Antifungal activity of caspofungin in combination with amphotericin B against *Candida glabrata*: Comparison of disk diffusion, Etest, and time-kill methods. *Antimicrob Agents Chemother*; **53**: 788-790.

Kishen, A., Upadya, M., Tegos, G. P. and Hamblin, M. R. (2010) Efflux pump inhibitor potentiates antimicrobial photodynamic inactivation of *Enterococcus faecalis* biofilm. *Phytochem Photobiol*; **86**: 1343-1349.

Kitajima, S. and Yamaguchi, K. (2009) Silybin from *Silybum Marianum* seeds inhibits confluent-induced keratinocytes differentiation as effectively as retinoic acid without inducing inflammatory cytokine. *J Clin Biochem Nutr*; **45**: 178-184.

Kjelleberg, S. and Molin, S. (2002) Is there a role for quorum sensing signals in bacterial biofilms? *Curr Opin Microbiol*; **5**: 254-258.

Kontoyiannis, D. P. and Lewis, R. E. (2003) Combination chemotherapy for invasive fungal infections: what laboratory and clinical studies tell us so far. *Drug Resist Updates*; **6**: 257-269.

Kourtesi, C., Ball, A. R., Huang, Y.-Y., Jachak, S. M., Vera, D. M. A., Khondkar, P., Gibbons, S., Hamblin, M. R. and Tegos, G. P. (2013) Microbial efflux systems and inhibitors: approaches to drug discovery and the challenge of clinical implementation. *Open Microbiol J*; **7**: 34-52.

Krishnan, T., Yin, W.-F. and Chan, K.-G. (2012) Inhibition of quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa* PAO1 by Ayurveda spice clove (*Syzygium aromaticum*) bud extract. *Sensors*; **12**: 4016-4030.

Kumar, A. and Schweizer, H. P. (2005) Bacterial resistance to antibiotics: Active efflux and reduced uptake. *Adv Drug Deliver Rev*; **57**: 1486-1513.

Kumar, S. and Pandey, A. K. (2013) Chemistry and biological activities of flavonoids: an overview. *Sci World J*; **2013**: Article ID 162750.

Kurek, A., Grudniak, A. M., Szwed, M., Klicka, A., Samluk, L., Wolska, K. I., Janiszowska, W. and Popowska, M. (2009) Oleanolic acid and ursolic acid affect peptidoglycan metabolism in *Listeria monocytogenes*. *Antonie van Leeuwenhoek*; **97**: 61-68.

Kurek, A., Nadkowska, P., Pliszka, S. and Wolska, K. I. (2012) Modulation of antibiotic resistance in bacterial pathogens by oleanolic acid and ursolic acid. *Phytomedicine*; **19**: 515-519.

Kvist, M., Hancock, V. and Klemm, P. (2008) Inactivation of efflux pumps abolishes bacterial biofilm formation. *Appl Environ Microbiol*; **74**: 7376-7382.

Kyaw, B. M., Arora, S. and Lim, C. S. (2012) Bactericidal antibiotic-phytochemical combinations against methicillin resistant *Staphylococcus aureus*. *Braz J Microbiol*; **43**: 938-945.

Lai, S., Tremblay, J. and Déziel, E. (2009) Swarming motility: a multicellular behaviour conferring antimicrobial resistance. *Environ Microbiol*; **11**: 126-136.

Lambert, P. A. (2005) Bacterial resistance to antibiotics: Modified target sites. *Adv Drug Deliver Rev*; **57**: 1471-1485.

Lambert, R. J. W., Johnston, M. D., Hanlon, G. W. and Denyer, S. P. (2003) Theory of antimicrobial combinations: biocide mixtures – synergy or addition? *J App Microbiol*; **94**: 747-759.

Lambert, R. J. W., Skandamis, P. N., Coote, P. J. and Nychas, G. J. E. (2001) A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J Appl Microbiol*; **91**: 453-462.

Landini, P., Antoniani, D., Burgess, J. G. and Nijland, R.. (2010) Molecular mechanisms of compounds affecting bacterial biofilm formation and dispersal. *Appl Microbiol Biotechnol*; **86**: 813-823.

Lang, K. S., Anderson, J. M., Schwarz, S., Williamson, L., Handelsman, J. and Singer, R. S. (2010) Novel florfenicol and chloramphenicol resistance gene discovered in Alaskan soil by using functional metagenomics. *Appl Environ Microbiol*; **76**: 5321-5326.

Larive, C. K., Barding, G. A. and Dinges, M. M. (2015) NMR spectroscopy for metabolomics and metabolic profiling. *Anal Chem*; **87**: 133-146.

Lawes, T., Edwards, B., López-Lozano, J.-M. and Gould, I. (2012) Trends in *Staphylococcus aureus* bacteraemia and impacts of infection control practices including universal MRSA admission screening in a hospital in Scotland, 2006–2010: retrospective cohort study and time-series intervention analysis. *BMJ Open*; **2**: e000797.

Lechner, D., Gibbons, S. and Bucar, F. (2008) Plant phenolic compounds as ethidium bromide efflux inhibitors in *Mycobacterium smegmatis*. *J Antimicrob Chemother*; **62**: 345-348.

Leclercq, R. (2002) Mechanisms of resistance to macrolides and lincosamides: Nature of the resistance elements and their clinical implications. *Clin Infect Dis*; **34**: 482-492.

Lee, J.-H., Park, J.-H., Cho, H. S., Joo, S. W., Cho, M. H. and Lee, J. (2013) Anti-biofilm activities of quercetin and tannic acid against *Staphylococcus aureus*. *Biofouling*; **29**: 491-499.

Lemkul, J. A. and Bevan, D. R. (2012) Morin inhibits the early stages of amyloid β-peptide aggregation by altering tertiary and quaternary interactions to produce "off-pathway" structures. *Biochemistry*; **51**: 5990-6009.

Leonard, S. N. (2012) Synergy between vancomycin and nafcillin against *Staphylococcus aureus* in an *in vitro* pharmacokinetic/pharmacodynamic model. *PLoS ONE*; **7**: Article ID e42103.

Levy, S. B. (2002) Active efflux, a common mechanism for biocide and antibiotic resistance. *J Appl Microbiol*; **92**: 65S-71S.

Lewis, K. (2013) Platforms for antibiotic discovery. *Nat Rev Drug Discov*; **12**: 371-387.

Lewis, K. and Ausubel, F. M. (2006) Prospects for plant-derived antibacterials. *Nat Biotechnol*; **24**: 1504-1507.

Lewis, R. E., Diekema, D. J., Messer, S. A., Pfaller, M. A. and Klepser, M. E. (2002) Comparison of Etest, chequerboard dilution and time–kill studies for the detection of synergy or antagonism between antifungal agents tested against Candida species. *J Antimicrob Chemother*; **49**: 345-351.

Li, X.-Z. and Nikaido, H. (2009) Efflux-mediated drug resistance in bacteria: An update. *Drugs*; **69**: 1555-1623.

Lila, M. A. and Raskin, I. (2005) Health-related interactions of phytochemicals. *J Food Sci*; **70**: 20-27.

Lin, R.-D., Chin, Y.-P. and Lee, M.-H. (2005) Antimicrobial activity of antibiotics in combination with natural flavonoids against clinical extended-spectrum β-lactamase (ESβL)-producing *Klebsiella pneumoniae*. *Phytother Res*; **19**: 612-617.

Ling, L. L., Schneider, T., Peoples, A. J., Spoering, A. L., Engels, I., Conlon, B. P., Mueller, A., Schaberle, T. F., Hughes, D. E., Epstein, S., Jones, M., Lazarides, L., Steadman, V. A., Cohen, D. R., Felix, C. R., Fetterman, K. A., Millett, W. P., Nitti, A. G., Zullo, A. M., Chen, C. and Lewis, K. (2015) A new antibiotic kills pathogens without detectable resistance. *Nature*; **517**: 455-459.

Liu, G., Liang, J.-C., Wang, X.-L., Li, Z.-H., Wang, W., Guo, N., Wu, X.-P., Shen, F.-G., Xing, M.-X., Liu, L.-H., Li, L., Liu, M.-Y. and Yu, L. (2011) *In vitro* synergy of biochanin A and ciprofloxacin against clinical isolates of *Staphylococcus aureus*. *Molecules*; **16**: 6656-6656.

Lo, A.-H., Liang, Y.-C., Lin-Shiau, S.-Y., Ho, C.-T. and Lin, J.-K. (2002) Carnosol, an antioxidant in rosemary, suppresses inducible nitric oxide synthase through down-regulating nuclear factor-κB in mouse macrophages. *Carcinogenesis*; **23**: 983-991.

Lomovskaya, O., Warren, M. S., Lee, A., Galazzo, J., Fronko, R., Lee, M., Blais, J., Cho, D., Chamberland, S., Renau, T., Leger, R., Hecker, S., Watkins, W., Hoshino, K., Ishida, H. and Lee, V. J. (2001) Identification and characterization of inhibitors of multidrug resistance efflux pumps

in *Pseudomonas aeruginosa*: Novel agents for combination therapy. *Antimicrob Agents Chemother*; **45**: 105-116.

Lorenzi, V., Muselli, A., Bernardini, A. F., Berti, L., Pagès, J.-M., Amaral, L. and Bolla, J.-M. (2009) Geraniol restores antibiotic activities against multidrug-resistant isolates from Gram-negative species. *Antimicrob Agents Chemother* **53**: 2209-2211.

Lu, T. K. and Collins, J. J. (2009) Engineered bacteriophage targeting gene networks as adjuvants for antibiotic therapy. *Proc Natl Acad Sci*; **106**: 4629-4634.

Lynch, A. S. (2006) Efflux systems in bacterial pathogens: An opportunity for therapeutic intervention? An industry view. *Biochem Pharmacol*; **71**: 949-956.

Mackay, M. L., Milne, K. and Gould, I. M. (2000) Comparison of methods for assessing synergic antibiotic interactions. *Int J Antimicrob Agents*; **15**: 125-129.

Madigan, M., Martinko, J. and Parker, J. (2004) *Brock biology of microorganisms*. 13th edition. São Paulo: Prentice Hall.

Madkour, A. E., Dabkowski, J. M., Nüsslein, K. and Tew, G. N. (2009) Fast disinfecting antimicrobial surfaces. *Langmuir*; **25**: 1060-1067.

Mah, T.-F. C. and O'Toole, G. A. (2001) Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol*; **9**: 34-39.

Mahamoud, A., Chevalier, J., Alibert-Franco, S., Kern, W. V. and Pagès, J.-M. (2007) Antibiotic efflux pumps in Gram-negative bacteria: the inhibitor response strategy. *J Antimicrob Chemother*; **59**: 1223-1229.

Mahmood, A., Ahmed, R. and Kosar, S. (2009) Phytochemical screening and biological activities of the oil components of *Prunus domestica* Linn. *J Saudi Chem Soc*; **13**: 273-277.

Maisnier-Patin, S. and Andersson, D. I. (2004) Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. *Res Microbiol*; **155**: 360-369.

Malléa, M., Mahamoud, A., Chevalier, J., Alibert-Franco, S., Brouant, P., Barbe, J. and Pagès, J.-M. (2003) Alkylaminoquinolines inhibit the bacterial antibiotic efflux pump in multidrug-resistant clinical isolates. *Biochem J*; **376**: 801-805.

Marín, L., Miguélez, E. M., Villar, C. J. and Lombó, F. (2015) Bioavailability of dietary polyphenols and gut microbiota metabolism: antimicrobial properties. *BioMed Res Int*; **2015**: Article ID 905215.

Markham, P. N., Westhaus, E., Klyachko, K., Johnson, M. E. and Neyfakh, A. A. (1999) Multiple novel inhibitors of the NorA multidrug transporter of *Staphylococcus aureus*. *Antimicrob Agents Chemother*; **43**: 2404-2408.

Marquez, B. (2005) Bacterial efflux systems and efflux pumps inhibitors. *Biochimie*; **87**: 1137-1147.

Marquez, B., Neuville, L., Moreau, N. J., Genet, J.-P., dos Santos, A. F., Caño de Andrade, M. C. and Goulart Sant'Ana, A. E. (2005) Multidrug resistance reversal agent from *Jatropha elliptica*. *Phytochemistry*; **66**: 1804-1811.

Martin, S. (2010) *In vitro* antimicrobial synergy testing of *Acinetobacter Baumannii*. Master thesis. *Department of Medical Microbiology*: University of Stellenbosch.

Martinez, A. l. and Lin, J. (2006) Effect of an efflux pump inhibitor on the function of the multidrug efflux pump CmeABC and antimicrobial resistance in Campylobacter. *Foodborne Pathog Dis*; **3**: 393-402.

Martini, N. D., Katerere, D. R. P. and Eloff, J. N. (2004) Biological activity of five antibacterial flavonoids from Combretum erythrophyllum (Combretaceae). *J Ethnopharmacol*; **93**: 207-12.

Mayer, I. and Nagy, E. (1999) Investigation of the synergic effects of aminoglycoside- fluoroquinolone and third-generation cephalosporin combinations against clinical isolates of *Pseudomonas* spp. *J Antimicrob Chemother*; **43**: 651-657.

Mayrhofer, S., Domig, K. J., Mair, C., Zitz, U., Huys, G. and Kneifel, W. (2008) Comparison of broth microdilution, Etest, and agar disk diffusion methods for antimicrobial susceptibility testing of *Lactobacillus acidophilus* group members [down-pointing small open triangle]. *Appl Environ Microbiol*; **74**: 3745-3748.

McCallum, N., Berger-Bächi, B. and Senn, M. M. (2010) Regulation of antibiotic resistance in *Staphylococcus aureus*. *Int J Med Microbiol*; **300**: 118-129.

McNulty, J., Nair, J. J., Bollareddy, E., Keskar, K., Thorat, A., Crankshaw, D. J., Holloway, A. C., Khan, G., Wright, G. D. and Ejim, L. (2009) Isolation of flavonoids from the heartwood and resin of *Prunus avium* and some preliminary biological investigations. *Phytochem Rev*; **70**: 2040-2046.

Mendonça-Filho, R. (2006) Bioactive phytocompounds: New approaches in the phytosciences. In: Ahmad I, Aqil F, Owais M, eds. *Modern Phytomedicine: Turning Medicinal Plants into Drugs*. Weinheim: Wiley; 1-24.

Middleton, P., Stewart, F., Al-Qahtani, S., Egan, P., O'Rourke, C., Abdulrahman, A., Byres, M., Middleton, M., Kumarasamy, Y., Shoeba, M., Lutfun, N., Delazar, A. and Dey Sarker, S. (2005) Antioxidant, Antibacterial Activities and General Toxicity of *Alnus glutinosa*, *Fraxinus excelsior* and *Papaver rhoeas*. *Iran J Pharm Res*; **2**: 81-86.

Mierziak, J., Kostyn, K. and Kulma, A. (2014) Flavonoids as important molecules of plant interactions with the environment. *Molecules*; **19**: 16240-16265.

Miller, J. S., *Economic Botany*. (2011) The discovery of medicines from plants: A current biological perspective. *Econ Bot*; **54**: 396-407.

Miller, L. A., Ratnam, K. and Payne, D. J. (2001) β-Lactamase-inhibitor combinations in the 21st century: current agents and new developments. *Curr Opin Pharmacol*; **1**: 451-458.

Mirza, Z. M., Kumar, A., Kalia, N. P., Zargar, A. and Khan, I. A. (2011) Piperine as an inhibitor of the MdeA efflux pump of *Staphylococcus aureus*. *J Med Microbiol*; **60**: 1472-1478.

Miyazaki, K. (2004) Novel approach for evaluation of estrogenic and anti-estrogenic activities of genistein and daidzein using B16 melanoma cells and dendricity assay. *Pigment Cell Res*; **17**: 407-412.

Monzón, M., Oteiza, C., Leiva, J. and Amorena, B. (2001) Synergy of different antibiotic combinations in biofilms of *Staphylococcus epidermidis*. *J Antimicrob Chemother*; **48**: 793-801.

Moosavian, M., Shoja, S., Rostami, S., Torabipour, M. and Farshadzadeh, Z. (2014) Inducible clindamycin resistance in clinical isolates of *Staphylococcus aureus* due to erm genes, Iran. *Iran J Microbiol*; **6**: 421-427.

Morán, A., Gutiérrez, S., Martínez-Blanco, H., Ferrero, M. A., Monteagudo-Mera, A. and Rodríguez-Aparicio, L. B. (2014) Non-toxic plant metabolites regulate *Staphylococcus* viability and biofilm formation: a natural therapeutic strategy useful in the treatment and prevention of skin infections. *Biofouling*; **30**: 1175-1182.

Morel, C., Stermitz, F. R., Tegos, G. and Lewis, K. (2003) Isoflavones as potentiators of antibacterial activity. *J Agr Food Chem*; **51**: 5677-5679.

Mukne, A. P., Viswanathan, V. and Phadatare, A. G. (2011) Structure pre-requisites for isoflavones as effective antibacterial agents. *Pharmacogn Rev*; **5**: 13-18.

Mullin, S., Mani, N. and Grossman, T. H. (2004) Inhibition of Antibiotic Efflux in Bacteria by the Novel Multidrug Resistance Inhibitors Biricodar (VX-710) and Timcodar (VX-853). *Antimicrob Agents Chemother*; **48**: 4171-4176.

Mulyaningsih, S., Sporer, F., Zimmermann, S., Reichling, J. and Wink, M. (2010) Synergistic properties of the terpenoids aromadendrene and 1,8-cineole from the essential oil of *Eucalyptus globulus* against antibiotic-susceptible and antibiotic-resistant pathogens. *Phytomedicine*; **17**: 1061-1066.

Musumeci, R., Speciale, A., Costanzo, R., Annino, A., Ragusa, S., Rapisarda, A., Pappalardo, M. S. and Iauk, L. (2003) *Berberis aetnensis* C. Presl. extracts: antimicrobial properties and interaction with ciprofloxacin. *Int J Antimicrob Agents*; **22**: 48-53.

Nascimento, G. G., Locatelli, J., Freitas, P. C. and Silva, G. L. (2000) Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. *Braz J Microbiol*; **31**: 247-256.

Natarajan, P., Katta, S., Andrei, I., Babu Rao Ambati, V., Leonida, M. and Haas, G. J. (2008) Positive antibacterial co-action between hop (*Humulus lupulus*) constituents and selected antibiotics. *Phytomedicine*; **15**: 194-201.

NCCLS/ CLSI. (1999) National Committee for Clinical Laboratory Standards: Methods for determining bactericidal activity of antimicrobial agents. Approved Standard M26-A; Wayne, Pa, USA.

Ngomuo, A. J. and Jones, R. S. (1996) Cytotoxicity studies of quercetin, shikimate, cyclohexanecarboxylate and ptaquiloside. *Vet Hum Toxicol*; **38**: 14-18.

Nicodemo, A. C., Araujo, M. R. E., Ruiz, A. S. and Gales, A. C. (2004) *In vitro* susceptibility of *Stenotrophomonas maltophilia* isolates: comparison of disc diffusion, Etest and agar dilution methods. *J Antimicrob Chemother*; **53**: 604-608.

Nicolson, K., Evans, G. and O'Toole, P. W. (1999) Potentiation of methicillin activity against methicillin-resistant *Staphylococcus aureus* by diterpenes. *FEMS Microbiol Lett*; **179**: 233-239.

Nikaido, H. (1998) Antibiotic resistance caused by Gram-negative multidrug efflux pumps. *Clin Infect Dis*; **27**: S32-S41.

Nikaido, H. (2003) Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol R*; **67**: 593-656.

Nikaido, H. and Vaara, M. (1985) Molecular basis of bacterial outer membrane permeability *Microbiol Rev*; **49**: 1-32.

Nohynek, L. J., Alakomi, H.-L., Kähkönen, M. P., Heinonen, M., Helander, I. M., Oksman-Caldentey, K.-M. and Puupponen-Pimiä, R. H. (2006) Berry phenolics: antimicrobial properties and mechanisms of action against severe human pathogens. *Nutr Cancer*; **54**: 18-32.

Noor, H., Cao, P. and Raleigh, D. P. (2012) Morin hydrate inhibits amyloid formation by islet amyloid polypeptide and disaggregates amyloid fibers. *Protein Sci*; **21**: 373-382.

Nordmann, P., Poirel, L., Carrër, A., Toleman, M. A. and Walsh, T. R. (2011) How to detect NDM-1 producers. *J Clin Microbiol*; **49**: 718-721.

O'Gorman, A., Gibbons, H. and Brennan, L. (2013) Metabolomics in the identification of biomarkers of dietary intake. *Comput Struct Biotechnol J*; **4**: Article ID e201301004.

O'Toole, G., Kaplan, H. B. and Kolter, R. (2000) Biofilm formation as microbial development. *Annu Rev Physiol*; **54**: 49-79.

Odds, F. C. (2003) Synergy, antagonism, and what the chequerboard puts between them. *J Antimicrob Chemother*; **52**, Article ID 1.

Okamoto, T. (2005) Safety of quercetin for clinical application (Review). *Int J Mol Med*; **16**: 275-278.

Olajuyigbe, O. O. and Afolayan, A. J. (2012) Synergistic interactions of methanolic extract of *Acacia mearnsii* De Wild. with antibiotics against bacteria of clinical relevance. *Int J Mol Sci*; **13**: 8915-8932.

Olson, M., Ceri, H., Morck, D., Buret, A. and Read, R. (2002) Biofilm bacteria: formation and comparative susceptibility to antibiotics. *Can J Vet Res*; **66**: 86-92.

Oluwatuyi, M., Kaatz, G. W. and Gibbons, S. (2004) Antibacterial and resistance modifying activity of *Rosmarinus officinalis*. *Phytochemistry*; **65**: 3249-3254.

Orhan, G., Bayram, A., Zer, Y. and Balci, I. (2005) Synergy tests by E test and checkerboard methods of antimicrobial combinations against *Brucella melitensis*. *J Clin Microbiol*; **43**: 140-143.

Orhan, I. E., Erdem, S. A., Senol, F. S., Kartal, M. and Sener, B. (2012) Exploration of cholinesterase and tyrosinase inhibitory, antiprotozoal and antioxidant effects of *Buxus sempervirens* L. (boxwood). *Ind Crops Prod*; **40**: 116-121.

Osawa, K., Yasuda, H., Maruyama, T., Morita, H., Takeya, K. and Itokawa, H. (1992) Isoflavanones from the heartwood of *Swartzia polyphylla* and their antibacterial activity against cariogenic bacteria. *Chem Pharm Bull (Tokyo)*; **40**: 2970-2974.

Ossola, B., Kääriäinen, T. M., Raasmaja, A. and Männistö, P. T. (2008) Time-dependent protective and harmful effects of quercetin on 6-OHDA-induced toxicity in neuronal SH-SY5Y cells. *Toxicology*; **250**: 1-8.

Otter, J. A., Yezli, S. and French, G. L. (2011) The role played by contaminated surfaces in the transmission of nosocomial pathogens. *Infect Cont Hosp Ep*; **32**: 687-699.

Pagès, J.-M. and Amaral, L. (2009) Mechanisms of drug efflux and strategies to combat them: Challenging the efflux pump of Gram-negative bacteria. *BBA-Proteins Proteom*; **1794**: 826-833.

Paixão, L., Rodrigues, L., Couto, I., Martins, M., Fernandes, P., de Carvalho, C. C., Monteiro, G. A., Sansonetty, F., Amaral, L. and Viveiros, M. (2009) Fluorometric determination of ethidium bromide efflux kinetics in *Escherichia coli*. *J Biol Eng*; **3**: 1-13.

Palaniappan, K. and Holley, R. A. (2010) Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria. *Int J Food Microbiol*; **140**: 164-168.

Palmer, A. C. and Kishony, R. (2014) Opposing effects of target overexpression reveal drug mechanisms. *Nat Commun*; **5**, Article ID 4296.

Pandey, K. B. and Rizvi, S. I. (2009) Plant polyphenols as dietary antioxidants in human health and disease. *Oxid Med Cell Longev*; **2**: 270-278.

Pandra, S. B., Otilia, D. B., Banji, J. F., Aavula, R. K. and Merugu, S..(2010) Antibiotic resistance: overview and mechanisms. *Int J Pharm Sci Res*; **1**: 17-27.

Park, J. H., Park, J. H., Hur, H. J., Woo, J. S. and Lee, H. J. (2012) Effects of silymarin and formulation on the oral bioavailability of paclitaxel in rats. *Eur J Pharm Sci*; **45**: 296-301.

Parkar, S. G., Stevenson, D. E. and Skinner, M. A. (2008) The potential influence of fruit polyphenols on colonic microflora and human gut health. *Int J Food Microbiol*; **124**: 295-298.

Patel, K. N., Patel, J. K., Patel, M. P., Rajput, G. C. and Patel, H. A. (2010) Introduction to hyphenated techniques and their applications in pharmacy. *Pharm Methods*; **1**: 2-13.

Pawlikowska-Pawlega, B. and Gawron, A. (1995) Effect of quercetin on the growth of mouse fibroblast cells *in vitro*. *Pol J Pharmacol*; **47**: 513-535.

Perumal, S. and Mahmud, R. (2013) Chemical analysis, inhibition of biofilm formation and biofilm eradication potential of *Euphorbia hirta* L. against clinical isolates and standard strains. *BMC Complement Altern Med*; **13**: 1-8.

PFAF (2016) Plants For A Future (PFAF) Database. http://www.pfaf.org/user/default.aspx (May 2016, last date accessed).

Phillipson, J. D. (2007) Phytochemistry and pharmacognosy. *Phytochemistry*; **68**: 2960-2972.

Piddock, L. J. V. (2006) Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin Microbiol Rev*; **19**: 382-402.

Piddock, L. J. V. (2015) Teixobactin, the first of a new class of antibiotics discovered by iChip technology? *J Antimicrob Chemother*; **70**: 2679-2680.

Piddock, L. J. V., Garvey, M. I., Rahman, M. M. and Gibbons, S. (2010) Natural and synthetic compounds such as trimethoprim behave as inhibitors of efflux in Gram-negative bacteria. *J Antimicrob Chemother*; **65**: 1215-1223.

Pinho, M., Lencastre, H. and Tomasz, A. (2001) An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci. *Proc Natl Acad Sci*; **98**: 10886-10891.

Pinto, M. S., McGahan, M. E., Steiner, W. W. and Priefer, R. (2011) The use of the pseudo-polyelectrolyte, poly(4-vinylphenol), in multilayered films as an antimicrobial surface coating. *Colloid Surface A*; **377**: 182-186.

Pintore, G., Usai, M., Bradesi, P., Juliano, C., Boatto, G., Tomi, F., Chessa, M., Cerri, R. and Casanova, J. (2002) Chemical composition and antimicrobial activity of *Rosmarinus officinalis* L. oils from Sardinia and Corsica. *Flavour Frag J*; **17**: 15-19.

Plaper, A., Golob, M., Hafner, I., Oblak, M., Šolmajer, T. and Jerala, R. (2003) Characterization of quercetin binding site on DNA gyrase. *Biochem Biophy Res Co*; **306**: 530-536.

Poole, K. (2012) Bacterial stress responses as determinants of antimicrobial resistance. *J Antimicrob Chemother*; **20**: 227-34.

Powers, J. H. (2004) Antimicrobial drug development – the past, the present, and the future. *Clin Microbiol Infect*; **10**: 23-31.

Prabhu, K., Rao, S. and Rao, V. (2011) Inducible clindamycin resistance in *Staphylococcus aureus* isolated from clinical samples. *J Lab Physicians*; **3**: 25-27.

Qin, N., Tan, X., Jiao, Y., Liu, L., Zhao, W., Yang, S. and Jia, A. (2014) RNA-Seq-based transcriptome analysis of methicillin-resistant *Staphylococcus aureus* biofilm inhibition by ursolic acid and resveratrol. *Sci Rep*; **4**: Article ID 5467.

Rand, K. H., Houck, H. J., Brown, P. and Bennet, D. (1996) Reproducibility of the microdilution checkerboard method for antibiotic synergy. *Antimicrob Agents Chemother*; **37**: 613-615.

Rattanachaikunsopon, P. and Phumkhachorn, P. (2010) Contents and antibacterial activity of flavonoids extracted from leaves of *Psidium guajava*. *J Med Plants Res*; **4**: 393-396.

Rauha, J.-P., Remes, S., Heinonen, M., Hopia, A., Kahkonen, M., Kujala, T., Pihlaja, K., Vuorela, H. and Vuorela, P. (2000) Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *Int J Food Microbiol*; **56**: 3-12.

Rausher, M. D. (2001) Co-evolution and plant resistance to natural enemies. *Nature*; **411**: 857-864.

Ren, D., Zuo, R., González Barrios, A. F., Bedzyk, L. A., Eldridge, G. R., Pasmore, M. E. and Wood, T. K. (2005) Differential gene expression for investigation of *Escherichia coli* biofilm inhibition by plant extract ursolic acid. *Appl Environ Microbiol*; **71**: 4022-4034.

Renau, T. E., Léger, R., Flamme, E. M., Sangalang, J., She, M. W., Yen, R., Gannon, C. L., Griffith, D., Chamberland, S., Lomovskaya, O., Hecker, S. J., Lee, V. J., Ohta, T. and Nakayama, K. (1999) Inhibitors of efflux pumps in *Pseudomonas aeruginosa* potentiate the activity of the fluoroquinolone antibacterial levofloxacin. *J Med Chem*; **42**: 4928-4931.

Renneberg, J. (1993) Definitions of antibacterial interactions in animal infection models. *J Antimicrob Chemother*; **31**: 167-175.

Rennie, R. P., Turnbull, L., Brosnikoff, C. and Cloke, J. (2012) First comprehensive evaluation of the M.I.C. evaluator device compared to Etest and CLSI broth microdilution for MIC testing of aerobic Gram-positive and Gram-negative bacterial species. *J Clin Microbiol*; **50**: 1147-1152.

Reynolds, R., Shackcloth, J., Felmingham, D. and MacGowan, A. (2003) Comparison of BSAC agar dilution and NCCLS broth microdilution MIC methods for in vitro susceptibility testing of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*: the BSAC respiratory resistance surveillance programme. *J Antimicrob Chemother*; **52**: 925-930.

Riesenfeld, C. S., Goodman, R. M. and Handelsman, J. (2004) Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. *Environ Microbiol*; **6**: 981-989.

Roccaro, A. S., Blanco, A. R., Giuliano, F., Rusciano, D. and Enea, V. (2004) Epigallocatechin-gallate enhances the activity of tetracycline in Staphylococci by inhibiting its efflux from bacterial cells. *Antimicrob Agents Chemother*; **48**: 1968-1973.

Rodrigues, L., Villellas, C., Bailo, R., Viveiros, M. and Aínsa, J. A. (2013) Role of the *Mmr* efflux pump in drug resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*; **57**: 751-757.

Römling, U. and Balsalobre, C. (2012) Biofilm infections, their resilience to therapy and innovative treatment strategies. *J Int Med*; **272**: 541-561.

Russell, A. D. (2003) Biocide use and antibiotic resistance: The relevance of laboratory findings to clinical and environmental situations. *Lancet Infect Dis*; **3**: 794-803.

Russo, P., Del Bufalo, A. and Cesario, A. (2012) Flavonoids acting on DNA topoisomerases: recent advances and future perspectives in cancer therapy. *Curr Med Chem*; **19**: 5287-5293.

Rutala, W. A. and Weber, D. J. (2001) Surface disinfection: should we do it? *J Hosp Infect*; **48**: S64-8.

Saavedra, M. J., Borges, A., Dias, C., Aires, A., Bennett, R. N., Rosa, E. S. and Simões, M. (2010) Antimicrobial activity of phenolics and glucosinolate hydrolysis products and their synergy with streptomycin against pathogenic bacteria. *Med Chem*; **6**: 174-183.

Sakanaka, S., Shimura, N., Aizawa, M., Kim, M. and Yamamoto, T. (1992) Preventive effect of green tea polyphenols against dental caries in conventional rats. *Biosci Biotechnol Biochem*; **56**: 592-594.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. M. and Yoga Latha, L. (2011) Extraction, Isolation and Characterization of Bioactive Compounds from Plants' Extracts. *Afr J Trad Compl Alt Med*; **8**: 1-10.

Sato, Y., Suzaki, S., Nishikawa, T., Kihara, M., Shibata, H. and Higuti, T. (2000) Phytochemical flavones isolated from *Scutellaria barbata* and antibacterial activity against methicillin-resistant *Staphylococcus aureus*. *J Ethnopharmacol*; **72**: 483-488.

Scalbert, A., Brennan, L., Fiehn, O., Hankemeier, T., Kristal, B. S., van Ommen, B., Pujos-Guillot, E., Verheij, E., Wishart, D. and Wopereis, S. (2009) Mass-spectrometry-based metabolomics: limitations and recommendations for future progress with particular focus on nutrition research. *Metabolomics*; **5**: 435-458.

Scambia, G., Ranelletti, F. O., Panici, P. B., De Vincenzo, R., Bonanno, G., Ferrandina, G., Piantelli, M., Bussa, S., Rumi, C., Cianfriglia, M. and Mancuso, S. (1994) Quercetin potentiates the effect of adriamycin in a multidrug-resistant MCF-7 human breast-cancer cell line: P-glycoprotein as a possible target. *Cancer Chemother Pharmacol*; **34**: 459-464.

Schelz, Z., Hohmann, J. and Molnar, J. (2010) Recent advances in research of antimicrobial effects of essential oils and plant derived compounds on bacteria. In: Chattopadhyay D, ed. *Ethnomedicine: A Source of Complementary Therapeutics*. Kerala: Research Signpost; 179-201.

Schmitz, F.-J., Sadurski, R., Kray, A., Boos, M., Geisel, R., Köhrer, K., Verhoef, J. and Fluit, A. C. (2000) Prevalence of macrolide-resistance genes in *Staphylococcus aureus* and *Enterococcus faecium* isolates from 24 European university hospitals. *J Antimicrob Chemother*; **45**: 891-894.

Schmitz, F. J., Fluit, A. C., Lückefahr, M., Engler, B., Hofmann, B., Verhoef, J., Heinz, H. P., Hadding, U. and Jones, M. E. (1998) The effect of reserpine, an inhibitor of multidrug efflux pumps, on

the *in vitro* activities of ciprofloxacin, sparfloxacin and moxifloxacin against clinical isolates of *Staphylococcus aureus*. *J Antimicrob Chemother*; **42**: 807-810.

Sebaugh, J. L. (2011) Guidelines for accurate EC50/IC50 estimation. **10**: 128-134.

Serrano, M. C., Morilla, D., Valverde, A., Chávez, M., Espinel-Ingroff, A., Claro, R., Ramírez, M. and Mazuelos, E. M. (2003) Comparison of Etest with modified broth microdilution method for testing susceptibility of *Aspergillus* spp. to voriconazole. *J Clin Microbiol*; **41**: 5270-5272.

Sexton, J. D., Tanner, B. D., Maxwell, S. L. and Gerba, C. P. (2011) Reduction in the microbial load on high-touch surfaces in hospital rooms by treatment with a portable saturated steam vapor disinfection system. *Am J Infect Control*; **39**: 655-662.

Shafahi, M. and Vafai, K. (2010) Synthesis of biofilm resistance characteristics against antibiotics. *Int J Heat Mass Transf*; **53**: 2943-2950.

Shahverdi, A. R., Monsef-Esfahani, H. R., Tavasoli, F., Zaheri, A. and Mirjani, R. (2007) Trans-cinnamaldehyde from *Cinnamomum zeylanicum* bark essential oil reduces the clindamycin resistance of *Clostridium difficile in vitro*. *J Food Sci*; **72**: S055-S8.

Shen, Y., Stojicic, S. and Haapasalo, M. (2011) Antimicrobial efficacy of chlorhexidine against bacteria in biofilms at different stages of development. *J Endod*; **37**: 657-661.

Shibata, H., Kondo, K., Katsuyama, R., Kawazoe, K., Sato, Y., Murakami, K., Takaishi, Y., Arakaki, N. and Higuti, T. (2005) Alkyl gallates, intensifiers of β-lactam susceptibility in methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother*; **49**: 549-555.

Shibata, H., Shirakata, C., Kawasaki, H., Sato, Y., Kuwahara, T., Ohnishi, Y., Arakaki, N. and Higuti, T. (2003) Flavone markedly affects phenotypic expression of β-lactam resistance in methicillin-resistant *Staphylococcus aureus* strains isolated clinically. *Biol Pharm Bull*; **26**: 1478-1483.

Shimizu, M., Shiota, S., Mizushima, T., Ito, H., Hatano, T., Yoshida, T. and Tsuchiya, T. (2001) Marked potentiation of activity of β-lactams against methicillin-resistant *Staphylococcus aureus* by corilagin. *Antimicrob Agents Chemother*; **45**: 3198-3201.

Shin, B. and Park, W. (2015) Synergistic effect of oleanolic acid on aminoglycoside antibiotics against *Acinetobacter baumannii*. *PLoS ONE*; **10**: Article ID e0137751.

Shiota, S., Shimizu, M., Mizusima, T., Ito, H., Hatano, T., Yoshida, T. and Tsuchiya, T. (2000) Restoration of effectiveness of β-lactams on methicillin-resistant *Staphylococcus aureus* by tellimagrandin I from rose red. *FEMS Microbiol Lett*; **185**: 135-138.

Shiota, S., Shimizu, M., Sugiyama, J., Morita, Y., Mizushima, T. and Tsuchiya, T. (2004) Mechanisms of action of corilagin and tellimagrandin I that remarkably potentiate the activity of β-lactams against methicillin-resistant *Staphylococcus aureus*. *Microbiol Immunol*; **48**: 67-73.

Si Si, W., Gong, J., Chanas, C., Cui, S., Yu, H., Caballero, C. and Friendship, R. M. (2006) *In vitro* assessment of antimicrobial activity of carvacrol, thymol and cinnamaldehyde towards

Salmonella serotype Typhimurium DT104: effects of pig diets and emulsification in hydrocolloids. *J Appl Microbiol*; **101**: 1282-1291.

Sibanda, T. (2007) Assessment of anti-bacterial potencials of *Garcinia Kola* seed extracts and their interactions with antibiotics. Master thesis. *Department of Biochemistry and Microbiology*. Alice: University of Fort Hare.

Sibanda, T. and Okoh, A. I. (2007) The challenges of overcoming antibiotic resistance: Plant extracts as potential sources of antimicrobial and resistance modifying agents. *Afr J Biotechnol*; **6**: 2886-2896.

Sibanda, T. and Okoh, A. I. (2008) *In vitro* evaluation of the interactions between acetone extracts of *Garcinia kola* seeds and some antibiotics. *Afr J Biotechnol*; **7**: 1672-1678.

Siebra, A. L. A., Oliveira, L. R., Martins, A. O. B. P. B., Siebra, D. C., Albuquerque, R. S., Lemos, I. C. S., Delmondes, G. A., Tintino, S. R., Figueredo, F. G., da Costa, J. G. M., Coutinho, H. D. M., Menezes, I. R. A., Felipe, C. F. B. and Kerntopf, M. R. (2016) Potentiation of antibiotic activity by *Passiflora cincinnata* Mast. front of strains *Staphylococcus aureus* and *Escherichia coli*. *Saudi J Biol Sci*. In press: (http://dx.doi.org/10.1016/j.sjbs.2016.01.019).

Simões, L. C., Simões, M. and Vieira, M. J. (2010) Influence of the diversity of bacterial isolates from drinking water on resistance of biofilms to disinfection. *Appl Environ Microbiol*; **76**: 6673-6679.

Simões, M. (2005) Use of biocides and surfactants to control *Pseudomonas fluorescens* biofilms: role of the hydrodynamic conditions. PhD Thesis. *Department of Biological Engineering*, Braga: University of Minho.

Simões, M., Bennett, R. N. and Rosa, E. A. S. (2009) Understanding antimicrobial activities of phytochemicals against multidrug resistant bacteria and biofilms. *Nat Prod Rep*; **26**: 746-757.

Simões, M., Rocha, R., Coimbra, M. A. and Vieira, M. (2008) Enhancement of *Escherichia coli* and *Staphylococcus aureus* antibiotic susceptibility using sesquiterpenoids. *Med Chem*; **4**: 616-623.

Singh, B. N., Singh, B. R., Singh, R. L., Prakash, D., Dhakarey, R., Upadhyay, G. and Singh, H. B. (2009) Oxidative DNA damage protective activity, antioxidant and anti-quorum sensing potentials of *Moringa oleifera*. *Food Chem Toxicol*; **47**: 1109-1116.

Skibola, C. F. and Smith, M. T. (2000) Potential health impacts of excessive flavonoid intake. *Free Radical Bio Med*; **29**: 375-383.

Skov, R., Smyth, R., Clausen, M., Larsen, A. R., Frimodt-Møller, N., Olsson-Liljequist, B. and Kahlmeter, G. (2003) Evaluation of a cefoxitin 30 µg disc on Iso-Sensitest agar for detection of methicillin-resistant *Staphylococcus aureus*. *J Antimicrob Chemother*; **52**: 204-207.

Smith, A. W. (2005) Biofilms and antibiotic therapy: Is there a role for combating bacterial resistance by the use of novel drug delivery systems? *Adv Drug Deliv Rev*; **57**: 1539-1550.

Smith, E., Williamson, M., Wareham, N., Kaatz, G. and Gibbons, S. (2007a) Antibacterial and modulators of bacterial resistance from the immature cones of *Chamaecyparis lawsoniana*. *Phytochemistry*; **68**: 210-217.

Smith, E. C., Kaatz, G. W., Seo, S. M., Wareham, N., Williamson, E. M. and Gibbons, S. (2007b) The phenolic diterpene totarol inhibits multidrug efflux pump activity in *Staphylococcus aureus*. *Antimicrob Agents Chemother*; **51**: 4480-4483.

Smolinska, A., Blanchet, L., Buydens, L. M. C. and Wijmenga, S. S. (2012) NMR and pattern recognition methods in metabolomics: From data acquisition to biomarker discovery: A review. *Anal Chim Acta*; **750**: 82-97.

Soler-Rivas, C., Espín, J. C. and Wichers, H. J. (2000) Oleuropein and related compounds. *J Sci Food Agr*; **80**: 1013-1023.

Song, Z. J., Johansen, H. K., Faber, V. and HØIby, N. (1997) Ginseng treatment enhances bacterial clearance and decreases lung pathology in athymic rats with chronic *P. aeruginosa* pneumonia. *APMIS*; **105**: 438-444.

Sopirala, M. M., Mangino, J. E., Gebreyes, W. A., Biller, B., Bannerman, T., Balada-Llasat, J.-M. and Pancholi, P. (2010) Synergy testing by Etest, microdilution checkerboard, and time-kill methods for pan-drug-resistant *Acinetobacter baumannii*. *Antimicrob Agents Chemother*; **54**: 4678-4683.

Spížek, J., Novotná, J., Řezanka, T. and Demain, A. (2010) Do we need new antibiotics? The search for new targets and new compounds. *J Ind Microbiol Biotechnol*; **37**: 1241-1248.

Srinivasan, D., Nathan, S., Suresh, T. and Lakshmana Perumalsamy, P. (2001) Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine. *J Ethnopharmacol*; **74**: 217-220.

Stapleton, P. D., Shah, S., Anderson, J. C., Hara, Y., Hamilton-Miller, J. M. T. and Taylor, P. W. (2004) Modulation of β-lactam resistance in *Staphylococcus aureus* by catechins and gallates. *Int J Antimicrob Agents*; **23**: 462-467.

Stavri, M., Piddock, L. J. V. and Gibbons, S. (2007) Bacterial efflux pump inhibitors from natural sources. *J Antimicrob Chemother*; **59**: 1247-1260.

StepanoviĆ, S., VukoviĆ, D., Hola, V., Bonaventura, G. D., DjukiĆ, S., ĆIrkoviĆ, I. and Ruzicka, F. (2007) Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *Acta Pathol Microbiol Immunol Scand*; **115**: 891-899.

Stermitz, F. R., Cashman, K. K., Halligan, K. M. et al. (2003) Polyacylated neohesperidosides from *Geranium caespitosum*: bacterial multidrug resistance pump inhibitors. *Bioorg Med Chem Lett*; **13**: 1915-1918.

Stermitz, F. R., Cashman, K. K., Halligan, K. M., Morel, C., Tegos, G. P. and Lewis, K. (2000a) Synergy in a medicinal plant: Antimicrobial action of berberine potentiated by 5′-methoxyhydnocarpin, a multidrug pump inhibitor. *P Natl A Sci*; **97**: 1433-1437.

Stermitz, F. R., Lorenz, P., Tawara, J. N., Zenewicz, L. A. and Lewis, K. (2000b) 5‘-Methoxyhydnocarpin-D and pheophorbide A: Berberis species components that potentiate berberine growth inhibition of resistant *Staphylococcus aureus*. *J Nat Prod*; **63**: 1146-1149.

Stewart, P. (1996) Theoretical aspects of antibiotic diffusion into microbial biofilms. *Antimicrob Agents Chemother*; **40**: 2517-2522.

Stewart, P. S. (2002) Mechanisms of antibiotic resistance in bacterial biofilms. *Int J Med Microbiol*; **292**: 107-113.

Stewart, P. S. and Costerton, J, W. (2001) Antibiotic resistance of bacteria in biofilms. *The Lancet*; **358**: 135-138.

Sueke, H., Kaye, S. B., Neal, T., Hall, A., Tuft, S. and Parry, C. M. (2010) An *in vitro* investigation of synergy or antagonism between antimicrobial combinations against isolates from bacterial *Keratitis*. *Invest Ophthalmol Vis Sci*; **51**: 4151-4155.

Sugimoto, M., Kawakami, M., Robert, M., Soga, T. and Tomita, M. (2012) Bioinformatics tools for mass spectroscopy-based metabolomic data processing and analysis. *Curr Bioinformatics*; **7**: 96-108.

Sullivan, Å., Edlund, C. and Nord, C. E. (2001) Effect of antimicrobial agents on the ecological balance of human microflora. *Lancet Infect Dis*; **1**: 101-114.

Sun, J., Deng, Z. and Yan, A. (2014) Bacterial multidrug efflux pumps: Mechanisms, physiology and pharmacological exploitations. *Biochem Biophys Res Commun*; **453**: 254-267.

Suzuki, N., Rivero, R. M., Shulaev, V., Blumwald, E. and Mittler, R. (2014) Abiotic and biotic stress combinations. *New Phytol*; **203**: 32-43.

Szczepaniak, S., Polanska, M., Van Assche, A., Moloney, R. and Willems, K. (2011) The synergism of natural compounds in the pursuit of safe and healthier food. *J Ind Microbiol Biotechnol*; **38**: 215-220.

Szekely, A., Johnson, E. M. and Warnock, D. W. (1999) Comparison of E-Test and broth microdilution methods for antifungal drug susceptibility testing of molds. *J Clin Microbiol*; **37**: 1480-1483.

Taguri, T., Tanaka, T. and Kouno, I. (2006) Antibacterial Spectrum of Plant Polyphenols and Extracts Depending upon Hydroxyphenyl Structure. *Biol Pharm Bull*; **29**: 2226-2235.

Talon, D. (1999) The role of the hospital environment in the epidemiology of multi-resistant bacteria. *J Hosp Infect*; **43**: 13-17.

Tan, T. Y., Lim, T. P., Lee, W. H. L., Sasikala, S., Hsu, L. H. and Kwa, A. L.-H. (2011) *In vitro* antibiotic synergy in extensively drug-resistant *Acinetobacter baumannii*: the effect of testing by time-kill, checkerboard, and Etest methods. *Antimicrob Agents Chemother*; **55**: 436-438.

Tanaka, H., Sato, M., Fujiwara, S., Hirata, M., Etoh, H. and Takeuchi, H. (2002) Antibacterial activity of isoflavonoids isolated from *Erythrina variegata* against methicillin-resistant *Staphylococcus aureus*. *Lett Appl Microbiol*; **35**: 494-498.

Tankeo, S. B., Tane, P. and Kuete, V. (2015) *In vitro* antibacterial and antibiotic-potentiation activities of the methanol extracts from *Beilschmiedia acuta*, *Clausena anisata*, *Newbouldia laevis* and *Polyscias fulva* against multidrug-resistant Gram-negative bacteria. *BMC Complement Altern Med*; **15**: 1-10.

Tegos, G., Stermitz, F. R., Lomovskaya, O. and Lewis, K. (2002) Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. *Antimicrob Agents Chemother*; **46**: 3133-3141.

Tegos, G. P., Haynes, M., Strouse, J. J., Khan, M. M. T., Bologa, C. G., Oprea, T. I. and Sklar, L. A. (2011) Microbial Efflux Pump Inhibition: Tactics and Strategies. *Curr Pharm Des*; **17**: 1291-1302.

Tegos, G. P., Masago, K., Aziz, F., Higginbotham, A., Stermitz, F. R. and Hamblin, M. R. (2008) Inhibitors of bacterial multidrug efflux pumps potentiate antimicrobial photoinactivation. *Antimicrob Agents Chemother*; **52**: 3202-3209.

The Pew Charitable Trusts (2016) Pew, CDC release national targets for reducing unnecessary antibiotic use. In: Hyun D, ed. The Pew Charitable Trusts, Press Release (accessed August 2016).

Thomas, C. M. and Nielsen K. (2005) Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nat Rev Micro*; **3**: 711-721.

Toda, M., Okubo, S., Ikigai, H., Suzuki, T., Suzuki, Y., Hara, Y. and Shimamura, T. (1992) The protective activity of tea catechins against experimental infection by *Vibrio cholerae* O1. *Microbiol Immunol*; **36**: 999-1001.

Toté, K., Berghe, D. V., Maes, L. and Cos, P. (2008) A new colorimetric microtitre model for the detection of *Staphylococcus aureus* biofilms. *Lett Appl Microbiol*; **46**: 249-254.

Touani, F. K., Seukep, A. J., Djeussi, D. E., Fankam, A. G., Noumedem, J. A. K. and Kuete, V. (2014) Antibiotic-potentiation activities of four *Cameroonian* dietary plants against multidrug-resistant Gram-negative bacteria expressing efflux pumps. *BMC Complement Altern Med*; **14**: 1-8.

Trombetta, D., Castelli, F., Sarpietro, M. G., Venuti, V., Cristani, M., Daniele, C., Saija, A., Mazzanti, G. and Bisignano, G. (2005) Mechanisms of antibacterial action of three monoterpenes. *Antimicrob Agents Chemother*; **49**: 2474-2478.

Tsuchiya, H., Sato, M., Miyazaki, T., Fujiwara, S., Tanigaki, S., Ohyama, M., Tanaka, T. and Iinuma, M. (1996) Comparative study on the antibacterial activity of phytochemical flavanones against methicillin-resistant *Staphylococcus aureus*. *J Ethnapharmacol*; **50**: 27-34.

Ulrich-Merzenich, G., D., P., Zeitler, H., Vetter, H. and Wagner, H. (2010) Drug development from natural products: Exploiting synergistic effects. *Ind J Exp Biol*; **48**: 208-219.

Vaara, M. (1992) Agents that increase the permeability of the outer membrane. *Microbiol Rev*; **56**: 395-411.

van der Heijden, I., Levin, A., De Pedri, E., Fung, L., Rossi, F., Duboc, G., Barone, A. and Costa, S. (2007) Comparison of disc diffusion, Etest and broth microdilution for testing susceptibility of carbapenem-resistant *P. aeruginosa* to polymyxins. *Ann Clin Microbiol Antimicrob*; **6**: Article ID 8.

van Vuuren, S. and Viljoen, A. (2011) Plant-based antimicrobial studies - methods and approaches to study the interaction between natural products. *Planta Med*; **77**: 1168-1182.

Vattem, D. A., Mihalik, K., Crixell, S. H. and McLean, R. J. C. (2007) Dietary phytochemicals as quorum sensing inhibitors. *Fitoterapia*; **78**: 302-310.

Ventola, C. L. (2015) The antibiotic resistance crisis: Part 1: Causes and threats. *Pharmacy and Therapeutics*; **40**: 277-283.

Verdrengh, M., Collins, L. V., Bergin, P. and Tarkowski, A. (2004) Phytoestrogen genistein as an anti-*Staphylococcal* agent. *Microb Infect*; **6**: 86-92.

Verstraeten, N., Braeken, K., Debkumari, B., Fauvart, M., Fransaer, J., Vermant, J. and Michiels, J. (2008) Living on a surface: swarming and biofilm formation. *Trends Microbiol*; **16**: 496-506.

Visalli, M. A., Jacobs, M. R. and Appelbaum, P. C. (1988) Determination of activities of levofloxacin, alone and combined with gentamicin, ceftazidime, cefpirome, and meropenem, against 124 strains of *Pseudomonas aeruginosa* by checkerboard and time-kill methodology. *Antimicrob Agents Chemother*; **42**: 953-955.

von Wintersdorff, C. J. H., Penders, J., van Niekerk, J. M., Mills, N. D., Majumder, S., van Alphen, L. B., Savelkoul, P. H. M. and Wolffs, P. F. G. (2016) Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Front Microbiol*; **7**, Article ID :173.

Wach, J.-Y., Bonazzi, S. and Gademann, K. (2008) Antimicrobial surfaces through natural product hybrids. *Angew Chem Int Ed*; **47**: 7123-7126.

Wagner, H. and Ulrich-Merzenich, G. (2009) Synergy research: Approaching a new generation of phytopharmaceuticals. *Phytomedicine*; **16**: 97-110.

Walberg, M., Gaustad, P. and Steen, H. B. (1999) Uptake kinetics of nucleic acid targeting dyes in *S. aureus*, *E. faecalis* and *B. cereus*: a flow cytometric study. *J Microbiol Methods*; **35**: 167-176.

Walker, E. S. and Levy, F. (2001) Genetic trends in a population evolving antibiotic resistance. *Evolution*; **55**: 1110-1122.

Wang, L. and Weller, C. L. (2006) Recent advances in extraction of nutraceuticals from plants. *Trends Food Sci Technol*; **17**: 300-312.

Wang, S.-Y., Sun, Z.-L., Liu, T., Gibbons, S., Zhang, W.-J. and Qing, M. (2014) Flavonoids from *Sophora moorcroftiana* and their Synergistic Antibacterial Effects on MRSA. *Phytother Res*; **28**: 1071-1076.

War, A. R., Paulraj, M. G., Ahmad, T., Buhroo, A. A., Hussain, B., Ignacimuthu, S. and Sharma, H. C. (2012) Mechanisms of plant defense against insect herbivores. *Plant Signal Behav*; **7**: 1306-1320.

Watkins, F., Pendry, B., Sanchez-Medina, A. and Corcoran, O. (2012) Antimicrobial assays of three native British plants used in Anglo-Saxon medicine for wound healing formulations in 10th century England. **144**: 408-415.

Weber, D. J., Rutala, W. A., Miller, M. B., Huslage, K. and Sickbert-Bennett, E. (2010) Role of hospital surfaces in the transmission of emerging health care-associated pathogens: *Norovirus*, *Clostridium difficile*, and *Acinetobacter* species. *Am J Infect Control*; **38**: 25-33.

White, R. L., Burgess, D. S., Manduru, M. and Bosso, J. A. (1996) Comparison of three different *in vitro* methods of detecting synergy: time-kill, checkerboard, and E test. *Antimicrob Agents Chemother*; **40**: 1914-1918.

WHO. (2011) World Health Organization (WHO)'s Report: European strategic action plan on antibiotic resistance. WHO Regional Committee for Europe, EUR/RC61/14, Copenhagen, Denmark.

WHO. (2015) World Health Organization (WHO)'s Report: Global Tuberculosis Control. WHO/HTM/TB/2015.22; 20th edition.

Wiegand, I., Hilpert, K. and Hancock, R. E. W. (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protoc*; **3**: 163-175.

Wishart, D. S. (2008) Quantitative metabolomics using NMR. *TrAC Trends Analyt Chem*; **27**: 228-237.

Wolf, R., Baroni, A., Greco, R., Donnarumma, G., Ruocco, E., Tufano, M. A. and Ruocco, V. (2002) Quinine sulfate and bacterial invasion. *Ann Clin Microbiol Antimicrob*; **1**: Article ID 5.

Wolf, R., Tufano, M. A., Ruocco, V., Grimaldi, E., Ruocco, E., Donnarumma, G. and Baroni, A. (2006) Quinine sulfate inhibits invasion of some bacterial skin pathogens. *Int J Dermatol*; **45**: 661-663.

Wolska, K., Grudniak, A., Fiecek, B., Kraczkiewicz-Dowjat, A. and Kurek, A. (2010) Antibacterial activity of oleanolic and ursolic acids and their derivatives. *Cent Eur J Biol*; **5**: 543 -553.

Woods, C. R. (2009) Macrolide-inducible resistance to clindamycin and the D-test. *Pediatric Infect Dis J*; **28**: 1115-1118.

Worley, B. and Powers, R. (2013) Multivariate analysis in metabolomics. *Curr Metabolomics*; **1**: 92-107.

Wright, G. D. (2003) Mechanisms of resistance to antibiotics. *Curr Opin Chem Biol*; **7**: 563-569.

Wright, G. D. (2005) Bacterial resistance to antibiotics: Enzymatic degradation and modification. *Adv Drug Deliver Rev*; **57**: 1451-1470.

Wright, G. D. (2007) The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat Rev Microbiol*; 5: 175-186.

Wright, G. D. (2010) Antibiotic resistance in the environment: a link to the clinic? *Curr Opin Microbiol*; **13**: 589-594.

Wu, T., He, M., Zang, X., Zhou, Y., Qiu, T., Pan, S. and Xu, X. (2013) A structure–activity relationship study of flavonoids as inhibitors of *E. coli* by membrane interaction effect. *BBA-Biomembranes*; **1828**: 2751-2756.

Xu, K. D., McFeters, G. A. and Stewart, P. S. (2000) Biofilm resistance to antimicrobial agents. *Microbiology*; **146**: 547-549.

Yam, T. S., Hamilton-Miller, J. M. and Shah, S. (1998) The effect of a component of tea (*Camellia sinensis*) on methicillin resistance, PBP2' synthesis, and beta-lactamase production in *Staphylococcus aureus*. *J Antimicrob Chemother*; **42**: 211-216.

Yang, Z.-C., Wang, B.-C., Yang, X.-S., Wang, Q. and Ran, L. (2005) The synergistic activity of antibiotics combined with eight traditional Chinese medicines against two different strains of *Staphylococcus aureus*. *Colloid Surface B*; **41**: 79-81.

Yap, P. S. X., Yiap, B. C., Ping, H. C. and Lim, S. H. E. (2014) Essential oils, a new horizon in combating bacterial antibiotic resistance. *Open Microbiol J*; **8**: 6-14.

Yu, H.-H., Kim, K.-J., Cha, J.-D., Kim, H.-K., Lee, Y.-E., Choi, N.-Y. and You, Y.-O. (2005) Antimicrobial activity of berberine alone and in combination with ampicillin or oxacillin against methicillin-resistant *Staphylococcus aureus*. *J Med Food*; **8**: 454-461.

Yuliana, N. D., Khatib, A., Choi, Y. H. and Verpoorte, R. (2011) Metabolomics for bioactivity assessment of natural products. *Phytother Res*; **25**: 157-169.

Zahin, M., Aqil, F., Khan, M. S. A. and Ahmad, I. (2010) Ethnomedicinal plants derived antibacterials and their prospects. In: Chattopadhyay D, ed. *Ethnomedicine: a source of complementary therapeutics*. Kerala: Research Signpost; 149-178.

Zdzisława, N. (2007) A review of anti-infective and anti-inflammatory chalcones. *Eur J Med Chem*; **42**: 125-137.

Zechini, B. and Versace, I. (2009) Inhibitors of multidrug resistant efflux systems in bacteria. *Recent Pat Antiinfect Drug Discov*; **4**: 37-50.

Zhang, A., Sun, H., Wang, P., Han, Y. and Wang, X. (2012) Recent and potential developments of biofluid analyses in metabolomics. *J Proteomics*; **75**: 1079-1088.

Zhang, D., Hu, H., Rao, Q. and Zhao, Z. (2011) Synergistic effects and physiological responses of selected bacterial isolates from animal feed to four natural antimicrobials and two antibiotics. *Foodborne Pathog Dis*; **8**: 1055-1062.

Zhao, W.-H., Hu, Z.-Q., Okubo, S., Hara, Y. and Shimamura, T. (2001) Mechanism of synergy between epigallocatechin gallate and β-lactams against methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother*; **45**: 1737-1742.

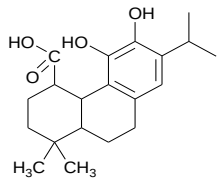
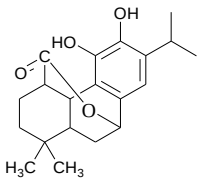
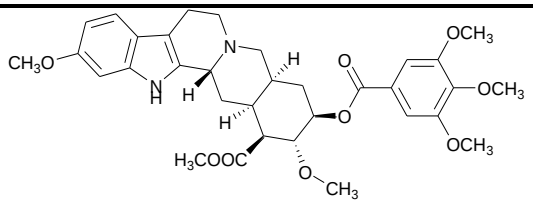
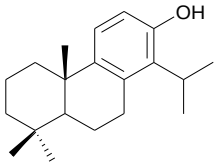
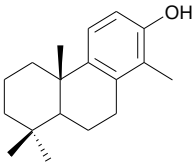
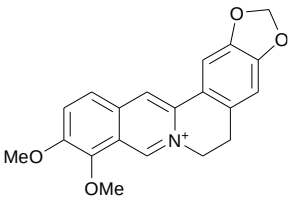
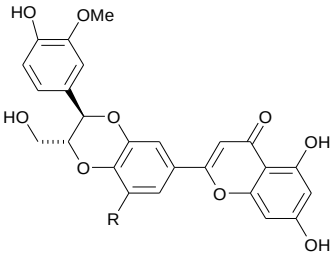
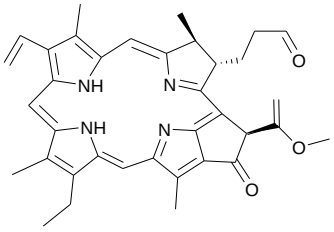
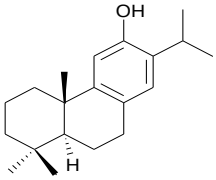
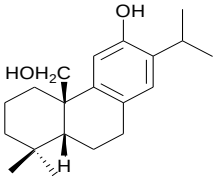
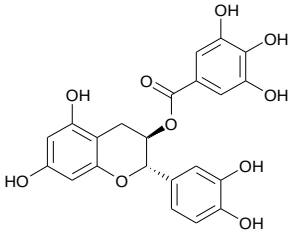
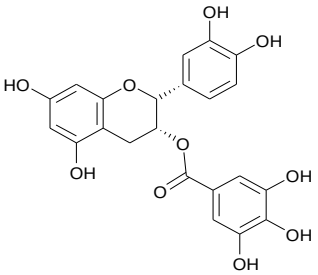
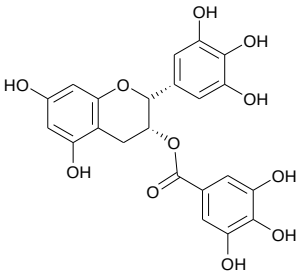
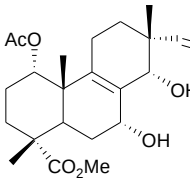
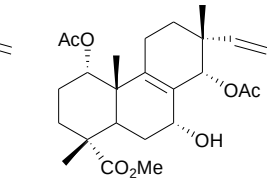
Zimmermann, G. R., Lehár, J. and Keith, C. T. (2007) Multi-target therapeutics: when the whole is greater than the sum of the parts. *Drug Discov Today*; **12**: 34-42.

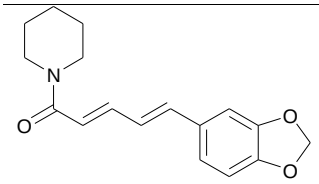
Zollfrank, C., Gutbrod, K., Wechsler, P. and Guggenbichler, J. P. (2012) Antimicrobial activity of transition metal acid MoO3 prevents microbial growth on material surfaces. *Mater Sci Eng C*; **32**: 47-54.

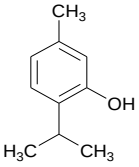
APPENDIX

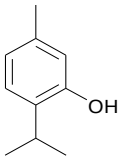
A1: CHEMICAL STRUCTURES OF IMPORTANT PHYTOCHEMICALS REPORTED FOR AN ANTIBIOTIC-POTENTIATING ACTIVITY

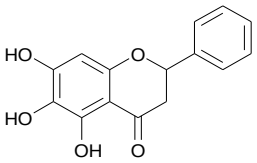
TABLE A.1 | Chemical structures of the several adjuvant molecules promoting antibiotic potentiation due to a resistance-modifying effect (described in *Section 2.6.2*)

			
Carnosic acid	Carnosol	Reserpine	
			
Totarol	Diterpene 416	Berberine	5*-methoxyhydnocarpin
			
Pheophorbide a	Ferruginol	5-Epipisiferol	Catechin gallate
			
Epicatechin gallate	Epigallocatechin gallate	Methyl-1α-aceto-xy-7α-14α-dihydroxy-8,15-isopimaradien-18-oate;	Methyl-1α,14α-diacetoxy-7α-hydroxy-8,15-isopimaradien-18-oate


Piperine


Thymol


Carvacrol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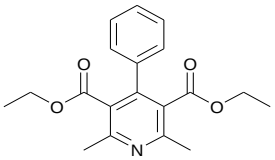
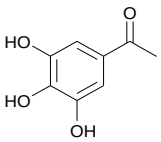
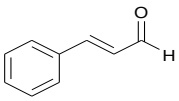
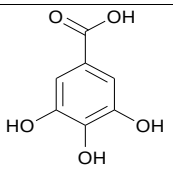
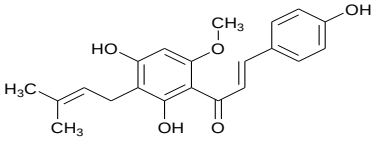
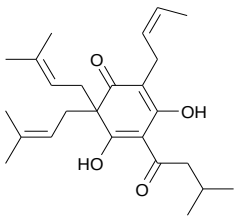

Dimethyl-4-phenyl-pyridi-ne-3,5-dicar-boxylic acid diethyl ester;

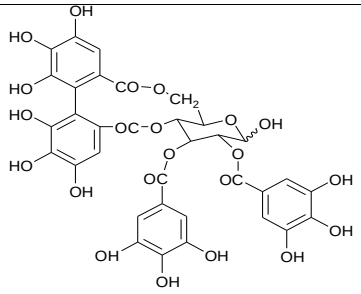

Ethyl gallate


Cinnamaldehyde


Gallic acid


Xanthohumol


Lupulon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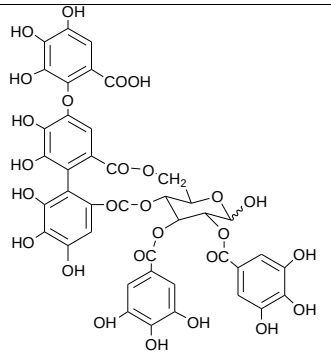
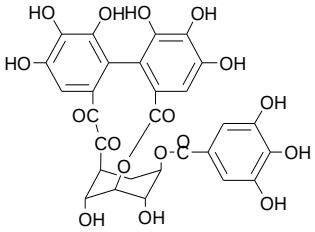
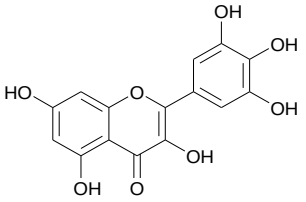
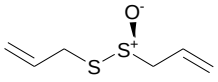
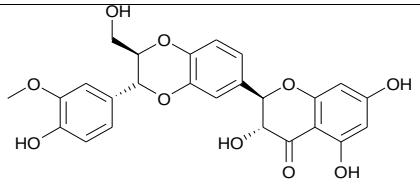
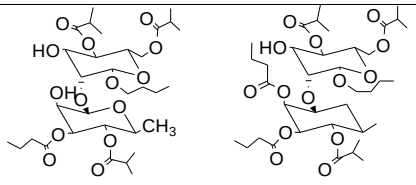

Rugosin B

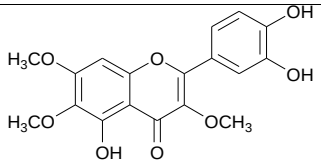

Corilagin

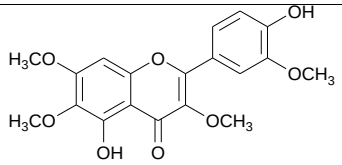

Myricetin

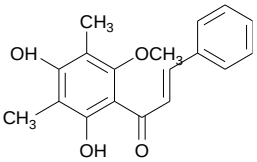

Allicin

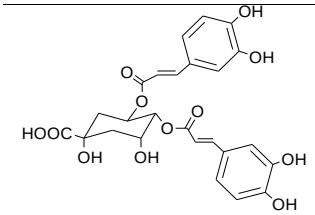

Silybin

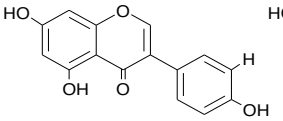

Polyacylated neohesperidoses

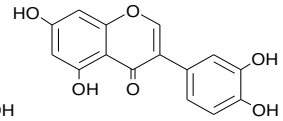

Chrysosplenol-D

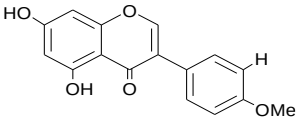

Chrysoplenetin


Chalcone


4’,5’-O-dicaffeoylquinic acid


Genistein


Orobol


Biochanin A

A2: ETHNOPHARMACOLOGICAL RELEVANCE OF THE PLANTS TESTED

TABLE A.2 | Classification and description of the plants tested for their ethnopharmacological relevance

Plant name	Class*	Ethnopharmacological relevance
Acacia dealbata	-	-
Cytisus striatus	-	-
Genista tridentata	-	-
Prunus domestica	2	Febrifuge, laxative, stomachic
Prunus avium	2	Antitussive, astringent, diuretic, tonic
Prunus persica	3	Astringent, demulcent, diuretic, expectorant, febrifuge, laxative, parasiticide and mildly sedative; used to treat gastritis, whooping cough, coughs and bronchitis and to help heal sores and wounds
Pyrus communis	1	-
Agrimonia eupatoria	3	Popular domestic remedy for diarrhea and to treat wounds and skin problems; antiaphonic, astringent, blood purifier, cholagogue, diuretic, tonic, vulnerary
Eriobotrya japonica	3	Analgesic, antibacterial, antiemetic, antitussive, antiviral, astringent, diuretic
Crataegus monogyna	5	Well-known in herbal folk medicine as heart tonic; astispasmidisc, astringent, cardiotonic, diuretic, hypotensive, sedative, tonic, vasodilator
Rubus idaeus	3	Anti-inflammatory, astringent, decongestant, ophthalmic, oxytocic and stimulant
Malus communis	2	Antibacterial; antidiarrhoeal; astringent; laxative; odontalgic; stoma chic
Eupatorium cannabinum	3	Detoxifying herb for fevers, colds, flu and other viral conditions; alterative, cholagogue, depurative, diuretic, emetic, expectorant, febrifuge, purgative, tonic
Centaurea nigra	2	Diaphoretic, diuretic, tonic, once had reputation as healer of wounds
Physalis angulata	1	Diuretic, expectorant, febrifuge
Cyphomandra betacea	-	-
Nerium oleander	2	Cardiotonic, diaphoretic, diuretic, emetic, expectorant and sternutatory
Trachelospermum jasminoides	2	Estorative and tonic
Eucalyptus globulus	4	Traditional Aboriginal herbal remedy used for coughs and colds, sore throats and other infections; antiperiodic, antiseptic, aromatic, deodorant, expectorant, febrifuge, hypoglycaemic and stimulant

Calluna vulgaris	2	Medicinal use in folk medicine as good urinary antiseptic and diuretic
Ficus carica	2	Stomachic
Castanea sativa	2	Treatment of fevers and ague, convulsive coughs such as whooping cough, anti-inflammatory, astringent, expectorant and tonic
Juglans regia	3	To treat constipation, chronic coughs, asthma, diarrhoea, dyspepsia, skin ailments and purify the blood; alterative, anthelmintic, anti-inflammatory, astringent and depurative;
Diospyros kaki	3	-
Vitis vinifera	2	Anti-inflammatory, astringent
Fraxinus excelsior	2	Cathartic, diaphoretic, mildly diuretic, laxative, purgative, treatment of fever and rheumatism
Actinidia chinensis	2	-
Buxus sempervirens	2	Sedative, alterative, antirheumatic, cathartic, cholagogue, diaphoretic, febrifuge, oxytocic and vermifuge; to treat syphilis, and also used as quinine substitute in the treatment of malaria
Pteridium aquilinum	2	Treatment for arthritis

(-) medicinal use not known (for the plant leaves); (*) classification according to the ethnopharmacological relevance described by the database PFAF (2016) as: 1 = very minor uses, 2 = reasonably useful plants, 3 = could be grown as standard crops, 4= very useful plants, 5 = great value.

A3: PHOTO-GALLERY

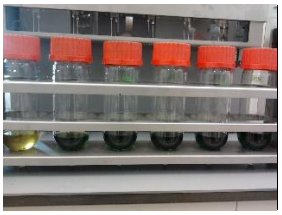
Cytisus striatus flower

Plant extracts

Fractionation of extracts (with water and ethyl acetate)

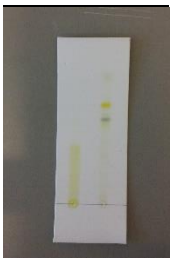
Rotaevaporation of plant extracts

Multi-extractor



Vials after one extraction cycle

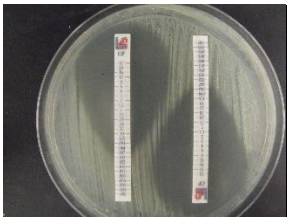
MPLC – medium pressure liquid chromatography (with different columns)



TLC plate

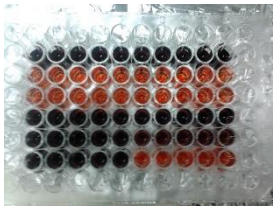
Plant-incorporated MH agar

Disc diffusion method

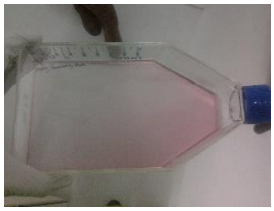


Etest

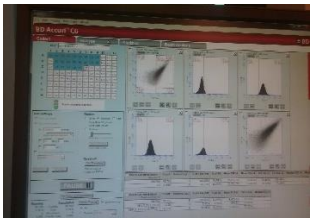
96-well microtiter plates for MIC determination or biofilm formation



Cytotoxicity assay with MTS



Growing mouse lung fibroblast cell culture



Flow cytometry

FIGURE A.1 | Photo-gallery of diverse methodologies performed along this project.