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Abstract: Background: The intensive use of antibacterial and antifungal drugs has dramatically increased the microbial resistance and has led to a higher number of difficult-to-eradicate infections. Combination therapy with two or more antimicrobial drugs has emerged some years ago to overcome the issue, but it has proven to be not completely effective. Natural secondary metabolites of MW ≤ 500 represent promising adjuvants for antimicrobials and have been the object of several researches which have increased in the last two decades.
Purpose: The purpose of this Review is to do a literature search of the natural compounds that showed high enhancing capacity of antibacterials' and antifungals' effects against bacteria and fungi and to analyze which are the natural products most used in combination with a focus on polyphenols and terpenoids.
Results: One hundred of papers were collected for reviewing. Fifty six (56) of them deal with combinations of low MW natural products with antibacterial drugs against bacteria and forty four (44) on natural products with antifungal drugs against fungi. Of the antibacterial adjuvants, 41 (73 %) were either polyphenols (27; 48%) or terpenes (14; 25 %). The remaining 15 papers (27%), deal with different class of natural products. Since most natural potentiators belong to the terpene or phenolic structural types, a more detailed description of the works dealing with these type of compounds is provided here. Bacterial and fungal resistance mechanisms, the modes of action of the main classes of antibacterial and antifungal drugs and the methodologies most used to assess the type of interactions in the combinations were included in the Review too.
Conclusions and perspectives
Several promising results on the potentiation of antifungal and antibacterial activities by low MW natural products mainly polyphenols and terpenes were reported in the literature and, in spite of that most

works included only in vitro assays, this knowledge opens a wide range of possibilities for the combination antimicrobial therapy. Further research including in vivo assays and clinical trials are required to determine the relevance of these antimicrobial enhancers in the clinical area and should be the focus of future studies in order to develop new antimicrobial combination agents that overpass the drawbacks of the existing antibiotics and antifungals in clinical use.

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Submission letter and authors agreement

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October 19th, 2017

Prof. Dr. Hildebert Wagner
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Editors of the Special Issue Hybrid Combinations
Phytomedicine

Dear Prof. Wagner and Prof. Efferth:

Please find attached the review article entitled "Plant phenolics and terpenoids as adjuvants of antibacterial and antifungal drugs**"** authored by Susana Zacchino, Estefanía Butassi, Melina Di Liberto, Marcela Raimondi, Agustina Postigo and Maximiliano Sortino with myself as corresponding author, which is submitted for publication in the special issue 'Hybrid Combinations' in Phytomedicine.

This work has not been published elsewhere and is not under active consideration by another Journal. All of the authors have read and approved the manuscript.

Sincerely yours,

Prof. Susana Zacchino
Corresponding author

AUTHOR DECLARATION TEMPLATE

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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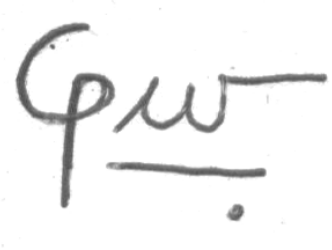

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Plant phenolics and terpenoids as adjuvants of antibacterial and antifungal drugs

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Keywords: Potentiation, Low molecular weight natural products, Combination, Antibacterial drugs; Antifungal drugs.

Abbreviations: ABC: ATP-Binding Cassette; AMPH: Amphotericin B; AMC: Amoxicillin:Clavulanic acid; AMP: ampicillin; CAZ: ceftazidine; CD: clerodane diterpene 16α-hydroxycleroda-3,13(14)-*Z*-dien-15,16-olide; CDR; *Candida* drug-resistance; CEP: cephapirin; CFU: Colonies forming units; CIP: Ciprofloxacin; CLSI: Clinical Laboratory Standards Institute; CPM: Carbapenem; CUR: Curcumin; DAP: Daptomycin; DRI: Dose Reduction Index; EGCg: Epigallocatechingallate; ECV: epidemiologic cut-off values; EPI: Efflux Pump Inhibitors; ERY: Erythromycin; ERSA: Erythromycin-resistant *Staphylococcus aureus*; EUCAST: European Committee on Antimicrobial Susceptibility Testing; FIC: Fractional Inhibitory Concentration; FICI: Fractional Inhibitory Concentration Index; FCZ: Fluconazole; ILSMR: Intensifiers Specifically of β-Lactam against Methicillin-resistant *Staphylococcus aureus*; IMP: Imipenem; ITZ: Itraconazole; KTZ: Ketoconazole; LVX: Levofloxacin; LZD: Linezolid; MCZ: Miconazole; MDR Multidrug resistant; MFS: Major facilitator superfamily; MIC: Minimum Inhibitory Concentration; MPM: Meropenem; MRSA: Methicillin-Resistant *Staphylococcus aureus*; MSSA: Methicillin Sensitive *Staphylococcus aureus*; MW: Molecular weight; Nor: Norfloxacin; OFL: Ofloxacin; OXA: Oxacillin; OXY: Oxytetracicline; PCZ: Posaconazole; PBP: Penicillin Binding Protein; PEN: Penicillin; PG: propyl gallate; PRSP: Penicillin resistant *Streptococcus pneumoniae*; PIP: Piperacillin; PPM: Panipenem; QUI: Quinolone; RNA: Ribonucleic acid; Sulb: Sulbactame; Str: Streptomycin; Ter: Terbinafine; TET: Tetracyclin; TRSA Tetracyclin-resistant *Staphylococcus aureus*; VCZ: Voriconazole; VRE: Vancomycin-resistant *Enterococcus spp.*; VRSA: Vancomycin-resistant *Staphylococcus aureus*.

ABSTRACT

Background: The intensive use of antibacterial and antifungal drugs has dramatically increased the microbial resistance and has led to a higher number of difficult-to-eradicate infections. Combination therapy with two or more antimicrobial drugs has emerged some years ago to overcome the issue, but it has proven to be not completely effective. Natural secondary metabolites of MW ≤ 500 represent promising adjuvants for antimicrobials and have been the object of several researches which have increased in the last two decades.

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Conclusions and perspectives

Several promising results on the potentiation of antifungal and antibacterial activities by low MW natural products mainly polyphenols and terpenes were reported in the literature and, in spite of that most works included only in vitro assays, this knowledge opens a wide range of possibilities for the combination antimicrobial therapy. Further research including *in vivo* assays and clinical trials are required to determine the relevance of these antimicrobial enhancers in the clinical area and should be the focus of future studies in order to develop new antimicrobial combination agents that overpass the drawbacks of the existing antibiotics and antifungals in clinical use.

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Introduction

The intensive use of antibacterial and antifungal drugs has dramatically increased the frequency of microbial resistance (Andersson and Diarmaid, 2010) and has led to an increase of difficult-to-eradicate infections. To overcome the issue, combination therapy with two or more antimicrobial drugs has emerged some years ago (Cuenca Estrella, 2004) in the belief that they can achieve a reversal of microbial resistance with lower quantities of each substance and can also lower the known antimicrobial drugs' toxic side-effects (Lewis and Kontoyiannis, 2001). In spite of the many advantages of combination therapy, several reports have proven that it has failed in several patients (Kristiansen et al., 2007) possibly due to the efficacy relies largely on the results of the *in vitro* studies and experimental animal models and evidences from well-designed clinical trials are lacking (Cuenca Estrella, 2004).

In the last years, the testing of combinations of antimicrobial drugs with non-antimicrobial compounds (therapeutic agents not originally designed for this purpose) appears to be a new promising strategy to cope with treatment failures (Bush et al., 2011; Ejim et al., 2011; Lehtinen and Lilius, 2007). As an example, Afeltra et al. (2004) reported the *in vitro* positive interactions between itraconazole (ITZ) and seven different non-antimicrobial membrane-active compounds against ITZ-susceptible and ITZ-resistant *Aspergillus fumigatus* strains.

Among the non-antimicrobial compounds, natural metabolites of MW ≤ 500 may represent promising adjuvants of antimicrobials' effects (Hemaiswarya et al., 2008; Langeveld et al., 2014).

According to previous reports (Wagner and Ulrich Merzenich, 2009) the potentiation of the antimicrobial activity by a natural product can be achieved by different mechanisms such as (i) multi-target effect, in which each compound targets a different site in the microbial cell; (ii) pharmacokinetic or physicochemical effects (i.e. improvement of solubility or bioavailability of the antimicrobial drug); (iii) targeting a specific resistance mechanism of microorganisms that is the major challenge of the combination therapy.

In this Review, we have made a literature search in order to have a look into the natural low MW metabolites that have shown enhancing microbial growth inhibition capacity of antibacterials (antibiotics) and antifungals against bacterial and fungal planktonic cells. Of them, a detailed analysis of the terpenoid or phenolic structures is provided. Previously, the most used methodologies to assess the antimicrobial effects of compounds alone or in combination was added to the Results section in order to a better comprehension of the results.

In addition, the main classes of antibacterial and antifungal drugs and their targets, the mechanisms of resistance for each type of drugs were included with the aim of facilitating the

understanding on how the combination of an antibacterial or antifungal drug with a low MW natural product can work.

Materials and methods

Search strategy

The search for suitable papers was performed in Internet databases (PubMed, Sciencedirect and other web pages, by using the following keywords: “bacterial infections”, “fungal infections” “planktonic cells”, “secondary metabolites”, “enhance”, “enhancers”, “synergism”, “natural products”, “potentiators”; “antifungal drugs”, “antibacterial drugs”, “chemosensitizing agents”, “in vitro”, “in vivo”. Additional papers were included in our collection after surveying the references from the selected articles. We explored articles that use *in vitro* and *in vivo* experimental systems.

Data extraction

The information gathered from the chosen articles included: the structures of natural potentiators; the concentrations at which they act as enhancers; the fungal or bacterial strains used; the *in vitro* and *in vivo* assays and the assessments of molecular mechanisms of the antimicrobial effects of the combinations. The information was divided into two groups: (a) Natural products in combination with antibacterial drugs against bacterial planktonic cells; (b) Natural products in combination with antifungal drugs against fungal planktonic cells.

Results and discussion

Methodologies most used to assess the type of interactions in the combinations

The analysis of adjuvancy in most of the reviewed works were carried out *in vitro* by using the microdilution assay in the checkerboard design which allows the calculation of the Fractional Inhibitory Concentration (FIC) of each partner and the Fractional Inhibitory Concentration Index (FICI) values for the combinations (see Supplementary material). In some of the works, isobolograms and time-kill studies (Berembaum, 1989; Martínez Irujo et al., 1996; Sun et al.; 2008; White et al., 1996) were also performed. It is worth to take into account that only few studies performed *in vivo* studies and the studies of the mechanism of action of the mixtures are scarce (Ballar and Coote, 2016; Campbell et al., 2012; Gupta et al., 2016; Han, 2007).

The Dose Reduction Index (DRI) (Chou, 2006; 2010), a measure on how many times the MIC of the antimicrobial drug is reduced by its partner when tested in combination (MIC antimicrobial alone/MIC antimicrobial in the mixture) was included in this Review when it was

possible. A greater DRI for an antimicrobial drug is indicative of a greater adjuvant capacity for a given effect level.

Modes of action of the main classes of antibacterial drugs

There are four proven targets for the main antibacterial drugs: (1) bacterial wall biosynthesis; (2) bacterial protein synthesis; (3) bacterial DNA replication and repair and (4) bacterial RNA synthesis (ECDC/EMEA, 2009; Kohanski et al.; 2010, Moore, 2013; Walsh, 2000). Most structural types that act for each mechanism of action are detailed in Table 1.

Insert Table 1

Antibacterial combinations

Bacterial resistance and its mechanisms

The resistance of a bacterium to a given antibiotic is assessed by determining the MIC of the antimicrobial substances against the microorganisms. This information, together with the known pharmacokinetic properties of the substance, allows the characterization of the bacteria as "susceptible", "intermediate" or "resistant" to a given antibiotic (Rodloff et al., 2008). The testing techniques for MIC determination must be standardized to make the test results reproducible, because parameters such as the culture medium, inoculum size, incubating temperature and time, all influence the results. The Clinical Laboratory Standards Institute (CLSI) of the United States and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) of the European Union have established guidelines that allow the harmonization of antimicrobial breakpoints throughout the world and define breakpoints for new agents (Brown et al., 2015).

The bacterial resistance can be classified as clinical and microbiological and, in turn, it can be primary (intrinsic) or secondary (acquired). Bacteria can show intrinsic resistance as a result of its own structural characteristics (Blair et al., 2015) (Fig. 1) or can also acquire it *via* mutations of chromosomal genes and by horizontal gene transfer (Andersson and Hughes, 2009; Sandegren and Andersson, 2009).

Insert Fig. 1

In general the bacterial resistance can be mediated by several mechanisms that fall into three main groups: (a) those that minimize the intracellular concentrations of the antibiotic as a result of efflux or poor penetration into the bacterium; (b) those that modify the antibiotic target by

genetic mutation or post-translational modification; (c) those that inactivate the antibiotic by hydrolysis or modification. All these mechanisms have been reviewed by Blair et al. (2015).

Regarding mechanism (a), the overexpression of efflux-pumps (that actively transport many antibiotics out of the cell) is a common mechanism that minimizes the intracellular concentration of the antibiotic. Also the limiting antibiotic entry into the bacterial cell is achieved by the downregulation of porins or by the replacement of porins with more-selective channels; i.e. the one that occurs in clinically relevant resistance to carbapenems (CPM) in enterobacteria when mutations reduce porin production or when mutant porin alleles are present (Baroud et al., 2013; Wozniak et al., 2012). Regarding mechanism (b), changes of the target structure can prevent efficient antibiotic binding, confer resistance although still enable the target to carry out its normal function. In mechanism (c), bacteria can destroy or modify antibiotics either by hydrolysis such as the action of penicillinase (β-lactamase) or by transfer of a chemical group, thus preventing the antibiotic from binding to its target protein as a result of steric hindrance. Several examples of resistant bacteria and the antibiotics to what they show resistance are listed in Table 2.

Insert Table 2

Out of the many antibiotic-resistant bacteria listed in Table 2, the methicillin-resistant *Staphylococcus aureus* (MRSA); vancomycin-resistant *S. aureus* (VRSA); vancomycin-resistant *Enterococcus* spp. (VRE); PEN-resistant *Streptococcus pneumoniae* (PRSP); cephalosporin-resistant *Escherichia* coli or *Klebsiella pneumoniae*; CPM-resistant *K. pneumonia* and CPM-resistant *Pseudomonas aeruginosa* have showed to be the responsible of the most frequently bloodstream difficult-to eradicate infections [ECDC/EMEA (2009)].

Of them, MRSA was the most used target for assessing the interactions between an antibiotic and a low MW natural product in the papers reviewed here, although tetracycline (TET), erythromycin (ERY) and quinolone (QUI)-resistant *S. aureus* were also used. Other target bacteria used in the reviewed papers were resistant strains of *E. coli*, *Enterobacter aerogenes, Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes*.

As it is well-known, *S. aureus* is a gram-(+) bacteria that can cause a wide range of clinical diseases, including skin and soft tissue infections, pneumonia, bloodstream infections, infective endocarditis and osteomyelitis and more severe infections such as necrotizing pneumonia, necrotizing fasciitis and sepsis (Wenzel and Perl, 1995; Weinke et al., 1992). Its ongoing ability to quickly acquire resistance to antimicrobials is a characteristic of *S. aureus* that was evidenced by

the appearance of MRSA in 1963, only three years later that methicillin was introduced in the market (1960) (Jevons et al., 1963).

Natural products in combination with antibacterial drugs against bacterial planktonic cells

A summary of the natural low MW compounds that potentiate the antibacterial activity of antibiotics is showed in Tables 3 and 4 and an amplified overview of some of them is detailed below. It is worth to take into account that of the natural products that proved adjuvancy inhibitory capacity of antibacterials, about 73% deal with polyphenols (48%) and terpenoids (25%). Of them, we have selected the most studied compounds and analyzed their potentiation capacity.

Insert Tables 3 and 4.

Combinations of polyphenols with antibacterial drugs

The structures of the main polyphenols that showed capacity for enhancing the activity of antibacterial drugs are presented in Fig. 2 and a summary of the main natural phenols that potentiate the capacity of different type of antibiotics is presented in Table 3.

Insert Fig. 2

Epigallocatechingallate

(-)-Epigallocatechingallate (EGCg) is the most extensively studied polyphenol in combination with antibiotics (Hu et al., 2001; 2002a; 2002b; Zhao et al., 2001, 2002; Novy et al., 2013; Sudano-Rocaro et al., 2004).

The group of Hu et al. published five papers on this issue. In the first paper, Hu et al. (2001) tested the activity of the mixture ampicillin (AMP):sulbactame (Sulb) (2:1) against 28 isolates of producing and non-producing β-lactamase MRSA from Fujigaoka and Hatanaoka hospitals of Showa University (Japan) in combination with different concentrations of EGCg. Results of this work showed that the activity of Amp:Sulb against β-lactamase-producing MRSA was potentiated 4-8 and 8-32 times when combined with 6.25 or 25 μg/ml of EGCg respectively, thus reaching the susceptibility breakpoint. Time-kill curves corroborated the synergistic activities of the mixtures against β-lactamase-producing and non-producing MRSA.

In a second work, the same group (Zhao et al., 2001) reported that MICs of penicillin (PEN) showed DRI values in the range 2-8, 2-16 and 8-32 when combined with 6.25, 12.5 and 25 μg/ml

of EGCg respectively, against 25 of the MRSA clinical isolates used in the previous paper. In addition, MICs of oxacillin (OXA) decreased 4-16, 4-32 and 8-64- fold respectively when combined with EGCg at the different concentrations. A lower drop was showed against Methicillin Sensitive *S. aureus* (MSSA) and no potentiation was observed against *E. coli.* Regarding the mechanism of action, the combinations between EGCg and β-lactam antibiotics induced damage of the bacterial cell-wall through direct binding to peptidoglycans and appeared not to have a direct relation with a PEN binding protein (PBP) synthesis or production, because it is not specific to MRSA.

In a third paper, Hu et al. (2002a) found that EGCg potentiates the anti-MRSA activity of the carbapenems (CPMs) imipenem (IMP), panipenem (PPM) and meropenem (MPM), against 24 clinical isolates of MRSA getting a range of DRIs = 2-512 in CPMs' MIC_{50} when combined with 1.56-25 μg/ml of EGCg. The MICs of IMP in the presence of EGCg were restored to the susceptibility breakpoint (≤ 4 μg/ml) in 8-75% of MRSA isolates. In two further papers (Zhao et al., 2002; Hu et al., 2002b), the group reported that the combination of EGCg with PEN showed synergism with 21 clinical isolates of penicillase-producing *S. aureus*. The results demonstrated that besides the effect of EGCg on the cell wall (Zhao et al., 2001), the direct inhibition of penicillinase activity by EGCg is responsible for the observed synergism. EGCg destroys the penicillinase activity, protecting PEN from inactivation.

In a further paper Sudano Rocaro et al. (2004) demonstrated that EGCg was able to reverse TET resistance in staphylococcal isolates expressing the specific efflux pump TET(K) and appeared to improve the MICs of TET with DRIs 256 and 16, for resistant and susceptible *Staphylococcus epidermidis* and *S. aureus* isolates, respectively.

A recent paper (Novy et al., 2013) explored the *in vitro* effect of EGCg in combination with oxytetracycline (OXY) against eight standard and clinical resistant isolates of *S. aureus*, including MRSA, TET-resistant (TRSA) and ERY-resistant (ERSA) *S. aureus* strains. Results showed a potentiation of 8-10 fold of EGCg on antibiotics against all *S. aureus* tested strains [two of them multidrug-resistant (MDR)]. Authors confirmed the enhancements through the construction of isobolograms (Berembaum, 1981; Wagner and Ulrich-Merzenich 2009). Some of the isoboles are shown in Fig. 3.

Insert Fig. 3

The fact that EGCg is easily available for natural sources [it is one of the major components of *Camellia sinensis* (L.) Kuntze (Theaceae) leaves] and is commercially available at low prices,

makes its combination with antibiotics highly promising for the future development of a new antibacterial two-components medicine.

Alkyl gallates

A series of alkyl gallates (from methyl to dodecyl gallates and stearyl gallate) demonstrated to be intensifiers specifically of β-lactam antibiotics (ILSMR) against MRSA when tested along with four β-lactam and nine non-β-lactam antibiotics (Shibata et al., 2005).

The lenght of the alkyl chain played a role on the intensifying activity, being C5 and C6 the optimum lenght (FICI=0.07-0.41), although the galloyl moiety showed to play a role too. Particulary isoamyl gallate enhances the MIC_{50} of PEN, AMP, cephapirin (CEP) and OXA against MRSA and MSSA in 32-256, and ~ 2-4-fold respectively. It also potentiates the MIC_{90} of the same antibiotics with DRIs 8->42 and 8-32 respectively. Interesting enough, FICI against MSSAs predominantly showed indifference.

In a further paper, Shibata et al. (2009) tested triple combinations of a short (propyl) and long (octyl) chains gallates plus OXA and FICIs ≤ 0.031 were obtained with 25 μg/ml propyl gallate (PG) and 12.5 μg/ml octyl gallate (OG) (DRIs > 32-256). Another gallates and alkyl gallates were tested in combination with antibiotics against MRSA (Stapleton et al., 2004).

Curcumin

Curcumin (CUR) isolated from the rhizome of *Curcuma longa* L. (Zingiberaceae), showed to possess a high capacity to enhance the antimicrobial activity of several antibiotics against MRSA and MSSA (Mun et al., 2013; 2014).

CUR showed to be a very good enhancer of OXA against MRSA and MSSA, although CUR potentiates also the activity of CIP, AMP and Norfloxacin (Nor), as well. The results were corroborated with time-kill assays. When used together, ½ MIC CUR + ½ MIC OXA caused an over 3 $\log_{10}$-fold in the Colonies-Forming Units *per* ml (CFU/ml) against the tested strains. Also CUR was tested in combination with IPM against *E. coli* and *K. pneumoniae* (Gunes et al., 2013).

In a recent paper, Joshi et al. (2014) tested the combination of CUR with CIP with the checkerboard assay and showed that CUR reverses the resistance of *S. aureus* to CIP, with 8-fold reduction in the MIC of CIP against a *S. aureus* strain that overexpress the NorA efflux pump. This is a trans-membrane pump in which two major binding cavities (site 1 and site 2) were observed. Site 1 is surrounded by a large number of transmembrane loops and is located deeper in the efflux pump. CIP optimally binds to site 1 through a strong H-bonding of the carboxyl

functionality with the cationic guanido group of Arg98. However, when combined with CUR, hydrophobic interactions as well as H-bonding interactions of CIP were mimicked by CUR which probably block the entry of CIP (a Nor A pump substrate) into site 1 (Fig. 4).

Insert Fig. 4

The antibacterial effect of CUR in combination with piperacillin (PIP), MPM and levofloxacin (LVX) was investigated In a more recent paper (Ballard and Coote, 2016) by employing both *in vitro* and *in vivo assays* against both a *P. aeruginosa* wild type and a strain that overexpresses efflux pump to PIP, MPM and LVX, with or without the presence of CUR. CUR demonstrated a restorative effect on the activity of PIP, MEM and LVX *versus* a *P. aeruginosa* strain with an efflux-pump-mediated MDR phenotype *in vitro*. Then, *in vivo* studies of CUR+PIP or CUR+LVX with *Galleria mellonella* larvae infected with MDR strains of *P aeruginosa* (possessing an efflux-pump-mediated phenotype) resulted in a significant increase in the survival of *G. mellonella*, compared to monotherapies (Fig. 5).

Insert Fig. 5

These combinations resulted in an enhanced therapeutic benefit that correlates with reduced larval burden of the infecting pathogen.

Limitations of CUR as antimicrobial potentiator

It has been reported that the poor bioavailability of CUR, and its concomitant low concentration in plasma, decrease its effectiveness. However, recent studies indicate that the use of piperine to prevent the glucuronidation of CUR (Sharma et al., 2010b), as well as its encapsulation in liposomes, can increase the absorption of CUR and thus its levels in plasma (Shukla et al., 2009). In addition, CUR is neither toxic to the cell nor it is transported by its target efflux pumps, and thus, its ability to sensitize cells to antimicrobials opens up the possibility to be developed in combination with conventional antibiotics (Sharma et al., 2009).

Other phenolic compounds

Many other studies reported the *in vitro* potentiation of natural low MW phenolic compounds on antibiotics. The xanthones α-, β- and γ- mangostins (Sakagami et al., 2005; Seesom et al., 2013) and isojacareubin (Zuo et al., 2012); the 3-benzylchromane brazilin (Zuo et al., 2014;); the

pholoroglucinol derivatives humulone and lupulone (Natarajan et al, 2008); the benzofuran derivative usnic acid (Segatore et al., 2012); the flavonoids luteolin, baicalein, and galangin (Eumkeb et al., 2010; Qian et al., 2015; Zhang et al., 2012); the biflavonoid isocryptomerin (Lee et al, 2009), a series of substituted chalcones (Tran et al, 2012), the kaempferol glycoside tiliroside (An et al., 2011; Falcão-Silva et al., 2009), ellagitannins such as corilagin (Shimizu et al., 2001); the phenylethanoid glycoside acteoside (Ali et al., 2011) and other phenols such as gallic, ferulic and chlorogenic acids, have showed to enhance the susceptibility of bacteria to antibiotics.

Combinations of terpenes with antibacterial drugs.

The main terpenes that showed capacity for enhancing the activity of antibacterial drugs are summarized in Fig. 6 and a summary of the published papers on the main natural terpenes that potentiate the capacity of different type of antibiotics is presented in Table 4.

Insert Fig. 6

Monoterpenes

The monoterpenes carvacrol (Carv), thymol (Thy), geraniol (Ger) and menthol have showed to potentiate the antibacterial activity of antibiotics against gram-(+) as well as gram-(-) bacteria. This subject has been thoroughly reviewed by Langeveld et al. (2014) who reported that Carv showed synergism with AMP, bacitracina, chloramphenicol, ERY, nalidixic acid, nitrofurantoin, novobiocin, PEN, Str, sulfamethoxazole and TET (Choi et al., 2009; Gallucci et al., 2006; Kollanoor Johny et al., 2010; Palaniappan and Holley, 2010; Zhang et al., 2011). Thy potentiated the activity of amikacin, AMP, bacitracin, chloramphenicol, erythromycin, gentamycin, neomycin, nitrofurantoin, Nor, novobiocin, PEN, Str, sulfamethoxazole, TET (Gallucci et al., 2006; Kollanoor Johny et al., 2010; Palaniappan and Holley, 2010; Schelz et al., 2006; Shin and Kim, 2005; Veras et al., 2012; Zhang et al., 2011); menthol interacts positively with AMP, ERY, gentamicin and OXY (Gallucci et al., 2006; Schelz et al., 2006) and Ger potentiated the activity of AMP, Nor , PEN and chloramphenicol. It is worth to take into account that Lorenzi et al. (2009) reported the efficacy of Ger in increasing susceptibility of the gram (-)-MDR- *E. aerogenes* (*acrAB* efflux pump-deficient strain) [but not of the strain that overexpress its *acrAB* efflux pumps (Du et al., 2014; Ma et al., 1996] towards the β-lactams AMP and PEN and the fluoroquinolone Nor, thus appearing to be a potent inhibitor of efflux mechanisms. This was an interesting finding since the vast majority of previously identified Efflux Pump Inhibitors (EPIs) have shown to be active against gram-(+) bacteria, particularly *S. aureus.* In addition, the very few EPIs that have shown

activity against gram-(-) bacteria, such as *Pseudomonas, Acinetobacter, Escherichia* and *Enterobacter* spp., were toxic (Nikaido, 1996).

Recently, Liu et al. (2015) reported that Thy enhanced the antibacterial activity of Str against planktonic cells of the foodborne pathogens *L. monocytogenes*, by decreasing the MIC of Str from 8 to 2 µg/ml (DRI = 4).

Carv, Thy, Ger and menthol are commercially available at very low prices, thus being good candidates for the development of antibacterial combinations.

Sesquiterpenes

Gonçalves et al. (2011) reported the antibacterial activity assessed with the disk diffusion assay of nine sesquiterpenes at different µg/plate, combined with TET_{10} (10 µg); amoxicillin/clavulanic acid $(AMC)_{30}$ (30 µg); ceftazidine $(CAZ)_{30}$ (30 µg); CAZ_{10} (10 µg); ERY_{10} (10 µg); CIP_5 (5 µg); PEN_{10} (10 µg); IMP_{10} (10 µg); and vancomycin $(VA)_{30}$ (30 µg) against *S. aureus* resistant to ERY_{10}; CIP_5; PEN_{10} and IMP_{10}, and *E. coli* resistant to TET_{10}; ERY_{10}; CIP_5; PEN_{10} and VA_{30}. Results showed that the largest effects against *S. aureus* were observed with the following combinations: *cis*-nerolidol with AMC_{30}, IMP_{10} and VA_{30}; guaiazulene with AMC_{30}, PEN_{10} and IMP_{10}; and *trans*-caryophyllene with PEN_{10} and IMP_{10} and (+)-aromadendrene with IMP_{10}.

In *E. coli*, the most pronounced effects were observed with the combinations *cis*-nerolidol-AMC_{30}, *cis*-nerolidol-CAZ_{30} and valencene-CAZ_{30}. In this study, a statistically significant larger diameter of the inhibition halo was formed when the sesquiterpene was added. These results with coincide those obtained by Simões et al. (2008).

Diterpenes

Several works demonstrated a potentiation capacity of diterpenes on antibiotics (Gupta et al., 2013; 2016). Among them, Gupta et al. (2013) showed that the natural clerodane diterpene 16α-hydroxycleroda-3,13(14)-*Z*-dien-15,16-olide (CD) isolated from the leaves of *Polyalthia longifolia* var. *pendula* (Sonn.) Thwaites (Annonaceae) enhanced the activity of OXA, TET, daptomycin (DAP) and linezolid (LZD) against clinical isolates of MRSA obtained in the Clinical Laboratory of Sanjay Gandhi Post Graduate Institute of Medical Science, Lucknow, India. The MICs of OXA, TET, DAP and LZD against the seven MRSA isolates tested, dropped 10-80, 4-16, 2-8 and 2-4-fold respectively.

In a further study, Gupta et al. (2016) investigated the *in vitro* and also the *in vivo* resistance-modifying potential of CD when it is combined with Nor, ciprofloxacin (CIP) and ofloxacin (OFL) against clinical isolates of MRSA. The most significant finding was that CD significantly reduced

MICs of Nor up to 16-fold against MRSA-ST2071. In qRT-PCR analysis, CD alone as well as in combination, significantly modulated the expression of various efflux pump genes including norA up to 2-fold in the same clinical isolate. Results of time-kill assays showed that CD in combination with Nor at ½MIC of each one significantly reduced the viability of bacterial cells in comparison to CD and Nor alone (Fig. 7).

Insert Fig. 7

The therapeutic efficacy of the combinations of CD with Nor was evaluated also *in vivo* in *S. aureus*- infected Swiss albino mice treated with the combinations Nor 0.3125 mg + CD 12.5 mg/kg and Nor 0.3125 + CD 25 mg/kg. The combinations significantly ($p < 0.01$, $p < 0.001$) lowered the systemic microbial burden in blood, liver, kidney, lung and spleen tissues in comparison to CD or Nor alone. Although these results are quite promising, CD is neither easily available from natural sources nor it is commercial, and thus it is difficult that this compound can be easily developed in a near future.

Triterpenes

Oleanolic acid (OA) showed positive interactions with aminoglycoside antibiotics gentamicin and kanamycin but not with other classes of antibiotics such as AMP, rifampicin, Nor, Chloramphenicol or TET against *Acinetobacter baumanii.* FICI values for the combinations OA-gentamicin and OA-kanamycin were < 0.313 and < 0.375 with DRIs = 4. According to authors, the potentiating effect of OA over gentamicin could be the increase of uptake of aminoglycosides *via* increased energy production and membrane permeability. In time-kill assays, results showed that bactericidal effects of combinations of gentamicin 1/16 MIC with OA <1/16 MIC were much higher than gentamicin and OA alone.

Other compounds with enhancing capacity of antibiotics

Other compounds also demonstrated enhancing capacity of the antibacterial activity of different type of antibiotics (reviewed by Hemaiswarya et al., 2008). Apart from phenolic or terpenoid compounds, the quinone β-lapachone (Macedo et al., 2013), the methyl xanthines caffeine, theobromine and theophylline (Esimone et al., 2008; Hosseinzadeh et al., 2006); anacardic acids (Muroi, et al., 2004) bisbenzylisoquinoline alkaloids (Zuo et al., 2011), glucosinolate hydrolysis products such as allylisothiocyanate and 2-phenylethylisothiocyanate (Saavedra, et al. 2010), gingerol (Nagoshi et al., 2006) phenylpropanoids (Basri et al., 2008;

Hemaiswarya and Doble, 2009; 2010; Kollanoor Johny et al., 2010; Moon et al., 2011, Palaniappan and Holley, 2010; Shahverdi et al., 2007; Zhang et al., 2011), among others, showed potentiation capacity of antibacterial drugs.

Concluding remarks on combinations of a natural product with an antibacterial drug

1) From the 56 papers on antibacterial combinations reviewed here, we could observe that 73% are either polyphenols and terpenes. Of them polyphenols constitute 48% and terpenes, 25%. Only 27% belong to other class of natural products. According to a previous report (Wagner and Ulrich Merzenich, 2009), this could be explained by the fact that polyphenols possess a strong ability to bind to different macromolecules like proteins or glycoproteins and in turn, terpenes have a great potential to traverse cell walls of bacteria due to their large lipophilicity.

2) The great majority of studies were performed *in vitro* and very few of them include *in vivo* assays. Most *in vitro* studies used the checkerboard design with allows the calculation of FICI and DRI values. Isobolograms were constructed in few studies (Muroi et al., 2004; Novy et al., 2013; Sakagami et al., 2005). Time-kill curves were extensively used (Gupta et al. 2013; 2016; Hu et al. 2001; Nagoshi et al., 2006; Sudano Rocaro et al., 2004; Zuo et al., 2014). Some works use diffusion assays (Gonçalves et al., 2011; Saavedra et al., 2010; Simões et al., 2008).

3) Polyphenols showed to be enhancers of antibiotics mainly against gram (+)-bacteria in particular against MRSA. Instead, monoterpenes such as Carv, Thy and Ger have showed to potentiate the antibacterial activity of antibiotics against gram-(+) as well as gram-(-) bacteria. Although sesqui- and diterpenes showed to be antibiotic-enhancers against MRSA, some examples demonstrated that sesquiterpenes can be good antibiotics' potentiators against gram (-) bacteria too.

4) Most natural potentiators can be available in sufficient amounts from natural sources as well as they are easily available from commercial sources, thus opening the way to future development of antibacterial combinations containing them.

5) Further developments of these preliminary studies such as structure-activity relationships, *in vivo* assays and clinical trials will be necessary for developing these natural potentiators in combination with currently used antibiotics.

Antifungal combinations

To understand how the combination of an antifungal drug with a low MW natural product can work, it is necessary to previously have a look to the main fungal spp that produce mycoses, the classes of antifungal drugs and their targets and the mechanisms of antifungal resistance.

Regarding the fungal spp that produce mycoses, about 90% of all life-threatening invasive fungal infections (IFI) are produced by species the genera *Cryptococcus, Candida, Aspergillus* and *Pneumocystis* (Darius et al., 2014; Kontoyannis et al., 2010). In turn, superficial infections are mainly produced by dermatophytes, a group of closely related filamentous fungi of *Microsporum*, *Trichophyton* and *Epidermophyton* genera (Weitzman and Summerbell, 1996).

Main classes of antifungal drugs and modes of action

Antifungal agents available for the management of fungal infections (Campoy and Adrio, 2017; Paiva and Pereyra, 2013; Lewis, 2011), include (A) the polyenes nystatin (Nys), amphotericin B (AMPH), and also lipid formulations of AMPH; (B), azoles such as imidazoles (ketoconazole (KTZ), miconazole (MCZ), econazole (ECZ) and clotrimazole) and triazoles (fluconazole (FCZ), ITZ, voriconazole (VCZ) and posaconazole (PCZ); (C) allylamines (terbinafine, naftifina) (D) echinocandins (caspofungin, micafungin and anidulafungin), and (E) 5-fluocytosine. Most of them (polyenes, azoles and allyamines) target the ergosterol of the fungal membrane by binding to it or by inhibiting some steps of its biosynthesis; 5-fluocytosine interferes with DNA and RNA synthesis; and the most recently appeared echinocandins target the fungal cell wall complex β(1,3)-D-glucan synthase. A summary of the antifungal agents and their mechanisms of action are listed in Table 5.

Insert Table 5

In spite of the several antifungal agents in clinical use, there is not any drug that meets all the desirable requirements for being a fully effective and non-toxic antifungal drug and all of them have notable drawbacks that are clearly described by Lewis (2011).

Antifungal resistance and its mechanisms

The fungal resistance produces clinical failures in antifungal chemotherapy that makes mycoses very difficult to eradicate. As in antibacterials, the resistance can be classified as microbiological and clinical and in turn, the resistance can be primary (intrinsic) or secondary (acquired) (Pemán et al., 2009). The strains are classified as resistant to an antifungal drug *in vitro* when the MIC of the drug exceeds the susceptibility breakpoint for that organism (Pfaller et al., 2010). In turn, in the clinical resistance, there is a negative response of a human being to the antifungal therapy regardless the MIC displayed by the fungal strain.

To determine MICs, standardized *in vitro* methods for antifungal susceptibility testing from the CLSI and EUCAST must be used. Data gathered by these standardized tests are useful (in

conjunction with other forms of obtaining data) for calculating clinical breakpoints and Epidemiologic Cut-off Values (ECVs) (Pfaller, 2012).

Strains of *Candida*, *Cryptococcus* and *Aspergillus* genera are the most prone to develop resistance. Of them, *C. albicans, C. parapsilopsis, C. tropicalis and C. glabrata* have showed mainly azole- and infrequently echinocandin- and AMPH- resistance (Arendrup and Perlin, 2014; Spampinatto and Leonardi, 2013). Azole-resistance mechanisms in *Candida* spp showed to be different from those in *Aspergillus* spp (Denning and Perlin, 2011; Kanafani and Perfect, 2008; Pemán et al., 2009; Pfaller, 2012). Although a detailed report of the resistance mechanisms of yeasts and molds have been extensively reviewed (Arendrup and Perlin, 2014; Denning and Perlin, 2011; Pemán et al., 2009; Pfaller, 2012, Spampinato and Leonardi, 2013), a brief account of the main azole-resistant mechanisms of yeasts is given below.

There are three described mechanisms of azole-resistance of yeasts: (i) decreased intracellular drug concentration by induction of efflux pumps; (ii) target site alteration and overexpression of target enzyme; (iii) development of bypass pathways (Pemán et al., 2009). Regarding point (i) upregulation of efflux pumps encoded by either MDR, or *Candida* drug resistance (CDR) genes, have been associated with resistance of *C. albicans, C. glabrata* and *C. dubliniensis* among others (Cannon et al., 2009). The CDR pumps belong to the superfamily of ATP-binding cassette (ABC) transporters and are able to extrude all azole antifungals. These pumps are encoded by the CDR1 and CDR2 genes in *C. albicans*. The other pump is a secondary transporter which utilizes proton gradient as a source of energy and is specific for FCZ. This pump belongs to the major facilitator superfamily (MFS) transporters and is encoded by the MDR1 gene in *C. albicans*. Respective of point (ii) the acquisition of point mutations in the gene encoding for the target enzyme *ERG11* leads to an altered target with reduced affinity to bind azoles. Point (iii) refers to the denial of the membrane-disorganization of the membrane of the azole drugs that is related to mutation of *ERG3.*

Combination therapy of two antifungal drugs has emerged as an attempt to prevent or delay the emergence of resistance and is currently used by medical Doctors to improve the results of monotherapy. However, the data on efficacy are sparse and consist largely of the results of *in vitro* studies since there are few reported in vivo studies and no data from clinical trials are available. In addition, the *in vitro* studies have yielded controversial results depending on the criteria used to evaluate the antifungal interaction. Several combinations that showed potentiation *in vitro* failed to do so in animal models (Cuenca Estrella, 2004). Due to these failures, other compounds such as natural products have been tested in combination with antifungal drugs with the aim to find new combinations that can eradicate fungal infections.

Combinations of low MW natural products with antifungals

Several types of natural compounds have been tested as adjuvants of antifungal drugs, but however, about 66% of the 44 papers revised here refer to polyphenols (34%) and terpenoids (32%) as the structures with the highest capacity of chemosensitizing yeasts or filamentous fungi towards antifungal drugs. Some selected examples are shown below.

Combination of polyphenols with antifungal drugs

The structures of the main polyphenols that showed capacity for enhancing the activity of antifungal drugs are summarized in Fig. 8 and the results are summarized in Table 6

Insert Fig. 8

Insert Table 6

Epigallocatechingallate

Similar to the effect in antibacterial combinations, EGCg showed an *in vitro* and *in vivo* enhancement of the antifungal drugs effects. Hirasawa and Takada (2004) showed that the combination of EGCg with AMPH or FCZ, each at sub-MIC concentrations, markedly decreased the growth of AMPH-resistant and FCZ-resistant *C. albicans* strains.

In a recent paper, Ning et al. (2015) reported that EGCg sensitized *C. albicans* (2 strains) and non-*albicans Candida* (5 strains) *in vitro,* when combined with MCZ, FCZ or AMPH. ECGg decreased 1.66-16-fold the MICs of MCZ; 1.66-8 fold the MICs of FCZ, and 3.93-15 fold the MICs of AMPH. No synergism was observed with EGCG-FCZ against *C. glabrata, C. krusei* and *C.kefyr.* Regarding *in vivo* studies, Han (2007) investigated the anti-*C. albicans* effect of the combination EGCg-AMPH (0.5-2 mg/kg) in a murine model of disseminated candidiasis. Mice administered with the combination had a mean survival time (MST) of 42.1 d, while each the AMPH and ECGg mice-receiving groups showed a MST of 11.7 d and 13.9 d respectively. In addition, the survivability of the combination-treated mice groups was much greater than AMPH alone-mice groups. These results confirmed the potentiation capacity of AMPH antifungal activity by ECGg previously found *in vitro*.

Curcumin

A series of papers (da Silva et al. 2015; García Gomes et al., 2012; Sharma et al., 2009; 2010a, 2010b; Tsao and Yin, 2000) reported the *in vitro* and *in vivo* potentiation of azoles by

CUR. In the first paper (Sharma et al., 2009), CUR showed to reverse the resistance of ABC transporters CaCdr1p and CaCdr2p expressing-*C. albicans* and the ScPdr5p-expressing *Saccharomyces cerevisiae* cells to KTZ, ITZ or MCZ.

Then, Sharma et al. (2010a) and previously Tsao and Yin (2000) demonstrated the *in vitro* potentiation of the antifungal activity of FCZ, MCZ, KTZ, ITZ and VCZ and the polyenes Nys and AMPH by CUR against one clinical sensitive and twenty-one FCZ-resistant strains of *C. albicans* (R-*Ca*). The authors assessed the interactions with FICI values and time-to-kill assays too.

Interesting enough, the potentiating effects of CUR-FCZ and CUR-AMPH could be associated with the accumulation of reactive oxygen species (ROS), which could be reversed by the addition of an antioxidant such as ascorbic acid (Sharma et al. 2010b). García Gomes et al. (2012) evaluated the interaction CUR-FCZ against a highly resistant strain of *C. albicans*. Results showed that 11 µM of CUR was able to reduce 80% of fungal growth when combined with 4 µg/ml FCZ with a very low FICI (~ 0.05). In addition, da Silva et al. (2015) tested *in vitro* the combination CUR-FCZ against *Cryptococcus gattii*, but the FICIs ranged from 0.79 to 2.23, thus indicating no potentiation *in vitro*. However, the *in vivo* study performed with mice, showed that CUR (400 mg/kg) enhances the effect of FCZ (10 mg/kg) in the treatment of cryptococcosis induced by *C. gattii*. (da Silva et al., 2015). The fungal burden in the brain was reduced in a higher extent by the combination CUR-FCZ ($p < 0.001$) than with CUR or FCZ alone. The survival time of animals treated with the combination was higher (21.5 d) than that of animals treated with FCZ alone (16 d) ($p < 0.05$).

Propyl gallates

Several studies reported the potentiation of antifungals' activities by natural low molecular weight polyphenols such as PG. D'Auria et al. (2001) showed that PG is a good FCZ and ITZ adjuvant against *C. albicans* resistant strains The DRI for PG-ITZ was 8 and for PG-FCZ DRI= >8. In another paper of the same group (Strippoli et al., 2000), PG was tested in combination with MCZ, ECZ and KTZ against 40 resistant strains of *C. albicans*, getting a high reduction of the antifungals MIC_{50}:. MCZ = 4 to MCZ-PG = 0.0062 µg/ml; ECZ = 8 to ECZ-PG = 1 and KTZ = 64 to KTZ-PG = 0.25.

Other polyphenols

Baicalein showed potentiation capacity of FCZ against FCZ-resistant *C. albicans* (Huang et al., 2008) and diorcinol D was tested in combination with FCZ against sensitive- and resistant- *C. albicans* planktonic cells (Li et al., 2015). The MICs of FCZ when combined with diorcinol D

631 decreased from 2- to 16-fold for sensitive isolates while decreased more than 250-fold against
632 resistant isolates. The time-killing assays confirmed the positive interactions.
633 In addition, the ellagitanin punicalagin enhanced the potency of FCZ against two strains of
634 *Candida* genus, namely *C. albicans* and *C. parapsilopsis*. The interaction was assessed with disk
635 diffusion assays, checkerboard design, isobologram and time-kill curves (Endo et al., 2010). Also
636 the lignan honokiol showed *in vitro* and *in vivo* positive interaction against 24 azole-resistant *C.*
637 *albicans* strains (Jin et al., 2010) and the phenylethanoid glycoside acteoside, showed
638 potentiation capacity of AMPH when acting against *Candida* spp., *C. neoformans* and *Aspergillus*
639 spp. The MIC of AMPH in combination with acteoside diminished 8-16-fold against *Candida*
640 strains; 64-fold against *C. neoformans* and 8-fold against *Aspergillus* spp.
641
642 *Combinations of terpenes with antifungals*
643 The structure of the main terpenes that showed capacity for enhancing the activity of
644 antifungal drugs are summarized in Fig. 9 and the results on their potentiatin capacity is showed
645 in Table 7.
646
647 Insert Fig. 9
648 Insert Table 7
649
650 *Monoterpenes*
651 The potentiation capacity of antifungal drugs by monoterpenes was reviewed by Campbell et
652 al. (2012). Furtherly, Ahmad et al. (2013) reported the potentiation of FCZ by the monoterpenes
653 Thy and Carv when tested against 38 FCZ-sensitive *C. albicans*, *C. tropicalis* and *C. glabrata* and
654 11 FCZ-resistant *C. albicans*, *C. krusei*, *C. glabrata*, *C. tropicalis* and *C. parapsilopsis*. The
655 combination FCZ-Thy showed FICI values ≤ 0.5 in 32/38 sensitive strains and 8/10 resistant
656 strains. FCZ-Carv showed FICI values ≤ 0.5 in 34/38 and 10/11 respectively. In addition, the
657 fungistatic activity of FCZ was transformed to fungicidal by both monoterpenes at sub-MIC values
658 of each one. Time-kill curves (Fig. 10) confirmed the potentiating fungicide activity.
659
660 Insert Fig. 10
661
662 The selective fungicidal characteristics and ability to restore FCZ-susceptibility to resistant
663 isolates make Thy and Carv in combination with FCZ as promising mixtures for candidiasis
664 treatments. Other antifungal combinations containing monoterpenes were reported by Khan and

Ahmad (2011) who found potentiation between FCZ and Ger against *T. rubrum.* Also the monoterpenes citronellol and Ger showed enhancement of KTZ activities against two *Trichophyton* spp. (*T. schoenleinii* and *T. soudanenese*) (Shin and Lim, 2004). Other monoterpenes in combination with antifungal drugs were comprehensively reviewed by Musiol et al. (2014).

Sesquiterpenes

Farnesol showed potentiation activity of FCZ against *C. albicans* and *C. dublininesis*. This was thoroughly reviewed by Campbell et al*.* (2012).

Diterpenes

The ent-clerodanes bacchotricuneatin, bacrispine and hawtriwaic acid isolated from *Baccharis* extracts, were tested in combination with terbinafine (Terb) against *Trichophyton rubrum* (Rodriguez et al., 2013). The three ent-clerodanes produced shifts of the dose-response curves of Terb towards lower concentrations (Fig. 11)

Insert Fig. 11

Terb MIC_{50} values decreased from 6.90 to 4.40 × 10^{-4} µg/ml (DRI = 1.55) when mixed with bacrispine; from 6.90 to 4.60 × 10^{-4} µg/ml (DRI = 1.50) when mixed with bacchotricuneatin and from 6.90 to 5.24 × 10^{-4} µg/ml (DRI = 1.32) when mixed with hawtriwaic acid. Isobolograms of bacrispine and bacchotricuneatin A with Terb showed the enhancement effects against *T. rubrum* Of them, bacchotricuneatin appears to exert the highest potentiating effect.

Other diterpene such as pseudolaric acid B from *Pseudolarix kaempferi* Gordon (Pinaceae) showed to enhance the *in vitro* activity of FCZ against a series of FCZ-resistant and FCZ-susceptible clinical isolates of *C. albicans* (Guo et al., 2010a). In 100% of the strains, potentiation was observed as determined by both the FICI values that ranged from 0.02 to 0.13 and the Bliss independence (BI) models.

Triterpenes

Retigeric acid from the lichen sp*. Lobaria kurokawae* Yoshim (Lobariaceae), combined with FCZ, KTZ, and ITZ showed strong potentiation against azole-resistant *C. albicans* strains, analyzed by both the FICI (their ranges were 0.1-0.75) and ∆E models obtained with 3-D plot

made by MATLAB7 (Sun et al., 2009). Regarding the mechanism of action, retigeric acid either facilitates the uptake of azoles or repairs the membrane damage associated with the action of the azoles.

Other type of compounds with enhancing capacity of antifungal drugs

Other natural products different from phenols and terpenoids showed chemosensitizing properties of fungal spp. Phenylpropanoids (Ahmad et al., 2010; Khan and Ahmad, 2011; Shin, 2004; Shin and Pyun, 2004); alkaloids (Han and Lee, 2005; Quan et al, 2006; Wei et al, 2011; Zhang et al., 2010); organosulfur compounds (Guo et al, 2010b; Iwazaki et al., 2010; Khodavandi et al, 2010; Ledezma et al, 2008; Shen et al., 1996; Wei et al., 2011); diphenylethers (Li et al., 2015); and others have showed to enhance the susceptibility of fungi to antifungals.

Concluding remarks of antifungal combinations

1) Twenty-nine (66%) out of the 44 revised papers showed that the most reported natural potentiators of antifungal drugs are polyphenols (34%) and terpenes (32%). Among phenols, the most studied compounds were ECGCg, CUR and alkylgallates and among terpenes, the monoterpenes were highly tested in combinations.

2) Most works were performed *in vitro* but very few combinations were tested *in vivo* (Campbell et al., 2012). No clinical trials on combinations of a natural product with an antifungal drug are found in the literature as has been already pointed out by Cuenca Estrella in 2004 (Cuenca Estrella, 2004).

3) AMPH and all azoles (mainly FCZ) were mostly tested in combination and the target fungi were mainly *C. albicans, C. neoformans* or *Aspergillus spp.* The most used was *C. albicans*

4) Most natural antifungal potentiators can be available in sufficient amounts from natural sources as well as they are easily available from commercial sources thus opening the way to future development of antifungal combinations containing them.

5) Well-designed clinical trials are highly needed in order to be able to eradicate the many times fatal fungal infections mainly in immunocompromised patients.

General conclusions and perspectives on the potentiation of an antimicrobial drug by low MW natural products

- The existing antimicrobial agents on their own have not met the expectations of eradicating the human microbial infections mainly in immunocompromised hosts. This drawback have led

731 doctors to use combination therapy that is the jointly administration of two antimicrobial drugs
732 with the aim of coping the microbial infections.
733 - Combinations of two antimicrobial drugs have several advantages*:* in addition to widen the
734 spectrum of activity and potency of each drug and lower the toxicity, they can achieve a more
735 rapid antimicrobial effect and allow a reduction in the doses of individual agents, thus preventing
736 emergence of antimicrobial resistance.
737 - In spite of the many advantages, several reports have proven that combination therapy have
738 failed in several patients mainly due to efficacy relies largely on the results of the *in vitro* studies
739 and experimental animal models, but evidences from well-designed clinical trials are lacking.
740 - In the last years, the testing of combinations of antimicrobial drugs with natural products has
741 opened hopeful perspectives for new antimicrobial combinations.
742 - Several promising results on the potentiation of antifungal and antibacterial activities by low MW
743 natural products were reported in the literature. Among the 56 works on antibacterial and the 44
744 on antifungal combinations revised in this review, 73% (antibacterial) and 66% (antifungal) of the
745 works include phenolic or terpenoid structures as potentiating compounds.
746 - In spite of the reported combinations were mostly performed *in vitro*, the potentiation capacity of
747 natural phenols and terpenoids on antimicrobial drugs should not be neglected, since this
748 knowledge opens a wide range of possibilities for the combination antimicrobial therapy that
749 needs to be taken into account. Further research including *in vivo* assays and clinical trials are
750 required to determine the relevance of these antimicrobial enhancers in the clinical area and
751 should be the focus of intensive studies in the next years in order to develop new combination
752 agents that overpass the drawbacks of the antibiotics and antifungals in clinical use.

753 **Conflicts of interest**

754 Authors declare that they do not have not any conflict of interest.

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Table 1: Main antibiotic classes grouped by mechanism of action

Inhibited process	Target	Class		Antibiotic	Resistance
Cell wall synthesis	Transpeptidases/transglycosylases (PBPs)	β-Lactams	Penicillins	Penicillin G, amoxicillin, ampicillin, oxacillin.	β-Lactamases, PBP mutants β-Lactamases inhibitors: Sulbactam Clavulanic acid
			Cephalosporins	*First generation:* cephalotin, cephapririn, cephalexin *Second generation*: cefacor, cefotetan *Thirth generation:* ceftriaxone *Fourth generation*: cefpirome, cefepime	
			Carbapenems	Imipenem Meropenem Panipenem	
	Peptidoglycan units (terminal D-Ala-D-Ala)	Glycopeptides and glycolipopeptides		Vancomycin Teicoplanin	Modified peptidoglycan precursors (low-binding affinity)
	Targeting cell membrane	Lipopeptides		Daptomycin Polymixin B	Spontaneous mutations
Protein synthesis	Peptidyl transferase (50s ribosome)	Macrolides		Azythromycin Chlarithromycin Clindamycin Dirithromycin Erythromycin	rRNA methylases, efflux, inactivating genes
		Lincosamides		Lincomycin Clindamycin	rRNA methylases, efflux, inactivating genes

		Streptogramins	Delfopristin Pritinamycin, Quinupristin	
		Phenicols	Chloranphenicol Florphenicol	Acetylation, target mutation, permeability barrier, efflux
		Oxazolidinone	Linezolid	
	Peptidyl transferase (30s ribosome)	Tetracyclines	Doxycycline Minocycline Oxytetracycline Tetracycline	Efflux, ribosomal protection protein, enzymatic inactivation
		Aminoglycosides	Amikacin Gentamicin Kanamycin Neomycin Streptomycin Trobamycin	Efflux, decreased permeability, ribosome alteration, amynoglicoside modification enzyme
DNA synthesis	DNA gyrase and topoisomerase II and IV	Fluoroquinolones	Ciprofloxacin Gemifloxacin Levofloxacin Nalidixic Adic Norfloxacin Ofloxacin Sarafloxacin	Loss of porins, efflux, DNA gyrase/topoisomerase mutations
	Tetrahydrofolic acid synthesis inhibition	Trimetroprim-sulfamethoxasole	Co-trimoxazole	
RNA synthesis	DNA-dependent RNA polymerase	Rifamycins	Rifampin Rifamycin Rifapentine	Mutations in gen that encodes RNA polymerase

Adapted and compiled from Walsh (2000), Frost (2007); ECDC/EMEA, 2009; Kohanski et al. (2010); Moore (2013).

Table 2: List of bacteria that mainly show resistance to antibiotics

Bacterial species	Antibiotics to whom the bacteria show antimicrobial resistance
Streptococcus pneumoniae	Penicilin, macrolides, lincosamidas, estreptogramina B, trimethoprim-sulphamethoxazole, tetracyclines, fluorquinolones
Streptococcus pyogenes	Macrolides, tetracyclines
Staphylococcus aureus Community-associated MRSA	Meticillin, cephalosporins, macrolides
Staphylococcus aureus	Meticillin, cephalosporins, quinolones, aminoglycosides, macrolides, ansamycins,
Enterococcus spp	Ampicillin, aminoglycosides (high level), glycopeptides, tetracycline.
Neisseria gonorrhoeae	Penicillin, cephalosporins, fluorquinolones tetracycline, macrolides
Salmonella spp. (non typhoidal)	Trimethoprim-sulphamethoxazole, fluorquinolones, ampicilin
Escherichia coli	Aminopenicilinas, cephalosporins, penicillin + β-lactamse inhibitors, monobactams, aminoglycosides, trimethoprim-sulphamethoxazole, quinolones
Klebsiella pneumoniae	Cephalosporins, penicillin + β-lactamse inhibitors, carbapenemes, monobactams, aminoglycosides, trimethoprim- sulphamethoxazole, quinolones
Enterobacter spp	Trimethoprim- sulphamethoxazole, quinolones, cephalosporins, penicillin + β-lactamse inhibitors, aminoglycosides
Pseudomonas aeruginosa	Aminoglycosides, carbapenemes, cephalosporins, antipseudomonadal penicillin + β-lactamse inhibitors, monobactams, fluorquinolones,
Acinetobacter baumanii	Aminoglycosides, carbapenemes, cephalosporins, antipseudomonadal penicillin + β-lactamse inhibitors fluorquinolones, trimethoprim-sulphamethoxazole, tetracyclines.

Adapted and compiled from Arias and Murray, 2015; Magiorakos et al., 2012; Singh et al., 2017; Soriano, 2008; Furuya and Lowy, 2006

Table 3: Summary of the main natural polyphenols that potentiate the capacity of different type of antibiotics. The Dose-Reduction Index (DRI) was calculated by authors of this Review based on data provided by the authors of the referenced papers.

Adjuvant	Antibiotic	Bacteria	Methods used	Results	Ref
EGCg	Ampicillin: sulbactam (2:1)	28 MRSA-strains	Checkerboard design and FICI determinations with Amp/Sulb (2:1) in combinations with 6.25 and 25 mg/l Time-kill studies	FICIS between 0.19-0.56. With 6.25 mg/l of EGCg the DRIs were 2-8 With 25 mg/l of EGCg, DRIs were 4-32 Time-kill studies: corroborated synergism with two strains, one of them β-lactamase producing and the other non β-lactamase producing.	Hu et al., 2001
	Penicillin Oxacillin	25 MRSA-strains	Checkerboard design and FICI For mechanisms of action: Test of direct binding to peptidoglycan.	Most striking result; EGCg reversed the high level resistance of the strain F-74 to oxacillin (DRI = 16). Mechanism: EGCg synergizes the activity of β-lactams against MRSA due to interference with the integrity of the cell wall through direct binding to peptidoglycan	Zhao et al., 2001
	Carbapenems (imipenem (IPM), panipenem and meropenem)	24 MRSA-strains	Checkerboard design and dose-response curves Time kill curves.	Best DRI was obtained for IPM (0.25 μg/ml with 25 μg/ml; DRI = 512. Time-kill curves showed synergism between IPM (16 μg/ml) and EGCg (12.5 μg/ml, 1/8 MIC) and IPM (32 μg/ml with EGCg (25 μg/ml, 1/4 MIC)	Hu et al., 2002a; 2002b
	Penicillin	21 penicillase-producing *S.aureus*	Checkerboard design Test of Direct Inhibition of penicillase	Penicillin was tested alone and with 3.12, 6.25, 12.5 and 25 μg/ml. All FICI values for the two highest concentrations were below 0.5 (0.14-0.38). The direct inhibition of penicillinase activity by EGCg is responsible for synergism	Zhao et al., 2002
	Tetracycline	Tet-K resistant- and sensitive- *S. epidermidis* and *S. aureus*	Checkerboard design and time-kill	EGCg sensitize staphylococci to tetracycline in strains in which the resistance is due to the expression of Tet(K) and also TetB efflux. DRI = 256 for resistant strains DRI = 16 for susceptible ones.	Sudano Roccaro et al. 2004
	Oxytetracicicline	ERSA; MRSA and TetRSA	Checkerboard design and isobolograms	EGCG showed marked synergistic activity in combination with oxytetracycline against various drug resistant *S. aureus* strains including MDR MRSA. DRI = 8-10	Novy et al. 2013
Alkylgallates	β-lactams: penicillin ampicillin; cephapirin; oxacillin and 9 non-β-lactams antibiotics	MRSA (n = 18) MSSA (n = 8)	Checkerboard design and FICI values	The synergistic activity of alkyl gallates appears to be specific to β-lactams against MRSA. The lenght of the alkyl chain played a role related to the intensifying activity, being the optimum lenght C5 and C6 (FICI = 0.07-0.41), although the galloyl moiety played a role too.	Shibata et al., 2005

	Oxacillin (OXA)	MRSA (n = 19) MSSA (n = 7)	Checkerboard design and FICI values	Triple combination of a short (propyl) and long chain (octyl) gallate plus OXA. FICIs ≤ 0.031) were obtained with 25 μg/ml PG and 12.5 μg/ml OG (DRIs > 32-256)	
Curcumin (CUR)	Oxacillin (OXA) Ampicillin (AMP); Ciproflozacin (CIP) Norfloxacin (Nor)	Two strains of MRSA (a clinical isolate DPS-1) and the ATCC 33591 and one MSSA ATCC 25923	Checkerboard design and FICI values. Time-kill assay	The combination of CUR the all antibiotics tested showed a FICI = 0.07-0.75 in the MSSA ATCC 25923. 0.31-0.75 against the MRSA ATCC 33591 and FICI = 0.245-0.75 against MRSA . DRIs = 2-128. The synergism was confirmed with time-kill curves	Mun et al., 2013
	Ciprofloxacin (CIP)	Four *S. aureus* (NorA, MdeA, TetK and MsrA overexpressed)	Checkerboiard design, DRI. Inhibition assay of S. aureus NorA efflux pump . Moleculardockin studies to identify binding sites.	When combined CUR and CIP, hydrophobic interactions as well as H-bonding interactions of CIP were mimicked by CUR which probably block the entry of CIP (a Nor A pump substrate)	Joshi et al., 2014
	PIperacillin (PIP) Meropenem (MPM) Levofloxacin (LVX)	MDR *P. aeruginosa* (That overexpressthe MexAB-OprM efflux pump	*In vitro*: Checkerboard design and FIC values *In vivo*: *Galleria mellonella*	In vitro, CUR restored the activity of PIP, MPM and LVX . In vivo, the combination therapy, showed an enhancement efficacy with a concomitant reduced bacterial burden	Ballard and Coote, 2016
	Imipenem (IPM)	Four *E. coli* and seven *K. pneumoniae* with (+) and (-) extended spectrum β-lactamase (ESBL) isolates	Checkerboard design and decrease of MICs (DRI)	DRIs between 2 and 64 were obtained	Gunes et al.,2013

Other phenolic compounds (Fig. 2)	
xanthones	Sakagami et al., 2005; Seesom et al., 2013; Zuo et al., 2012
3-benzylchromane	Zuo et al., 2014
pholoroglucinol derivatives	Natarajan et al, 2008
usnic acid	Segatore et al., 2012
Flavonoids, flavonoids glycosides, biflavonoids and chalcones	Eumkeb et al., 2010; Zhang et al., 2012; Qian et al., 2015 Lee et al, 2009, Tran et al, 2012, Falcão-Silva et al., 2009; An et al., 2011
ellagitannins	Shimizu et al., 2001
phenylethanoid glycoside acteoside	Ali et al., 2011

Table 4: Summary of the main natural terpenes that potentiate the capacity of different type of antibiotics.

Adjuvant	Antibiotic	Bacteria	Methods used	Results	Ref
Monoterpenes					
Carvacrol	Ampicillin, Bacitracin, Chloramphenicol, Erythromycin, Nalidixic acid, Nitrofurantoin, Novobiocin, Penicillin, Streptomycin, Sulfamethoxazole, Tetracyclin	Reviewed by Langeveld et al., 2013			Palaniappan and Holley, 2010; Zhang et al., 2011; Kollanoor Johny et al., 2010; Choi et al., 2009; Gallucci et al., 2006
Thymol	Amikacin, Ampicillin, Bacitracin, Chloramphenicol, Erythromycin, Gentamycin, Neomycin, Nitrofurantoin, Norfloxacin, Novobiocin, Penicillin, Streptomycin, Sulfamethoxazole, Tetraciclin	Reviewed by Langeveld et al., 2013			Veras et al., 2012; Palaniappan and Holley, 2010 Zhang et al., 2011 Kollanoor Johny et al., 2010 Shin and Kim, 2005 Gallucci et al., 2006 Schelz et al., 2006
	Streptomycin	*L. monocytogenes*	Checkerboard design, FICI values	FICI = 0.375 DRI = 4	Liu et al., 2015
Menthol	Ampicillin, Erythromycin, Gentamicin and Oxacillin	Reviewed by Langeveld et al., 2013			Schelz et al., 2006 Gallucci et al., 2006
Geraniol	Ampiciliin, Norfloxacin, Penicillin, Chloramphenicol	Reviewed by Langeveld et al., 2013			Schelz et al., 2006 Gallucci et al., 2006 Lorenzi et al., 2009
Sesquiterpenes					
Cis-nerolidol	Amoxicillin/Clavulanic acid $(AMC)_{30}$ 30 µg Imipenem $(IMP)_{10}$ 10 µg Vancomycin $(VA)_{30}$ 30 µg	*S. aureus* resistant to erythromycin 10 µg (ERY_{10}); Ciporfloxacin (5 µg (CIP_5); penicillin 10 µg (PEN_{10}) and imipenem (IMP_{10})	Disk diffusion assays	A statistically significant larger diameter of the inhibition halo was formed when the sesquiterpene was added	Gonçalves et al., 2011
Guaiazulene	Amoxicillin/Clavulanic acid $(AMC)_{30}$ Penicillin 10 µg $(PEN)_{10}$ Imipenem 10 µg (IMP_{10})				
Trans-caryophyllene	PEN_{10} IMP_{10}				
(+)-Aromadendrene	IMP_{10}.				
Cis-nerolidol	AMC_{30}	*E. coli* resistant	Disk diffusion assays	A statistically significant larger diameter of	Gonçalves et al., 2011

	CAZ_{30}	to TET_{10}; ERY_{10}; CIP_5; PEN_{10} and VA_{30}		the inhibition halo was formed when the sesquiterpene was added	
Valencene	CAZ_3				
Diterpenes					
Clerodane diterpene: 16α-hydroxycleroda-3,13(14)-*Z*-dien-15,16-olide (CD)	Oxacillin (OXA), Tetracyclin (TET), Daptomycin (DAP) Linezolid (LZD)	Clinical isolates of MRSA	Checkerboard design and FICI values and DRI	CD in combination - with OXA, FICIs = 0.22-0.40; DRIs=10-80 - with TET, FICIs = 0.32-0.44; DRIs = 4-16 - with DAP, FICIs: 0.32-0.68; DRIs = 4-8 - with LZD, FICIs = 0.34-0.69; DRIs = 2-4	Gupta et al., 2013
	Fluoroquinolone antibiotics Norfloxacin (Nor) Ciprofloxacin (CIP) Ofloxacin (OFL)	Clinical isolates of MRSA	In vitro: checkerboard design, FICI values and time-kill curves Expression of efflux-pump genes (*norA, norB, norC, mepA, mdeA*) In vivo assays with Swiss albino mice	CD-Nor, FICIs 0.315-0.50 in 9/15 strains. DRIS= 4-16. CD-CIP, FICIs 0.32 in 6 strains. DRIs = 4-8. CD-OFL: FICIs: 0.32 in 8 strains, DRIs 4-8 Time-kill: CD (0.5 MIC) + Nor (0.5 MIC) : diminished the viability 4-fold respective to both partners alone that diminished 2-fold Down regulation of efflux-pump genes was observed for the combination IN vivo: CD-Nor showed 1.8-fold staphylococcal load reduction in different tissues and blood compared with untreated controls	Gupta et al., 2016
Triterpenes					
Oleanolic acid	Gentamicin Kanamycin	*Acinetobacter baumanii*	Checkerboard design, FICI values and time-kill curves	FICI values for the combinations OA-gentamicin and OA-kanamycin were < 0.313 and < 0.375 with DRIs = 4. In time-kill assays, bactericidal effects of combinations of gentamicin 1/16 MIC with OA <1/16 MIC were much higher than gentamicin and OA alone.	Shin and Park, 2015

Table 5: Main antifungal classes grouped by mechanism of action

	Target		Class		Antibiotic	
Cell membrane	Ergosterol synthesis	Lanosterol 14-α-demetilase inhibitor	Azoles	Imidazoles	Ketoconazole Miconazole Econazole Clotrimazole	
				Triazoles	First generation	Fluconazole Itraconazole
					Second generation	Voriconazole Ravuconazole Posaconazole
		Squalene monooxygenase inhibitors	Allylkamines		Terbinafine Amorolfine Naftifine	
			Thiocarbamates		Tolnaftate Tolcinate	
	Ergosterol binding		Polyenes		Amphotericin B Nystatin	
Nucleous	DNA/RNA synthesis inhibitor		Pyrimidine analogue		5-fluocytosine	
	Mitotic inhibitor – interaction with β-tubulin		Benzofuran		Griseofulvin	
Cell wall	β-1,3-D-glucan synthase inhibitors		Echinocandins		Anidulafungin Caspofungin Micafungin	
	Chitin synthase inhibitors		Peptide		Nikkomycin	

Adapted and compiled from Paiva and Pereyra, 2013; Lewis, 2011; Campoy and Adrio, 2016.

Table 6. Summary of the main natural polyphenols that potentiate the activity of antifungal drugs

Adjuvant	Antifungal drug	Fungal strain	Methods used	Results	Ref
EGCg	AMPH	- AMPH-Resistant *C. albicans* - AMPH-sensitive *C. albicans*	Broth dilution assays at fixed ratios of both partners	EGCg enhances the antifungal effect of AMPH against antimycotic-susceptible and -resistant C. albicans.	Hirasawa and Takada, 2004
	FCZ	-FLZ- resistant *C. albicans* -FCZ sensitive *C.albicans*		EGCg enhances the antifungal effect or FCZ against antimycotic-susceptible and -resistant C. albicans.	
	MCZ, FCZ, AMPH	C. albicans SC5314 C. albicans ATCC10231 C. parapsilopsis ATCC 22019. C. tropicalis ATCC 13803, C. glabrata ATCC 66032, C. kefyr ATCC 46764 C. krusei ATCC14243	Checkerboard design and determination of FICI values	Against all *Candida* planktonic cells EGCG_MCZ, MICs of MCZ reduced from 0.25–1 to 0.031–0.25 µg/ml; EGCG-AMPH MICs of AMPH reduced from 0.063-0.25 to 0.016-0.063 µg/ml). EGCG-FCZ no synergism was observed against *C. glabrata, C. krusei* and *C.kefyr*.	Ning et al., 2015
	AMPH	*C. albicans*	In vivo: murine model of disseminated candidiasis	The combination had a mean survival time (MST) of 42.1 d, while the AMPH- and ECGg- alone mice-receiving groups showed a MST of 11.7 d and 13.9 d respectively.	Han, 2007
Curcumin (CUR)	KTZ, ITZ, MCZ; FCZ; VCZ	*C. albicans* and *S. cerevisiae* overexpressing ABC drug transporters (CaCdr1p, CaCdr2p, ScPdr5p)	Spot assay Checkerboard design. FICI values.	CUR selectively synergizes KTC, MCZ and ITZ (FICIs = 0.25, 0.13, 0.25 respectively), but not FCZ, VCZ. CUR modulates only ABC multidrug transporters	Sharma et al., 2009
	KTZ, ITZ, MCZ, FCZ VCZ, Nys, AMPH	A clinical sensitive *C. albicans* strain (S-Ca) and 21 Resistant *C. albicans* strains (R-Ca)	Spot assay; Checkerboard design. FICI values, Time kill curves. DRIs	CUR interacts positively with FCZ, KTZ, MCZ ITZ, VCZ, AMPH, and Nys with FICI values between 0.093-0.375. For S-Ca, DRI values varied from 7.9-64 and for R-Ca, DRIs = 4-16. Time-kill assays corroborated the findings.	Sharma et al., 2010a Sao and Yin, 2000
	FCZ	*C. albicans* highly resistant to FCZ.	Checkerboard design, FICI	CUR at 11 µM reverse the resistance of FCZ at increasing concentrations. FICI = 0.05	García-Gomes et al., 2012
	FCZ	*Cryptococcus gattii*	In vitro: checkerboard and FICI values *In vivo* assays with mice	FICIs = 0.79 to 2.23, thus in vitro, no potentiation *In vivo*: CUR (400 mg/kg)- FCZ (10 mg/kg) *reduced the fungal* burden in the brain and get a higher survival time	Da Silva et al., 2015
Propylgallate (PG)	FCZ ITZ	Resistant *C. albicans* strains	Broth dilution assays: antifungal agents were two-fold diluted and PG were added	The DRI for PG-ITZ was 8 and for PG-FCZ DRI = >8.	D'Auria et al., 2001

			at a fixed concentration		
	MCZ ECZ KTZ	*40 C. albicans* strains	Idem than above	High reduction of the $MICs_0$ when acting in combination:. For example $MICs_{50}$ of MCZ = 4; MCZ-PG = 0.0062 µg/ml; ECZ = 8; ECZ-PG = 1; KTZ = 64; KTZ-PG = 0.25	Strippoli et al., 2000
Baicalein	FCZ	30 FCZ-resistant *C. albicans*	Checkerboard design. FICI values. Time-kill curves	FICI for MIC_{80} = 0.069; DRI of FLZ = 256 Time-kill studies confirmed the interaction	Huang et al., 2008
Diorcinol D	FCZ	Eleven sensitive- and resistant-*C. albicans* cells	Checkerboard design. FICI values. Time-kill curves	The MICs of FCZ decreased from 2 to 16-fold for sensitive isolates while dcreased more than 250-fold against resistant isolates. The time-killing assays confirmed the positive interactions	Li et al., 2015
Punicalagin	FCZ	One strain of C. albicans and one of C. parapsilopsis	Checkerboard design. FICI values. Isobologram. Time-kill curves	FICI = 0.25; The isobologram and the time-kill curves confirm the positive interaction	Endo et al., 2010
Honokiol	FCZ	24 azole-resistant *C. albicans* strains	In vitro: checkerboard FICI values. Time-kill curves In vivo:	FICI values: 0.25-0.5; DRIs for FCZ = 128-512. Time-kill studies confirmed the positive interactions with one strain. The in vivo results showed a prolonged survival (80 to 100 %) of mice and a greateer efficacy in clearing Candida fro the kidneys	Jin et al., 2010
Acteoside	AMPH	One strain of each: C. albicans, C. glabrata, C. krusei, C. parapsilopsis; C. tropicalis, C. neoformans, A. flavus, A fumgatus, A. niger and A. parasiticus	Checkerboard assay, effects on cell viability, membrane permeabilty, bunding to ergosterol	The MIC of AMPH in combination with acteoside diminished 8-16-fold against *Candida* strains; 64-fold against *C. neoformans* and 8-fold against *Aspergillus* spp. There was decreased viability, of the cells, increased uptoke of Propidium iodide and enhance leakage f 260nm-absorbing material when cells were exposed to AMPH + acteoside	Ali et al., 2011
AMPH: Ampohtericin B, FCZ: fluconazole; KTZ ketoconazole; ECZ: Econazole; MCZ: miconazole; ITZ: itraconazole; VCZ: voriconazole; Nys: nystitin;					

Table 7. Summary of the main natural phenols with two or more phenolic OH that potentiate the capacity of antifungals					
Adjuvant	Antifungal drug	Fungal strain	Methods used	Results	Ref
Monoterpenes					
Thymol (Thy)	FCZ, KTZ, AMPH, ITZ	Reviewed by Campbell et al. 2012			Braga et al., 2007; Kim et al., 2008a,b; Guo et al., 2009; Faria et al, 2011
Thymol (Thy) Carvacrol (Carv)	FCZ	38 FCZ-sensitive *Candida* strains 11 FCZ-resistant *Candida* strains		FICI FCZ-Thy; = 0.42-1.25 (FICI values ≤ 0.5 in 32/38 and in 8/10 in susceptible and resistant strains respectively). FICI FCZ-Carv = 0.25-1 (FICI values ≤ 0.5 in 34/38 and 10/11 in susceptibel and resistant strains respectively). This potentiations was confirmed by time-kill assays. FCZ was converted in fungicide.	Ahmad et al., 2013
Geraniol (Ger)	FCZ	One strain of *T. rubrum*	Checkerboard design and FICI values	FICI = 0.312	Kahn and Ahmad, 2011
Geraniol (Ger) Citronellol	KTZ	*2 Trichophyton* spp (*T. schoenleinii* and *T. soudanenense*)	Checkerboard design, isobolograms and FICI values	FICI range = 0.18-0.38	Shin and Lim, 2004
Sesquiterpenes					
Farnesol	FCZ	Reviewed by Campbell et al. 2012			Hornby et al., 2001 Hornby and Nickerson, 2004 Jabra-Rizk et al., 2006 Yu et al. 2012
Diterpenes					
Bacchotricuneatin Bacrispin Hawtriwaic acid	Terbinafine	One strain of *T. rubrum*	Checkerboard design, isobolograms	Terb MIC_{50} decreased from 6.9 to 4.4 × 10^{-4} µg/ml (DRI = 1.55); to 4.6 × 10^{-4} µg/ml (DRI = 1.50) and to 5.24 × 10^{-4} µg/ml (DRI = 1.32) when combined with bacrispine, bacchotricuneatin and hawtriwaic acid respectively	Rodríguez et al., 2013
Triterpenes					
Retigeric acid	FCZ, KTZ ITZ	10 azole-resistant *C. albicans*	Checkerboard design, and ΔE model	FICI ranges = 0.1-0.75. AE model confirmed the potentiation	Sun et al., 2009
AMPH: Ampohtericin B, FCZ: fluconazole; KTZ ketoconazole; ECZ: Econazole; MCZ: miconazole; ITZ: itraconazole; VCZ: voriconazole; Nys: nystitin;					

Figure legends

Legends for Fig.

Fig. 1: Intrinsic mechanisms of resistance. The example shows β-lactam antibiotics targeting a penicillin-binding protein (PBP). Antibiotic A can enter the cell *via* a membrane-spanning porin protein, reach its target and inhibit peptidoglycan synthesis. Antibiotic B can also enter the cell *via* a porin, but unlike antibiotic A, it is efficiently removed by efflux. Antibiotic C cannot cross the outer membrane and so is unable to access the target PBP. Reproduced from Blair et al. 2015, with permission # 4083101005380 from Nature Publishing Group.

Fig. 2. Structure of the polyphenols that showed potentiation capacity of antibacterial drugs

Fig. 3. Three out of the seven isobolograms showing the interaction of epigallocatechingallate (EGCG) and oxytetracyclin (OXY) against *Staphylococcus aureus* strains. Reproduced from Novy et al. (2013) with permission # 3955710170823 from Elsevier.

Fig. 4. Proposed hypothetical binding site for a NorA inhibitor. NorA binding interactions of CUR at site 1. Reproduced from Joshi et al., 2014 with permission 4085631105081 from The Royal Society of Chemistry

Fig. 5. Effect of treatment with combination of (a) piperacillin (PIP) + CUR; (b) levofloxacin (LVX) + CUR on survival of *Galleria mellonella* larvae infected with *P. aeruginosa* PAM1020 or PAM1032. Reproduced from Ballard and Coote, 2016 (open access).

Fig. 6. Terpenes that showed potentiation capacity of antibacterial drugs

Fig. 7. Time-to-kill studies of the clerodane diterpene 16α-hydroxycleroda-3,13 (14)-*Z*-dien-15,16-olide (CD) alone, of norfloxacine (Nor) alone and of the combination of (CD + Nor) at sub-inihibitory concentrations (1/2 MIC each) using the clinical isolate MRSA ST2071. Reproduced from Gupta et al (2016) with permission # 3955710722191 from Elsevier.

Fig. 8. Structures of polyphenols that showed potentiation capacity of antifungal drugs

Fig. 9. Terpenes that showed potentiation capacity of antifungal drugs

Fig. 10. Representative time-kill curves of sensitive (left) or resistant (right) *Candida* isolates following the exposure to (b) ½ Minimum Inhibitory Concentration (MIC) of thymol; (c) ½ MIC of carvacrol; (d): ½ MIC of Fluconazole (FCZ); (e) ½ MIC of FCZ combined with ½ MIC of thymol; (f) ½ MIC of FCZ combined with ½ MIC of carvacrol. (a) represents the untreated *Candida* cells (control). Reproduced from Ahmad et al. 2013 with permission # 3955710881284 from Elsevier.

Fig. 11. Dose-response curves of terbinafine in combination with a fixed amount (31.25 μg/ml) of each ent-clerodane bacrispine (A); bacchotricuneatin (B) and hawtriwaic acid (C)

isolated from *Baccharis* extracts. Reproduced from Rodriguez et al., 2013, with permission # 3955710548737 from Elsevier.

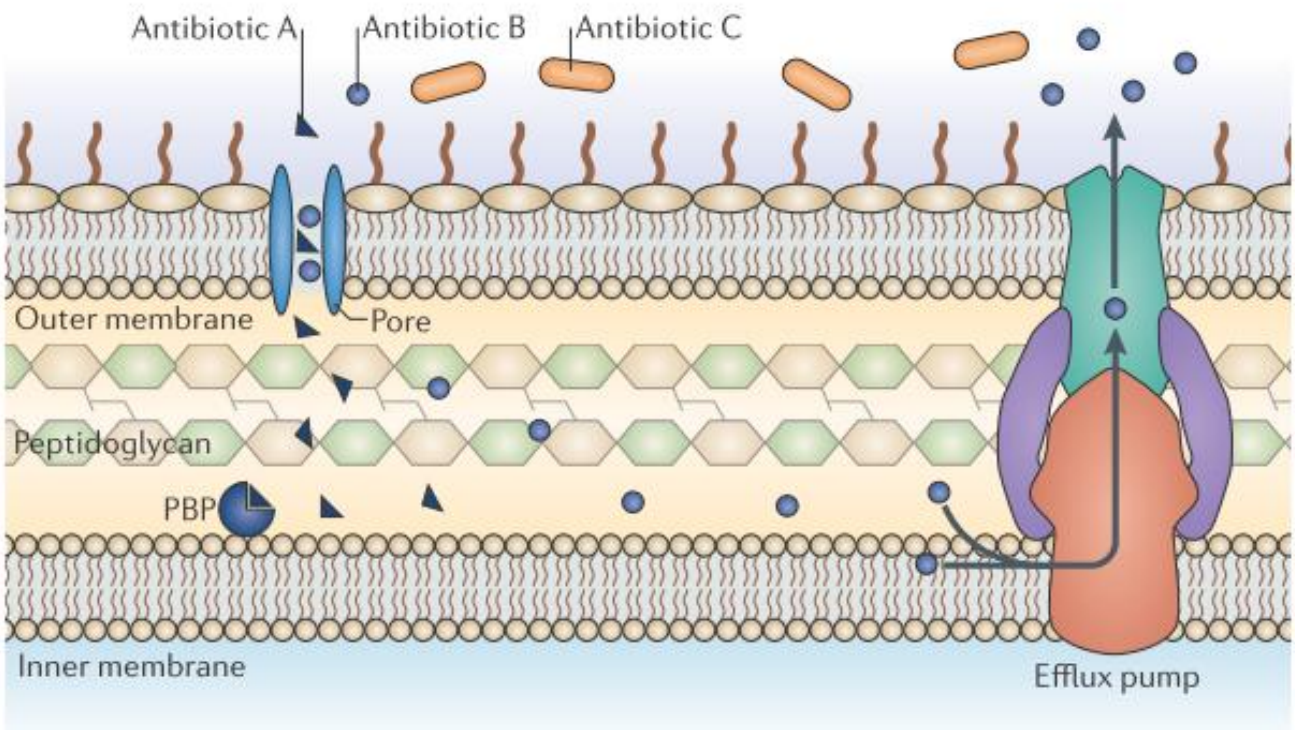
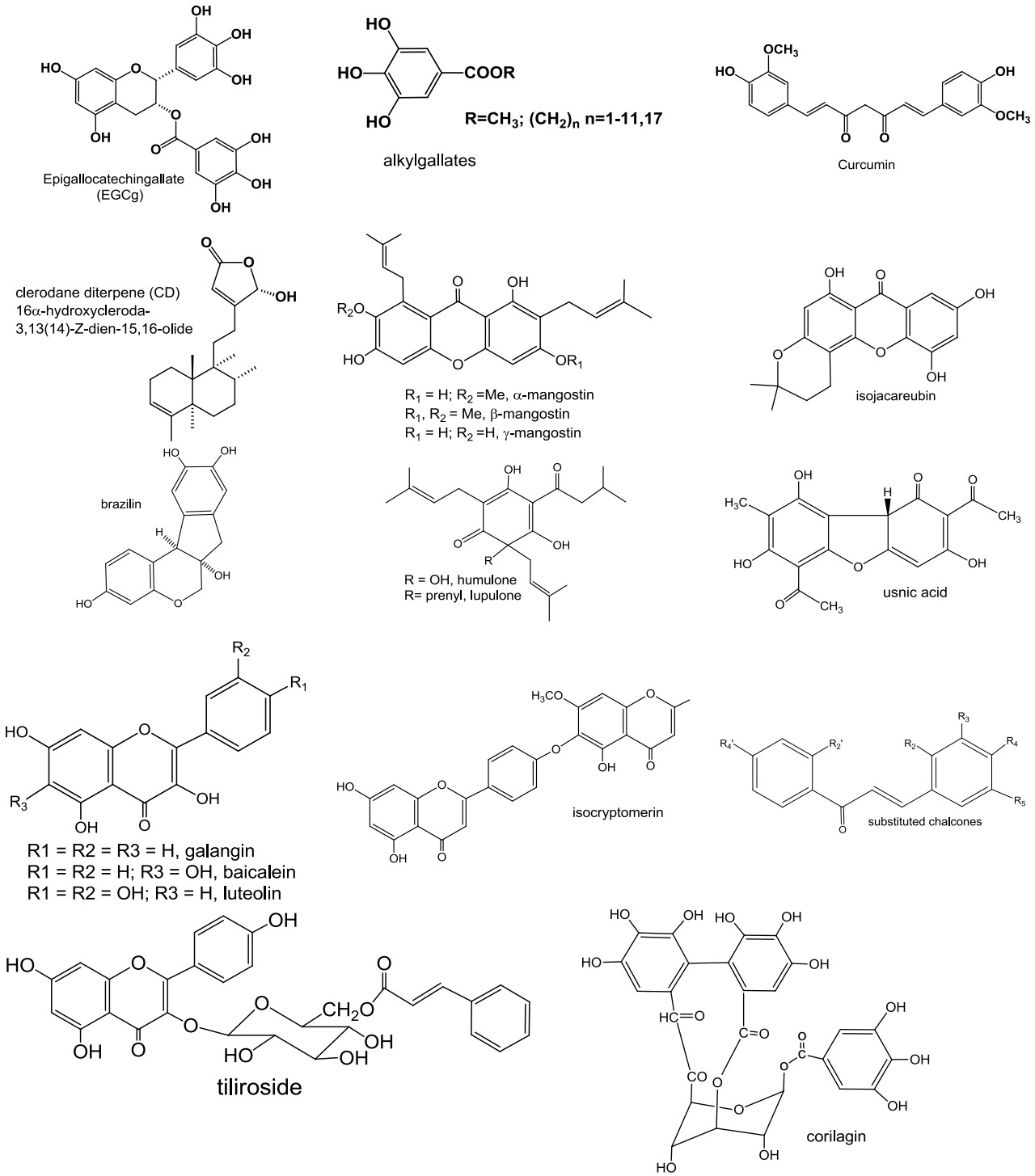
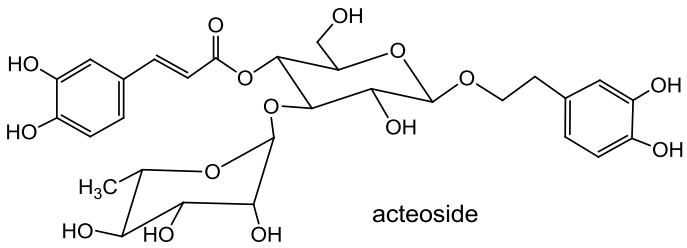

Antibiotic A
Antibiotic B
Antibiotic C
Outer membrane
Pore
Peptidoglycan
PBP
Inner membrane
Efflux pump

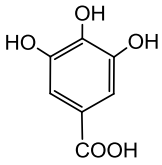
Fig. 1

Fig. 2

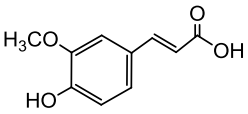

Epigallocatechingallate
(EGCg)
R=CH3; (CH2)n n=1-11,17
alkylgallates
Curcumin
clerodane diterpene (CD)
16α-hydroxycleroda-
3,13(14)-Z-dien-15,16-olide
R1 = H; R2 =Me, α-mangostin
R1, R2 = Me, β-mangostin
R1 = H; R2 =H, γ-mangostin
isojacareubin
brazilin
R = OH, humulone
R= prenyl, lupulone
usnic acid
R1 = R2 = R3 = H, galangin
R1 = R2 = H; R3 = OH, baicalein
R1 = R2 = OH; R3 = H, luteolin
isocryptomerin
substituted chalcones
tiliroside
corilagin



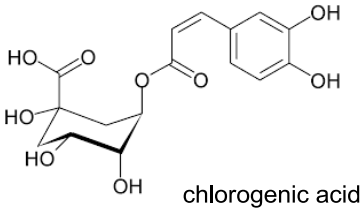
acteoside



gallic acid



ferulic acid



chlorogenic acid

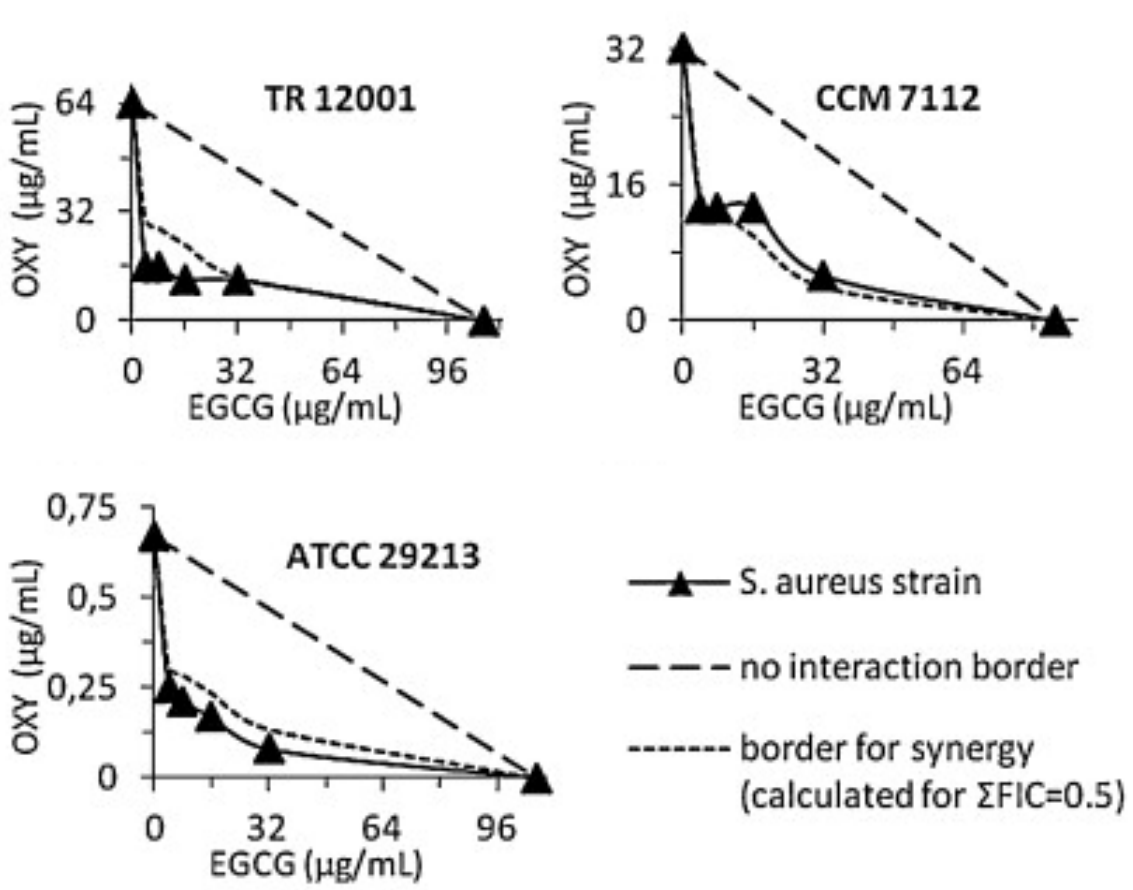

TR 12001
CCM 7112
ATCC 29213
OXY (µg/mL)
EGCG (µg/mL)
64
32
0
16
0,75
0,5
0,25
0
32
64
96
S. aureus strain
no interaction border
border for synergy
(calculated for ΣFIC=0.5)

Fig. 3

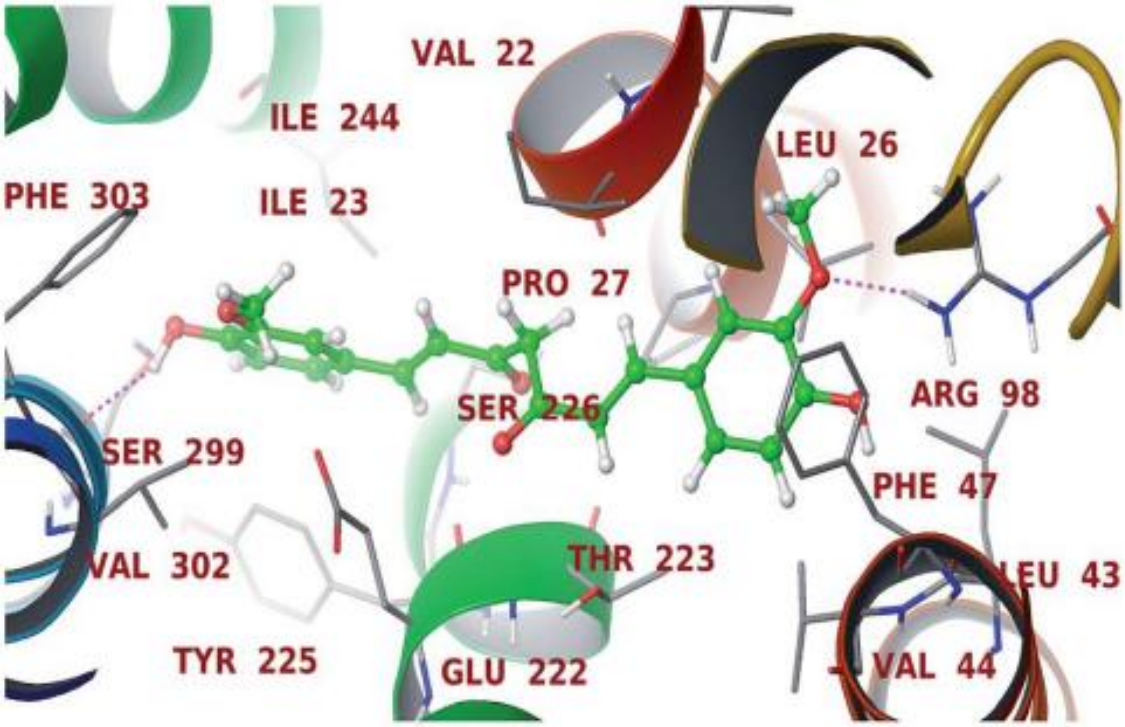

VAL 22
ILE 244
LEU 26
PHE 303
ILE 23
PRO 27
ARG 98
SER 226
SER 299
PHE 47
VAL 302
THR 223
LEU 43
TYR 225
GLU 222
VAL 44

Fig. 4

Zacchino, S., Fig. 5

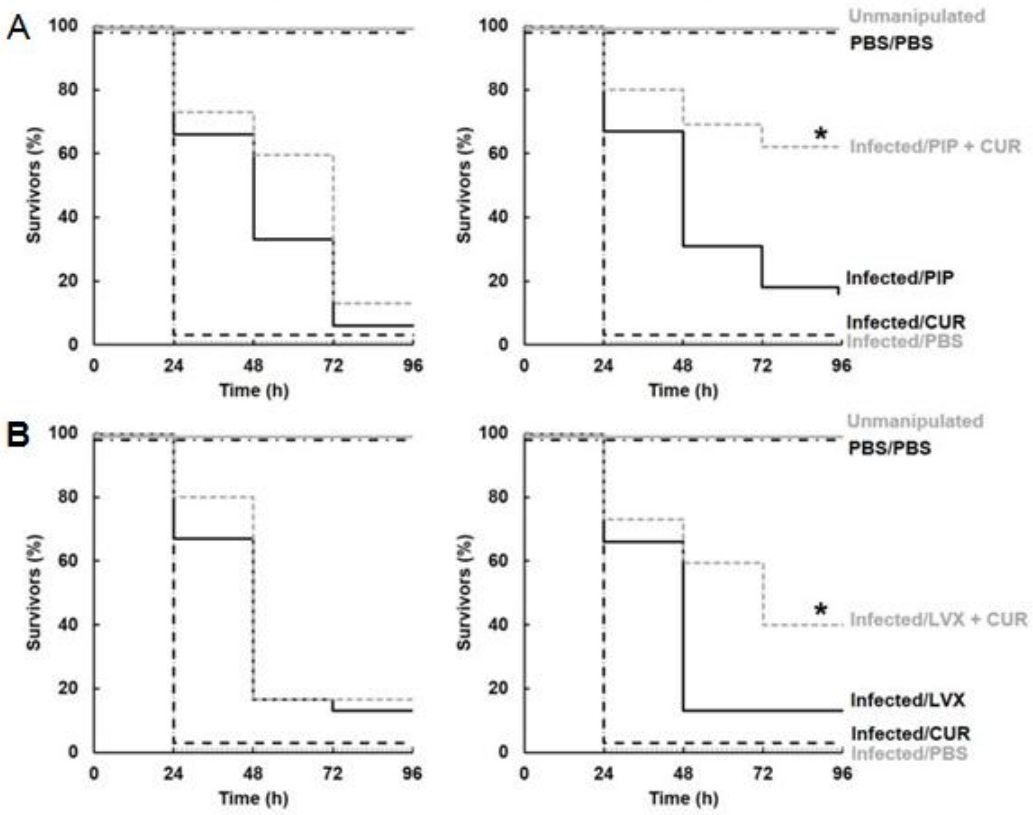
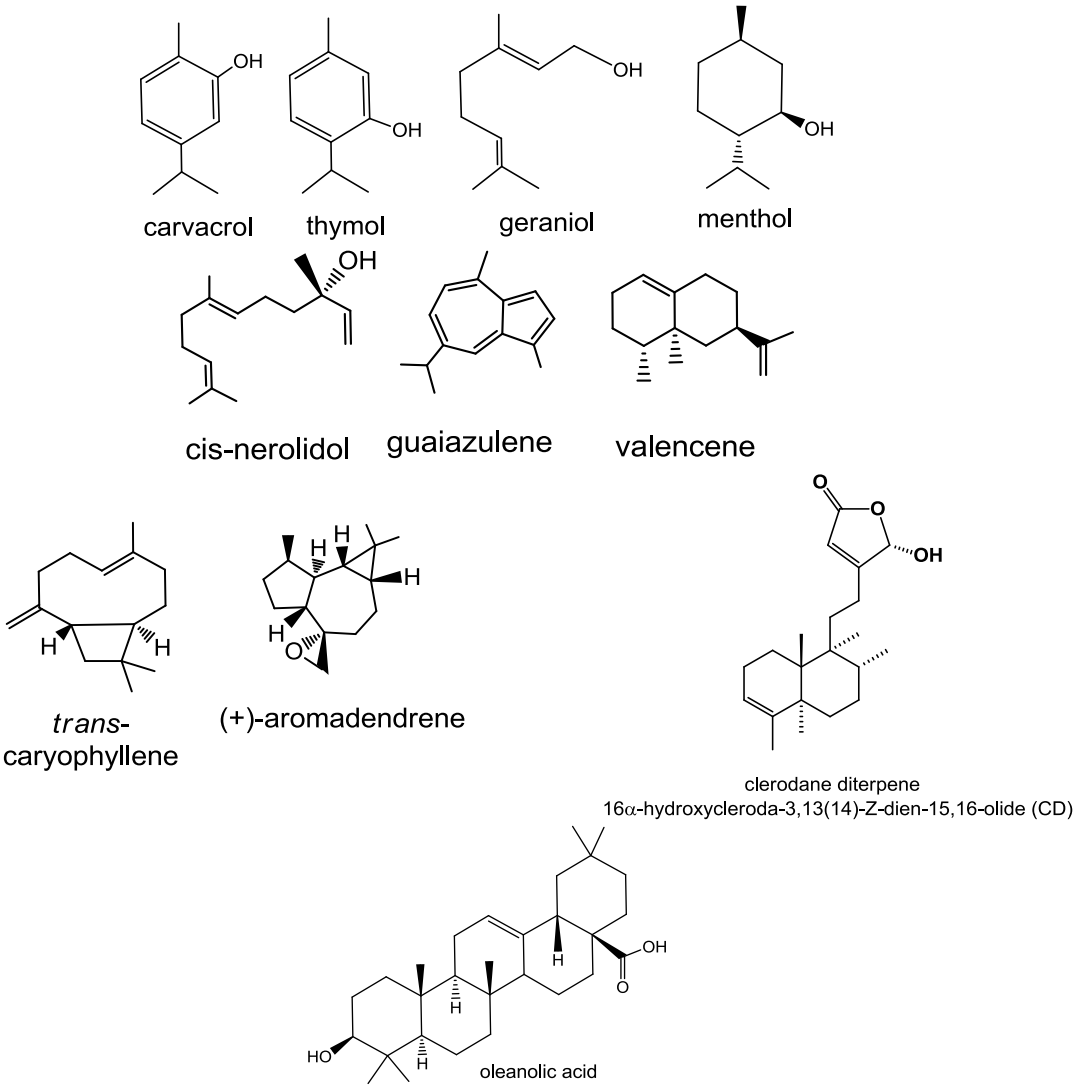


Fig. 5

Fig. 6


carvacrol
thymol
geraniol
menthol
cis-nerolidol
guaiazulene
valencene
trans-caryophyllene
(+)-aromadendrene
clerodane diterpene
16α-hydroxycleroda-3,13(14)-Z-dien-15,16-olide (CD)
oleanolic acid

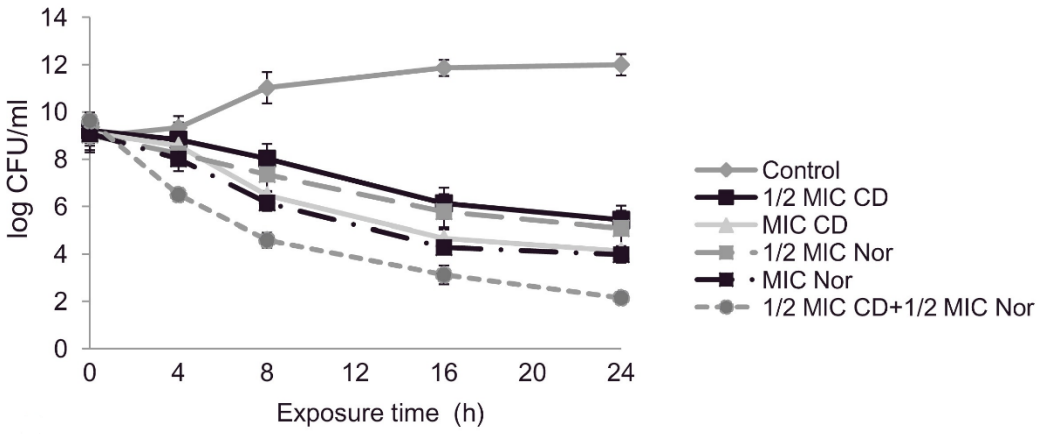

14
12
10
8
6
4
2
0
log CFU/ml
0
4
8
12
16
20
24
Exposure time (h)
Control
1/2 MIC CD
MIC CD
1/2 MIC Nor
MIC Nor
1/2 MIC CD+1/2 MIC Nor

Fig. 7

Fig. 8

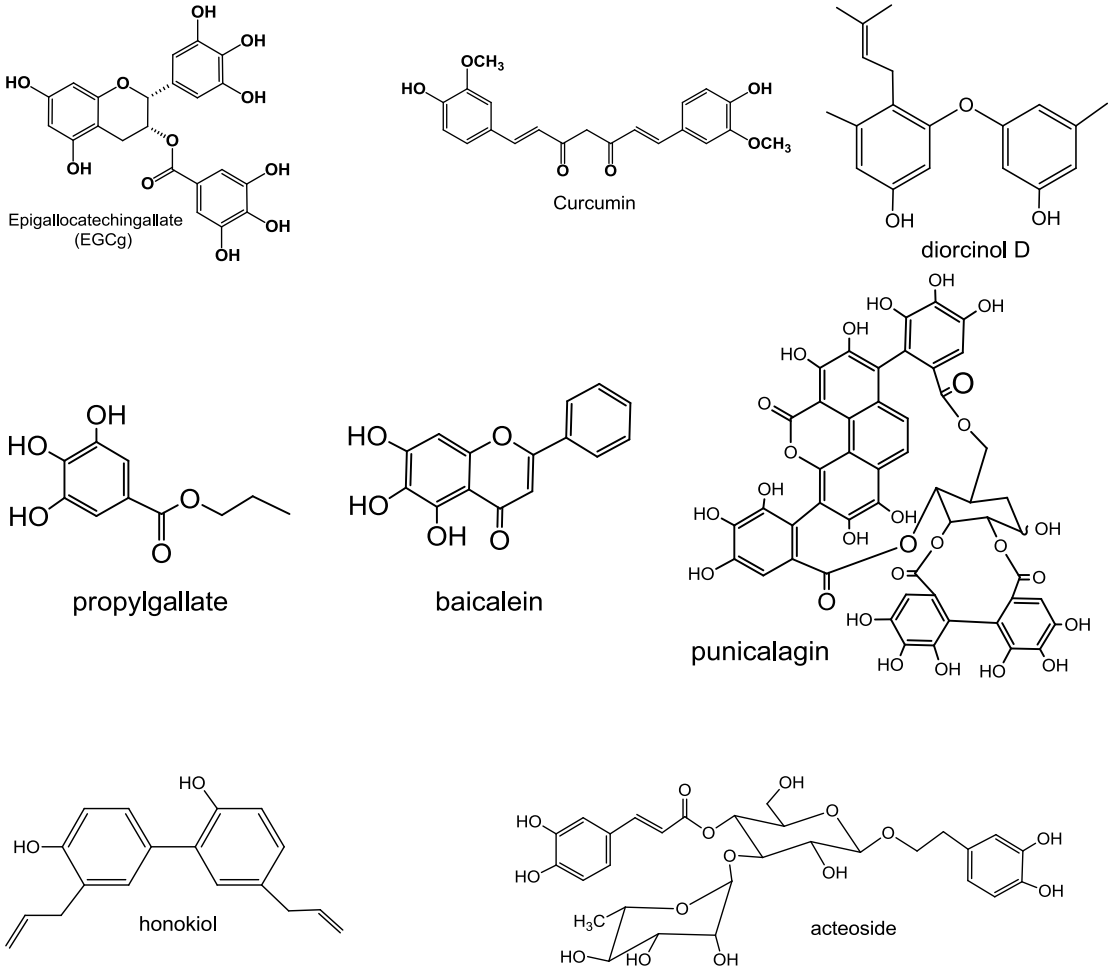
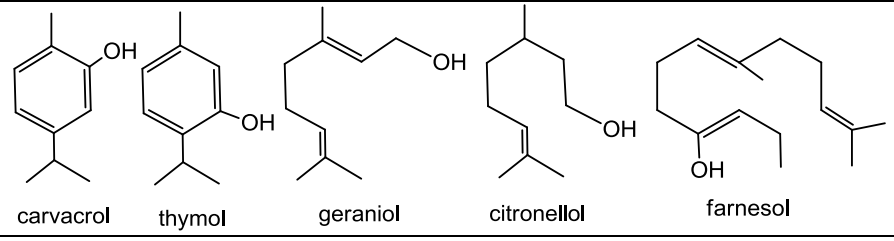
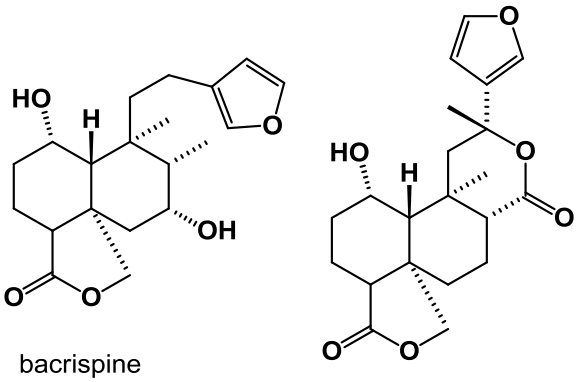

Epigallocatechingallate
(EGCg)
Curcumin
diorcinol D
propylgallate
baicalein
punicalagin
honokiol
acteoside

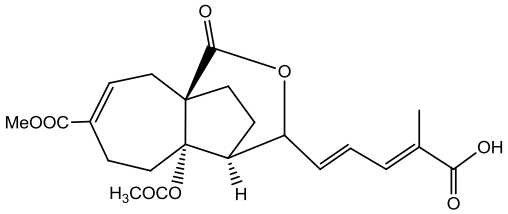
Fig. 9



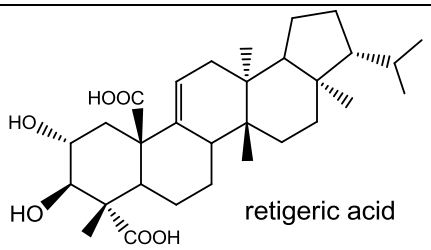
carvacrol thymol geraniol citronellol farnesol



bacrispine bacchotricuneatin hawtriwaic acid



pseudolaric acid B



retigeric acid

Zacchino, S., Fig. 10

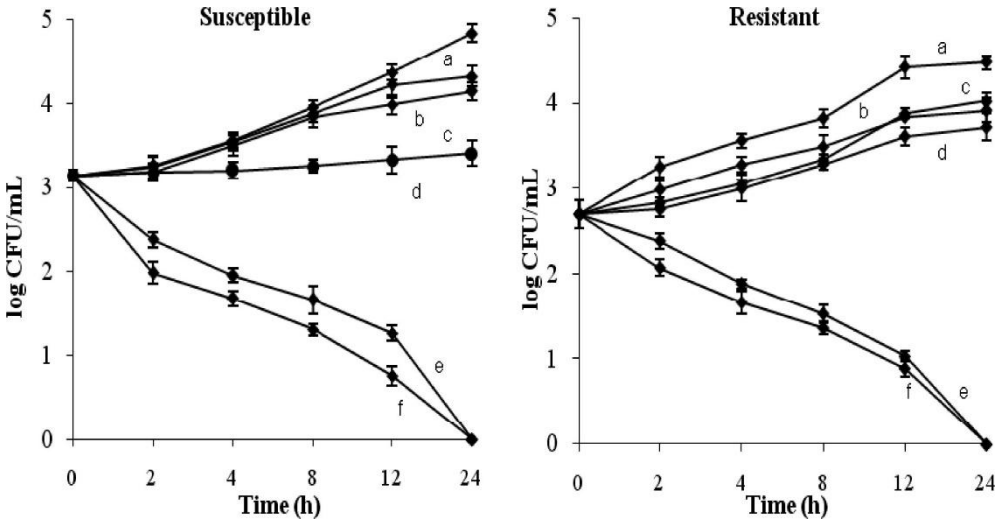


Fig. 10

Zacchino, S., Fig. 11

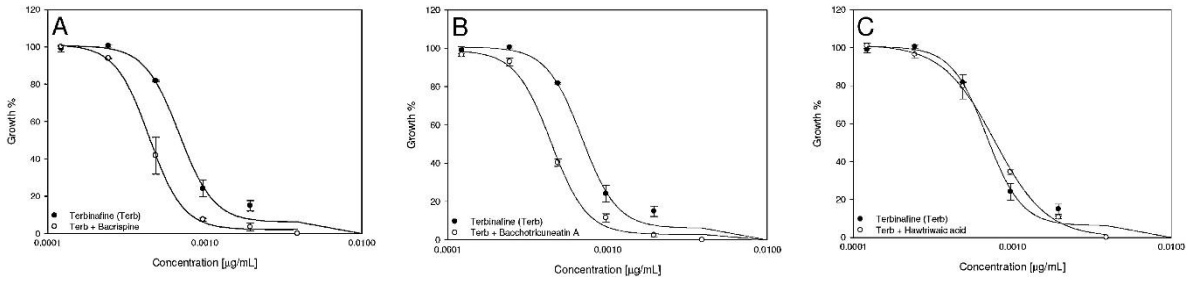


Fig. 11

Supplementary material

Methods for quantifying the potentiation in the combinations

Quantification of interactions have been usually done in the examples commented in this review with *in vitro* methods.

Checkerboard design

This assay is performed in 96-well microplates in which each row and each column contain two-fold serial dilutions of substance X and Y respectively, at concentrations around its MICs; reaching a unique combination of the two substances in each well. Then, a quantified inoculum of the microbial strain is added to each well and the microplate is incubated at a proper temperature during a suitable time for each microbial sp. The concentrations of the first wells without visible growth along the stepwise boundary between inhibition and growth were used to calculate the Fractional Inhibitory Concentration (FIC) and the Fractional Inhibitory Concentration Index (FICI) (Bonapace et al., 2002). The FICI is calculated by the sum of the values of Fractional Inhibitory concentration (FIC) (Eq. 1).

$$\mathrm{FICI} = \mathrm{FIC_X} + \mathrm{FIC_Y} = \frac{\mathrm{MIC_X\ in\ combination}}{\mathrm{MIC_X\ alone}} + \frac{\mathrm{MIC_Y\ in\ combination}}{\mathrm{MIC_Y\ alone}} \quad \text{(Eq. 1)}$$

According to Odds (2003) a FICI ≤ 0.5 is indicative of 'synergism'; a FICI > 4.0 indicates antagonism and a FICI in the range >0.5 – 4.0 is indicative of 'no interaction'. Another limits for FICI and different expressions for the term "no interaction" such as "additive", "summation" and "indifference" are found in the literature (Martinez Irujo, 1996; Schelz et al., 2006).

Isobolograms

An isobole is an "iso-effect" curve (Fig. S1) obtained in a two dimensional graphic in which the *x* and *y* axes represent FIC_X and FIC_Y (obtained in the checkerboard assay) respectively. The line connecting the MICs of both compounds represents the line of no interaction. Synergistic mixtures fall below the line of indifference (FICI ≤ 0.5) and antagonistic ones fall above the line of no interaction. (FICI ≥ 4) (Wagner and Ulrich Merzenich, 2009).

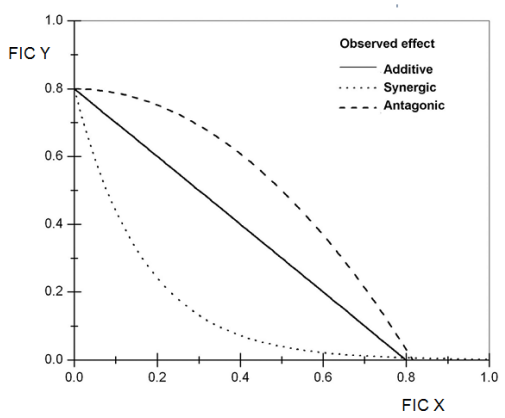


Fig. S1: Representation of an isobologram

Time-kill studies

The time-kill method is used to get information about the time-dependent progression of the antimicrobial activity. In this method, synergy is defined as a 100-fold or 2-$\log_{10}$ decrease in colony count at 24 h produced by the combination, compared with the line produced by the most active single agent (Kiraz et al., 2010). Fig. S2 shows a time-kill graphic of a synergistic combination of A and B.

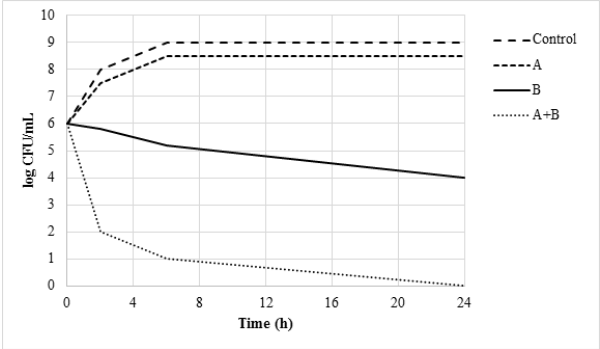


Fig. S2: Time-kill curves of two partners A and B

Disk diffusion assays

The disk diffusion assay can be performed in two ways: (a) two disks are placed at 20 mm one each other (center to center) and after a suitable time of incubation, an inhibition zone is formed between both disks if synergistic effect was present (b) two sterile paper disks were embedded each with one of the drugs alone and a third disk is impregnated with a prepared sample containing the mixture of both drugs. Inhibition zone diameters were measured after incubation for a time according to the microbial growth (Fig. S3) (Kiraz et al., 2010).

Fig. S3: Disk diffusion assay for interaction assessments

References for Supplementary material

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