Carotenoids and flavonoids can impair the effectiveness of some antimicrobial drugs against clinical isolates of *Escherichia coli* and *Staphylococcus aureus*

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**Abstract**

Patients are taking more control over their health, and attracted by contributions to human nutrition, the use of phytonutrients raised. Carotenoids and flavonoids are among the most studied and consumed chemical entities in this sense. However, patients are often unaware of the potential risks associated to drug-herbal interactions. In this study, four drugs were tested combined to lycopene, β-carotene, resveratrol and rutine in an *in vitro* model against clinical isolates of *E. coli* and *S. aureus*. Statistically significant antagonism was detected within most of the tested interactions for at least one isolate. Few events of synergism were detected. For *E. coli*, the antimicrobial activity of Cephalexin and Amoxicillin was significantly reduced. For *S. aureus*, strong reduction of the antimicrobial activity was seen for all drugs. Our data suggest that there might be risks of impairment of the antimicrobial activity of these drugs if co-administered with the tested phytonutrients. Further complementary *in vivo* studies are needed for full assessment of the biological effects of these interactions.

**Introduction**

In the latest decades, more individuals are taking control over their health for treating and preventing diseases, especially if they are chronic or if they do not necessarily demand treatments in hospital settings. One of the most adopted health promotion measures for these purposes has been the rise on the use of phytonutrients, which are specific components obtained from plant food that can be employed for several health benefits through oral or topical use. Varied pharmacological activities of interest have been described for many phytonutrients, and several pharmaceutical formulations have been developed with these compounds in order to explore them clinically, as herbal remedies or phytomedications (Mouly *et al*., 2015; Kreft *et al*., 2015).

Many phytonutrients-based products are sold without need of medical prescription worldwide. The advances of media technologies favored a wide dissemination of the outstanding (and often unique) physiologic and biochemical contributions of phytonutrients to human nutrition, what helps to explain the rise in consumption. Carotenoids and flavonoids are among the most studied and consumed chemical entities nowadays as both nutrients and phytomedication (Pápai *et al*., 2010; Rodrigues *et al*., 2013).

Carotenoids are tetraterpenic pigments lacking symmetry, produced by plants with a vital role in photosynthesis, protection from photo-oxidation and in plant–animal communication. Some bacteria and fungi are also producers of these molecules. Lycopene and β-carotene are among the most studied substances of the group. Carotenoids are responsible for the color of many fruits and flowers, and currently, their most important biological activities are the antioxidant potential and being precursors of vitamin A and its derivatives retinoic acid and retinal. Recent studies also suggests that the antitumor and antioxidant activities of these carotenoids consists in quenching singlet oxygen and scavenging reactive oxygen species (ROS), minimizing cell damage and preventing the development of different types of cancer (Boullata *et al*., 2012; Berginc *et al*., 2015).

Flavonoids are present in many plant foods and have assumed great relevance in the latest years due to antioxidant, anti-inflammatory, antimicrobial, anti-platelet, cardioprotective and antitumor properties. Some chemical subgroups are more commonly detected in plant food, like flavanones, anthocyanidins, isoflavones, flavones and flavonols. Different works have provided evidence that the biological properties of flavonoids and their ingestion
can have a protective effect against the progression of several diseases like hypertension, diabetes and malignant neoplasms (Won et al., 2012; Conner et al., 2013).

Despite the advantages of the ingestion of phytonutrients, a concern arises when patients are using medications combined to natural products. Although considerable quantities of flavonoids and carotenoids are consumed daily in human vegetable diet and adverse effects have not been described so far, many interactions of drugs and molecules of natural sources remain speculative and poorly described; therefore, predicting positive or negative effects of the combined use of drugs and molecules of natural sources becomes a difficult endeavor. Antimicrobial drugs represent a particular problem in this subject matter: many bacterial infections are of difficult treatment because of multidrug resistance, and negative effects due to interactions can lead to complex clinical pictures of difficult treatment because of bacterial growth with strong drug resistance (Conner et al., 2013; Dias-Souza et al., 2013).

Considering the limited knowledge on interactions of phytonutrients with antimicrobial drugs, we investigated the effects of the combined use of lycopene, resveratrol and rutine with certain antimicrobials in an in vitro model against clinical isolates of Escherichia coli and Staphylococcus aureus. Here we show by the first time that despite these phytonutrients had no antimicrobial activity against the clinical isolates, the tested interactions resulted in strong reduction of the pharmacological activity of the antimicrobial drugs, with rare occurrences of synergism in clinical samples. Our data open doors for clinical counseling of patients and professionals regarding such interactions.

Materials and Methods

Microorganisms

Ethical approval was obtained prior to the commencement of the study (University Vale do Rio Doce Ethics Committee, PQ 024/10-10). Isolates of E. coli consisted of uropathogenic samples and isolates of S. aureus were from catheter tips, both obtained of adult patients. Samples (10 of each species) were kindly provided by Dr Pedro Marçal from the clinical isolates collection from the Microbiology Research Laboratory, University Vale do Rio Doce. All isolates were cultured overnight in BHI broth (Difco) at 35±2°C for activation, and tested with Gram-positive and Gram-negative bacteria identification cards for VITEK 2 system (bioMérieux) for identity confirmation. Each card was inoculated with a bacterial suspension prepared in saline solution from manufacturer’s kit and analyzed according to the manufacturer’s instructions.

Interference test

The possible interference of phytonutrients on the antimicrobial drugs was assessed as described by Dias-Souza et al. (2013), with some modifications. Lycopene, β-carotene, resveratrol and rutine 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 et al., 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. Agar plates were prepared with Mueller-Hinton agar (Difco). Antimicrobial disks (gentamycin 10 μg, amoxicillin 10 μg, cephalexin 30 μg and ciprofloxacin 5 μg against E. coli - and nitrofurantoin 300 μg, penicillin 10 U, erythromycin 15 μg and oxacillin 5 μg against S. aureus, all from Sensifar) were distributed as for performing an antimicrobial susceptibility assay as recommended by the CLSI (2009). 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.

Assessment of the antimicrobial activity of the phytonutrients

This assay was performed in duplicate. 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 0.1% resazurine solution (Sigma). 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**

All analyses were carried out in Minitab 17 statistical package for Windows. Homocedasticity and normality were assessed through Bartlett’s test and through Shapiro-Wilk test respectively. Mean diameters of the inhibition zones with and without addition of phytonutrients were analyzed using ANOVA followed by post-hoc Tukey test. The significance level was set at p ≤ 0.05.

**Results**

For both species, statistically significant antagonism was detected within most of the tested interactions for at least one isolate in each association. On the other hand, events of statistically significant synergism of phytonutrients and drugs were rare, and some tendencies (i.e., differences statistically not significant when compared to the controls) of synergism and antagonism were registered. For *E. coli* strains, the antimicrobial activity of Cephalexin and Amoxicillin was significantly reduced in most of the tested strains when any of the tested interfering phytonutrients was combined to them. However, few antagonistic interference events were detected for Gentamycin and Ciprofloxacin, and most of the results of synergism and antagonism tendencies were observed for these drugs (Table 1). For *S. aureus*, strong reduction of the antimicrobial activity was seen for all tested drugs, and all drugs had at least one isolate without formation of inhibition zones. No synergism was detected for any of the tested combinations against the strains (Table 2).

Most of the published data on the pharmacological activity of the phytonutrients we tested are related to antioxidant and anti-proliferative activities, and to protective effects on varied biological tissues (Arabbi et al., 2004; Fisberg et al., 2008). Therefore, considering our interference data, we sought to investigate if the phytonutrients used in this study would have any antimicrobial activity against the clinical isolates. As the results of this experiment would give us some directions on the understanding of the interference test, we cultured each isolate with solutions of 100 and 500 μg/mL of each phytonutrient, and assessed bacterial growth through resazurine staining (data not shown). Interestingly, none of the phytonutrients showed antimicrobial activity against any strain in the tested concentrations.

**Discussion**

The majority of the tested drug-phytonutrient interactions were characterized as antagonism (Figure 1), and significant differences were found when compared with the control. Our data suggest that there may be risks of impairment of the antimicrobial activity of such drugs if these phytonutrients are used in combination to them. This becomes even more important for both nutritional and pharmaceutical counseling on drug-nutrient interaction risks. The research on interactions of natural products and synthetic antimicrobials has been carried out mostly

**Table 1. Interference of flavonoids and carotenoids over antimicrobial drugs against *E. coli* isolates**

<table>
<thead>
<tr>
<th>Strain</th>
<th>GEN</th>
<th>GEN+LYC</th>
<th>GEN+β</th>
<th>AMOX</th>
<th>AMOX+LYC</th>
<th>AMOX+β</th>
<th>CPH</th>
<th>CPH+LYC</th>
<th>CPH+β</th>
<th>CIP</th>
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<td>10</td>
<td>10</td>
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<td>15±0.7</td>
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<tr>
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<tr>
<td>Escherichia coli 10</td>
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</table>

Data is presented ±Standard deviation. GEN: Gentamycin; AMOX: Amoxicillin; CPH: Cephalexin; CIP: Ciprofloxacin. +LYC: Addition of Lycopene; +β: Addition of β-carotene; +RES: Addition of Resveratrol; +RUT: Addition of Rutine; *: Synergism, statistically significant; †: Antagonism, statistically significant; Δ: Synergism tendency (no statistical significance); †: Antagonism tendency (no statistical significance); Absence of signals indicate no difference when compared to the control group.
with plant extracts, and few works have investigated the possible effects of isolated molecules in this context. In this work, we have shown for the first time that lycopene, β-carotene, resveratrol and rutine, which are phytonutrients present in food widely consumed in Brazil like tomatoes, carrots, grapes and apple (respectively), and are also used as phytomedications for varied therapeutic purposes, can interfere in the antimicrobial activity of some drugs of clinical interest.

The rise in the concomitant use of medications and herbal products or nutritional supplements can lead to considerably increased risks of adverse effects due to negative interactions. Currently, our understanding on the potential of interactions between molecules from natural sources and synthetic drugs is very limited, primarily due to difficulties in elucidating the possible mechanisms involved, what also impairs predicting such events. Patients who choose to use herbal products and are following a drug therapy scheme are often not aware of possible adverse reactions. The large use of certain herbal products has pointed out that they are not free of risks as commonly stated among many patients worldwide (Mouly et al., 2015).

Sato et al. (2006) have shown synergism of isoflavones isolated from *Erythrina poeppigiana* combined to vancomycin against methicillin-resistant *S. aureus* (MRSA). Of the isolated isoflavones, isolupalbigenin and erythrinin B exhibited the highest anti-MRSA activity. Moreover, Mun et al. (2014) investigated the antimicrobial activity of carvone enantiomers R-car and S-car combined to Gentamicin against MRSA. Carvone is a naturally occurring monoterpane widely distributed in several plants. The combined molecules R-car/S-car, R-car/ Gentamicin and S-car/Gentamicin exhibited significant synergistic activity against MRSA. Minimal inhibitory concentration values for R- and S-car against six different strains of *S. aureus* ranged between 0.5 and 1 mg/ml.

Gould et al. (2009) reported the antimicrobial activities of pomegranate rind extract free and combined to cupric sulphate against methicillin-sensitive and resistant *S. aureus*, and Panton-Valentine Leukocidin positive community acquired MSSA. Pomegranate rind extract showed limited efficacy against MRSA and MSSA strains when used alone. Exposure to copper (II) ions alone for 2 hours resulted in moderate antimicrobial activity against the isolates, which was enhanced by the addition of pomegranate rind extract. Reduction in growth was observed for 80% of the isolates.

Jayaraman et al. (2010) assessed the interference in vitro of the phytonutrients protocatechuic acid, gallic acid, ellagic acid, rutin, berberine and myricetin in the antimicrobial activity of ciprofloxacin, ceftazidime, tetracycline, trimethoprim, sulfamethoxazole, polymyxin B and piperacillin, against five *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 clinical

### Table 2. Interference of flavonoids and carotenoids over antimicrobial drugs against *S. aureus* isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>NIT</th>
<th>NIT+LYC</th>
<th>NIT+β</th>
<th>PEN</th>
<th>PEN+LYC</th>
<th>PEN+β</th>
<th>ERI</th>
<th>ERI+LYC</th>
<th>ERI+β</th>
<th>OXA</th>
<th>OXA+LYC</th>
<th>OXA+β</th>
</tr>
</thead>
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<td><em>Staphylococcus aureus</em> 1</td>
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<td><em>Staphylococcus aureus</em> 7</td>
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<td><em>Staphylococcus aureus</em> 8</td>
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<td><em>Staphylococcus aureus</em> 9</td>
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Data is presented as Mean ±Standard deviation; NIT: nitrofurantoin; PEN: penicillin; ERI: erythromycin; OXA: oxacillin; +LYC: Addition of Lycopene; +β: Addition of β-carotene; +RES: Addition of Resveratrol; +RUT: Addition of Rutine; *: Synergism, statistically significant; ‡: Antagonism, statistically significant; Δ: Synergism tendency (no statistical significance); †: Antagonism tendency (no statistical significance); Absence of signals indicate no statistical difference when compared to antimicrobial discs of the control group.
isolates.

The in vitro antimicrobial activity of pannarin, a phenolic molecule (from the depsidone family) isolated from lichens, was evaluated by Celenza et al. (2012) in combination with some antimicrobials against MRSA. The authors observed moderate synergistic action when pannarin was combined to Gentamycin, whilst antagonism was observed when combined to levofloxacin.

Interactions between the methanolic extract of Acacia mearnsii and eight antimicrobials were investigated by Olajuyigbe and Afolayan (2012). The synergistic interaction was most expressed by combining the extract with tetracycline, metronidazole, amoxicillin, ciprofloxacin, chloramphenicol and nalidixic acid against E. coli (ATCC 6538), and erythromycin, metronidazole, amoxicillin, chloramphenicol and kanamycin against S. aureus (ATCC 25922).

Previous work of our group have shown that the hydroethanolic extract of cashew (Anacardium occidentale) stem bark, which is rich in flavonoids, saponins, anacardic acid and tannins and has also antibiofilm activity, can also reduce the antimicrobial activity of varied drugs against clinical isolates of S. aureus strains (Dias-Souza et al., 2013). Furthermore, Hussin and El-Sayed (2011) explored the interactions of dichloromethane/methanol extracts of Punica granatum, Thymus vulgaris, Commiphora molmol and Achillea fragrantissima combined to tetracycline, against S. aureus (ATCC 25923), Bacillus megaterium (ATCC 14591), E. coli (ATCC 25922), P. aeruginosa (ATCC 27853) and Klebsiella pneumonia (ATCC 700603) strains. All extracts had significant antimicrobial activity against all tested bacteria and increased significantly the post antibiotic effect.

This study, nevertheless, has some limitations. The concentrations of the tested phytonutrients are related to their consumption in Brazil; therefore, different concentrations should be tested for more generalisable results. Also, our data was related to four drugs which are widely used in hospital settings, but other drugs can be considered. Although we could provide statistical support for our observations, the method we explored in this research is qualitative; quantitative approaches such as the checkerboard method are being explored by our group to conduct further researches based on the results we described in this paper.

Conclusions

We have shown for the first time that lycopene, β-carotene, resveratrol and rutine can interfere in the antimicrobial activity of determined drugs against E. coli and S. aureus clinical strains. Our initial studies indicate that there can be risks of impairment of clinical treatments with the tested antimicrobial drugs if they are co-administered with the tested phytonutrients, what can contribute
to bacterial resistance. Further complementary in vivo studies are needed for a complete assessment of the biological effects of these interactions, and the molecular mechanisms that are responsible for the observed effects remains to be determined. Even so, these technical limitations have not impaired our measuring of the responsiveness of the methods used in this study, neither their potential of predicting the pharmacological results of interactions expressed in synergism and antagonism.

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References


