Interference of flavonoids and carotenoids on the antimicrobial activity of some drugs against clinical isolates of *Pseudomonas aeruginosa*

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Abstract

The use of phytonutrients in pharmaceutical dosage forms aiming a healthier lifestyle has increased worldwide, and in spite of being generally an useful healthcare measure, this is not free of risks when concomitant medication use is considered. The combined use of phytonutrients and medication remains poorly investigated, and represents a serious problem in drug therapy, given that side effects due to drug-nutrient interactions are poorly predictable. In this study, three drugs were tested in combination to lycopene β-carotene (carotenoids), diosmin and curcumin (flavonoids) against clinical isolates of *P. aeruginosa*. Strains identity was confirmed by automated methods and the interference of phytomolecules on the pharmacological activity of the chosen drugs was assessed in vitro using antimicrobial disks. Here we show that lycopene, β-carotene, diosmin and curcumin can act synergistically when combined to antimicrobial drugs against clinical isolates of *P. aeruginosa*. *In vivo* studies are necessary for assessing the biological effects of these interactions considering clinical contexts..

Introduction

The use of phytomolecules of nutritional value – phytonutrients – in healthcare has increased worldwide in the latest years, and many formulations of pure compounds or blends are sold in several countries as dietary supplements, what generally can be done without any prescription. Different diseases have been successfully treated with phytonutrients such as obesity, insomnia and constipation. Moreover, phytonutrients have been largely explored in skin care cosmetic formulations and as nutraceutics. An important consideration on this topic is that they are also promising alternatives on drug discovery for diseases that are currently focused in many health policies worldwide, such as the neglected infectious diseases, cancer, and neuropsychological, immunological and endocrine disorders (Gruenwald, 2004; Custodio *et al*., 2008; Dias-Souza *et al.*, 2015).

Nevertheless, differently from the perception reported by patients in several studies, the use of phytonutrients is not free of risks. Some wrong ideas commonly diffused among patients may help to explain why the number of reports of side effects such as hepatotoxicity, blood disorders and renal failure

are steadily increasing worldwide due to natural products. These include: 1) phytonutrients and other natural products always offer superior safety of use when compared to synthetic drugs; 2) phytonutrients and other natural products can accelerate the expected therapeutic effects of synthetic drugs; 3) phytonutrients have no specific way to be prepared and consumed, and that 4) phytonutrients and other natural products are naturally superior to synthetic drugs (Gruenwald, 2004; Rodríguez-Fragoso *et al*., 2011; Dos Santos *et al*., 2015).

An important point in this topic is the combined use of phytonutrients and medication: scientific information about safe and effective use of combinations of phytonutrients and synthetic drugs is still limited because of the scarcity toxicological studies. Antimicrobial drugs (AD) represent a critical pharmacotherapeutic class in this context, because: a) they are not targeted to human receptors, but to bacterial targets; b) among the most popular and reachable scientific data about phytonutrients for patients are antimicrobial activity studies, and combined to traditional knowledge, this somehow seems to influence patients to use phytonutrients as alternatives to synthetic drugs (combined or not to

them) (Seden *et al.*, 2010) and c) bacterial resistance is growing worldwide, and negative consequences of interactions of phytonutrients would only contribute to this critical picture (Ioannides, 2003).

Pseudomonas aeruginosa is an ubiquitous Gram-negative microorganism that belongs to the microbiota of humans and several animals. Infections in healthy individuals rarely lead to disease, but the bacterium may offer health risks to immunocompromised patients. This opportunistic pathogen is highly resistant to environmental factors and may exhibit multidrug-resistant (MDR) phenotype, what explains its clinical importance. Previous data published by our group have described resistance from Brazilian clinical isolates to different antimicrobial drugs, consistent with other recent observations in Brazil and worldwide (Rodrigues *et al.*, 2014).

In this paper, we show for the first time that lycopene, β-carotene, diosmin and curcumin can interfere on the activity of antimicrobial drugs against clinical isolates of *Pseudomonas aeruginosa*. Different drugs were tested in combination to each of these phytomolecules in an *in vitro* model using antimicrobial disks. Moreover, we assessed the antimicrobial potential of the tested phytomolecules. The lacking of research data in this field makes our study even more relevant.

Materials and Methods

Microorganisms

Samples of *P. aeruginosa* consisted of tracheal secretion isolates obtained of adult patients, kindly provided by Dr Pedro Marçal from the clinical isolates collection from University Vale do Rio Doce. All isolates were cultured overnight in BHI broth (Difco) at 35±2°C for activation and then cultivated in Cetrimide agar overnight. Samples were then tested in VITEK 2 system (bioMérieux), using Gram-negative bacteria identification cards according to the manufacturer's instructions. Identification scores were accepted if results were superior to 98%.

Interference test

The possible interference of the phytomolecules on antimicrobial drugs was assessed as recently described by our group (Dias Souza *et al.*, 2013; Santos *et al.*, 2015). Lycopene, β-carotene, diosmin and curcumin were purchased from Fagron (Brazil) in analytical grade, and solutions were prepared in hot DMSO (analytical grade) based in common concentrations of these nutrients consumed in Brazil as manufactured formulations: 15 mg/mL for lycopene (Moritz and Tramonte, 2006), 30 mg/mL

for β-carotene (Fisberg *et al.*, 2008) and 60 mg/mL for flavonoids (Arabbi *et al.*, 2004).

The interference assay was performed in duplicate as described in our previous study (Dos Santos *et al.*, 2015). Agar plates were prepared with Mueller-Hinton agar (Difco). Antimicrobial disks (chloramphenicol 30 µg, aztreonam 30 µg, meropenem 10 µg, all from Sensifar) were distributed as for performing standard antimicrobial susceptibility assays (CLSI, 2012). Following, briefly, 10 µL of each phytonutrient solution was then dispensed in each disk. Plates were incubated overnight at 35±2°C, and the inhibition zone mean diameter was compared with control plates (disks free of phytonutrients). Synergism was considered if the inhibition zone mean diameter was at least 2 mm larger than the control, and antagonism was considered if the inhibition zone mean diameter was at least 2 mm shorter than the control. If the inhibition zone mean diameters were larger or shorter than the control but no significant difference was seen, data was described as tendency of synergism or antagonism (Dias-Souza *et al.*, 2013; Dos Santos *et al.*, 2015).

Assessment of the antimicrobial activity of the phytonutrients

This assay was performed in duplicate as previously described (Dos Santos *et al.*, 2015). Overnight strains cultured in BHI agar (Difco) were transferred to Mueller Hinton Broth (Oxoid) and the turbidity was adjusted as a 0.5 McFarland standard. Strains were then dispensed in 96-wells polystyrene plates, using 100 µL in three wells for each bacterial sample. For each repetition of three wells, 100 µL of solutions of each phytonutrient in the concentrations of 100 and 500 µg/mL were dispensed. The plates were then incubated overnight at 35±2°C. Bacterial growth was analyzed through viability staining using 50 µL of a 0.01% resazurine solution. Pink color indicated bacterial growth, and blue color indicated effective antimicrobial activity. Wells with cultures free of phytonutrients and fresh media were used as positive and negative controls respectively.

Statistical analysis

Homocedasticity was assessed through Bartlett's test. Normality of data was assessed through Shapiro-Wilk test. Mean diameters of the inhibition zones with and without the phytonutrients were analyzed using ANOVA followed by Tukey test. The significance level was set at $p<0.05$. All analyses were carried out in Minitab 17 software for Windows.

Table 1. Interference of flavonoids and carotenoids over antimicrobial drugs against *P. aeruginosa* isolates

Isolates	CHLO	CHLO+LYC	CLO+β	ATM	ATM+LYC	ATM+β	MER	MER+LYC	MER+β
P. aeruginosa 1	15	18 Δ	17±1.41 Δ	10	21.5±2.12 Δ	20.5±0.7 Δ	12.5±0.7	11φ	12
P. aeruginosa 2	15	12±0.7	20 Δ	0 ♦	25 Δ	24Δ	30	30	31±1.41 δ
P. aeruginosa 3	11	16.5±0.7	15±1.41 Δ	20.5±0.7	21±1.41 Δ	12 ♦	27.5±0.7	27.5±0.7	27
P. aeruginosa 4	10.5±0.7	13.5±0.7	13 Δ	0 ♦	14.5±0.7 Δ	13±1.41 Δ	31.5±0.7	30	32 δ
P. aeruginosa 5	9	11.5±0.7	14 Δ	20±2.8	28Δ	29 Δ	20	19.5±0.7	17±1.41
P. aeruginosa 6	11.5±0.7	12 δ	14 Δ	17.5±0.7	28±2.82 Δ	23.5±0.7 Δ	31±1.41	29	29
P. aeruginosa 7	14	25 Δ	17 Δ	20	27±2.82 Δ	20	27.5±0.7	25 ♦	27.5±0.7δ
P. aeruginosa 8	9.5±0.7	13 Δ	13 Δ	20.5±0.7	25±1.41 Δ	26 Δ	31±1.41	24.5±0.7	24.5±0.7
P. aeruginosa 9	13±1.41	16 Δ	15±0.7 Δ	19.5±0.7	22 Δ	22 Δ	30	27.5±0.7	27.5±0.7
P. aeruginosa 10	13	11±1.41 ♦	16 Δ	28	28	29±0.7 Δ	28.5±0.7	30.5±0.7	30.5±0.7
Isolates	**CHLO**	**CHLO+C**	**CLO+D**	**ATM**	**ATM+C**	**ATM+D**	**MER**	**MER+C**	**MER+D**
P. aeruginosa 1	15	18Δ	19±0.7 Δ	10	20Δ	20.5±0.7Δ	12.5±0.7	30 Δ	11.5±0.7
P. aeruginosa 2	15	19±1.41Δ	16	0	25Δ	20Δ	30	29.5±0.7	32 δ
P. aeruginosa 3	11	16Δ	14.5±0.7Δ	20.5±0.7	22 δ	23 δ	27.5±0.7	29.5±0.7	31±1.41δ
P. aeruginosa 4	10.5±0.7	13Δ	14.5±0.7Δ	0	0	28Δ	31.5±0.7	28.5±0.7	28.5±0.7
P. aeruginosa 5	9	12.5±0.7Δ	22Δ	20±2.8	33Δ	29±1.41 Δ	20	19±0.7	19.5±0.7
P. aeruginosa 6	11.5±0.7	0 ♦	14.5±0.Δ7	17.5±0.7	24Δ	22 Δ	31±1.41	28.5±0.7	28.5±2.1
P. aeruginosa 7	14	0 ♦	17Δ	20	21±1.41 δ	20.5±0.7	27.5±0.7	24.5±0.7	24.5±0.7
P. aeruginosa 8	9.5±0.7	13.5±0.7Δ	12Δ	20.5±0.7	20	24 Δ	31±1.41	25.5±0.7	30.5±0.7Δ
P. aeruginosa 9	13±1.41	0 ♦	19.5±0.7Δ	19.5±0.7	22.5±0.7Δ	22 Δ	30	29.5±0.7	28.5±0.7
P. aeruginosa 10	13	17.5±0.7Δ	15 Δ	28	25 ♦	24 ♦	28.5±0.7	29	27.5±0.7φ

Data is presented as inhibition zones in mm±standard deviation. CHLO: Chloranphenicol, ATM: Aztreonan, MER: Meropenem. +LYC: Addition of Lycopene; +β: Addition of β-carotene; +C: Addition of Curcumine; +D: Addition of Diosmine; Δ Synergism, statistically significant; δ Synergism tendency (no statistical significance); ♦: Antagonism, statistically significant, φ: Antagonism tendency (no statistical significance); Absence of signals indicate no difference when compared to the control group.

Results

Lack of antimicrobial effect of the tested phytomolecules against the clinical strains

In this experiment, we cultured each isolate with solutions of 100 and 500 µg/mL of each phytonutrient in polystyrene 96 wells non-treated plates, and assessed bacterial growth through resazurine staining (data not shown). Pink color was detected in all wells of test, indicating that the phytonutrients had no antimicrobial activity against any strain in the tested concentrations.

Interference effects of the phytomolecules on antimicrobials was mostly synergic

Lycopene and β-carotene enhanced the antimicrobial activity of aztreonam and chloramphenicol for most of the strains, and this synergism is reported with statistical support by the first time (Table 1). The percentage of observations of statistically significant synergism (SSS) ($p < 0.05$) in these combinations was higher than antagonism (and no interference) observations. These phytonutrients, nevertheless, had no interference on the antimicrobial activity of meropenem (Figure 1).

We performed the same experiment to know the behavior of curcumin and diosmin over the tested drugs. SSS was observed when curcumin was combined to chloramphenicol and aztreonam, and also when diosmin was combined to all tested drugs ($p < 0.05$) (Table 1). Diosmin was the only phytonutrient that presented SSS when combined to meropenem against the clinical isolates of this study (Figure 1).

Discussion

Here we have shown that lycopene, β-carotene, diosmin and curcumin can interfere in different levels on the pharmacological activity of chloramphenicol and aztreonam and meropenem against clinical isolates of *P. aeruginosa*. For chloramphenicol and aztreonam, most of our results provided evidence of SSS of both carotenoids and flavonoids in most of the tested strains in the *in vitro* model we used ($p < 0.05$). Diosmin was the only phytomolecule that presented SSS when combined to meropenem.

Moreover, we assessed the bactericidal potential of the tested phytomolecules. As the results of this experiment would give us some directions on the understanding of the interference test, and because most of the published data on the pharmacological potential of these phytonutrients is associated to protective effects primarily due to scavenging mechanisms (Arabbi *et al.*, 2004; Fisberg *et al.*, 2008), we performed antimicrobial activity assays in 96 wells plates against the clinical isolates. However, none of the phytomolecules we tested had antimicrobial activity against these strains.
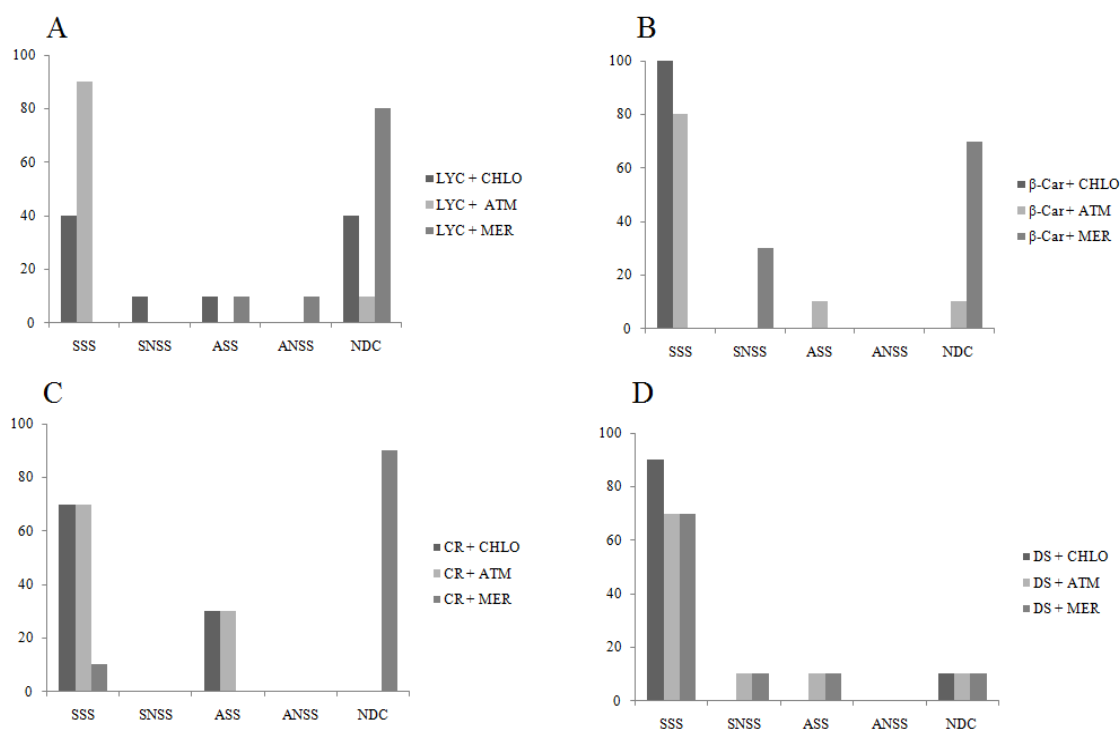
Figure 1. Summarization of interference parameters results. Data is presented as percentage of *P. aeruginosa* isolates included in each interference parameter for Lycopene (A), β-carotene (B), Curcumin (C) and Diosmin (D). LYC: Lycopene; β-car: β-carotene; CR: Curcumin; DS: Diosmin; CHLO: Chloramphenicol; ATM: Aztreonam; MER: Meropenem. SSS: statistically significant synergism; SNSS: Synergism, no statistical significance (considered synergism tendency); ASS: antagonism, statistically significant; ANSS: antagonism, no statistical significance (considered antagonism tendency); NDC: no difference when compared to the control group (considered no interference)

Studies on phytomolecules-AD interactions are very scarce. Recently, our group described the interference of these carotenoids and other flavonoids on the antimicrobial activity of different drugs against clinical isolates of *S. aureus* and *E. coli*, which are also important pathogens regarding food safety (Dos Santos *et al.*, 2015). Most of the interference effects were antagonistic for the tested drugs against these pathogens, and the phytonutrients tested also failed to show antimicrobial activity. Conversely, in the present paper, we have demonstrated that synergic antimicrobial effects are possible against *P. aeruginosa* strains in most of the tested combinations. Possible mechanisms that help to explain our observations might be associated to membrane binding and/or its modification, or modification of resistance mechanisms like efflux pumps, drug transporter channels and/or inactivating enzymes such as β-lactamases. Studies on the impact of these combinations on gene expression associated to these elements are necessary for confirmation of such hypotheses.

Several medicinal food, which are important sources of phytonutrients, have been described with broad antimicrobial activity. Extracts of *Annona squamosa* (custard apple) prepared in chloroform, petroleum ether, and ethanol, presented antimicrobial properties against clinical isolates of *P. aeruginosa* (Gajalakshmi *et al.*, 2011). In another study, protocatechuic acid, gallic acid, ellagic acid, rutin, berberine and myricetin, were combined to ciprofloxacin, ceftazidime, tetracycline, trimethoprim, sulfamethoxazole, polymyxin B and piperacillin against *P. aeruginosa* isolates. The combinations of sulfamethoxazole and protocatechuic acid, sulfamethoxazole and ellagic acid, sulfamethoxazole and gallic acid, and tetracycline and gallic acid demonstrated inhibitory activity against the isolates (Jayamaran *et al.*, 2010).

Regarding possible mechanisms of action, it was described that the phytomolecules protocatechuic acid, gallic acid, quercetin and myricetin, could possibly bind to *P. aeruginosa* dihydrofolate reductase and in combination with sulfamethoxazole, could inhibit different steps of folate synthesis and result in synergism. However, indifferent interactions of trimethoprim and these phytochemicals could be due to the binding of these molecules into the active site

of dihydrofolate reductase, resulting in competitive inhibition of the enzyme and no inhibition of bacterial growth (Jayaraman *et al.,* 2011).

Although our results are mostly related to synergism when combining the phytomolecules we tested, it is not possible, however, to suggest that our data is fully representative of *in vivo* administration of these combinations. Pharmacological effects of drugs and phytonutrients, and their interactions effects in individuals, are dependent on factors like age, genetics, concomitant diseases, drug dosage and cytochrome P450-mediated biotransformation (Ioannides, 2003). Thus, researches using *in vivo* models are, thus, necessary.

Conclusion

We have shown that lycopene, β-carotene, diosmin and curcumin can have synergic effect of interference in the antimicrobial activity of determined drugs against *P. aeruginosa* clinical strains. However, this study is not withouth limitations. We used common concentrations of the tested phytonutrients in formulations for consumption in Brazil, and as these can vary worldwide due to dietary habits, consumption concentrations from other parts of the world should be tested to provide more generalisable results. Also, other drugs can be considered for tests. We provided statistical support for our observations, but further complementary *in vivo* studies are needed for a complete assessment of the biological effects of these interactions, and the molecular mechanisms that are involved in the observed effects remains to be determined. Nevertheless, these limitations have not impaired our measuring of the responsiveness of the methods used in this study, neither their potential of suggesting the effects of such interactions on the efficacy of antimicrobial drugs.

Acknowledgements

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