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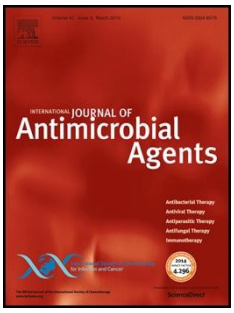

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Resveratrol enhancement on survival of *Staphylococcus aureus* under levofloxacin and photodynamic treatments

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Highlights

- Reactive oxygen species based antimicrobial treatment may be hindered by natural antioxidants.
- Administration of antioxidants should be avoid or limited during photodynamic inactivation and antibiotic therapy.
- Resverastrol co-administred with levofloxacin increases 20-fold the bacterial survival compared with levofloxacin alone
- Bacterial eradication by photodynamic inactivation therapy fails in the presence of resverastrol

Abstract

Reactive oxygen species (ROS) are an efficient tool to eradicate microorganisms, due to the capacity of these species to damage almost all types of biomolecules and to kill cells. The increment in the resistance mechanisms to antibiotics leads to the exploration of new strategies to eliminate microorganisms that involves production of ROS, such as superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). ROS are produced during several antimicrobial treatments, including antibiotic and photodynamic therapies. Among natural antioxidants, resveratrol (RSV) is efficient to prevent damage from ROS, and every day more people incorporate it as a dietary or cosmetic supplement. However, the consequences of the administration of RSV during an antimicrobial treatment are unknown. To investigate possible antagonistic or synergistic effects of RSV during antibiotic therapy (levofloxacin, LVX) or a photodynamic therapy (visible radiation and methylene blue, MB), the elimination of *S. aureus* of a planktonic culture was evaluated in the presence of RSV. Results shows that the antimicrobial capacity of these therapies is significantly diminished when LVX or MB are co-administered with RSV, indicating that the consumption of RSV during an antimicrobial

treatment must be, at least, warned. Moreover, considering that the ROS antimicrobial activity of antibacterial agents, the topical addition of RSV, may also affect the control of pathogens of the human body. The results presented in this article highlight the importance of the evaluation of possible antagonistic effect when an antimicrobial agent with ROS-mediated action is co-administrated with RSV.

Keywords: antibiotics, levofloxacin, photodynamic inactivation, resveratrol, *Staphylococcus aureus*

1. Introduction

Resveratrol (trans-3,5,4'-trihydroxy-stilbene, RSV, Figure 1) is a polyphenolic phytoalexin found in plant species such as grapes, peanuts and berries, and it is produced in response to UV radiation, fungal infection and mechanical injury [1]. This compound was first isolated in 1939 from *Polygonum cuspidatum*, a plant used in traditional Asian medicine during hundreds of years [2]. The therapeutic potential of RSV and its close derivatives has been widely investigated due to their antioxidant, antiviral, cardioprotective and anti-inflammatory properties, and also cancer chemoprevention an treatment [1,3,4]. Moreover, this compound has been proposed to prevent atherosclerosis, aging and diabetes [5–7], and is well known as scavenger of Reactive Oxygen Species (ROS). ROS can be grouped into oxygen-centered radicals: hydroxyl radical ($^{\bullet}OH$), peroxyl radical ($ROO^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), superoxide anion ($O_2^{\bullet-}$) and oxygen-centered non-radicals: singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) [8]. These species are involved in aging, cancer, multiple sclerosis, heart diseases.

Antibiotic treatment is the conventional therapy to eradicate bacterial infections. Levofloxacin (LVX, Figure 1) is one of the drugs widely employed because it is a broad-spectrum antibiotic of the third generation fluoroquinolones, family of antibiotics that generates ROS after inhibition of DNA gyrase, enhancing the bacterial death [9,10]. However, in the last decades the development of multidrug-resistant strains has promoted the search for novel treatments, as an alternative to conventional antibiotic therapy.

The combined action of natural products as RSV and antimicrobial agents has been proposed as an improved approach to kill different species of pathogenic microorganisms [11,12]. However, in most of the reported research works, since the concentrations of RSV needed for bacterial eradication broadly exceed the solubility limit of this compound in water, RSV is

dissolved in methanol-H_2O solutions. This fact limits the application of RSV as antimicrobial agent in topic treatments. It is noteworthy that it has also been postulated that RSV promotes a cytotoxic and prooxidant action depending on its concentration and time of exposure [13]. At the same time, several pharmaceuticals containing RSV promise to provide chemopreventive and therapeutic effects against many diseases and skin disorders [1].

Photodynamic therapy (PDI) is an innovative ROS-mediated antimicrobial treatment that combines a suitable chromophore with UV or visible radiation to generate ROS or radicals able to eradicate microorganisms in a localized area [14,15]. Methylene Blue (MB, Figure 1) has been widely used in the PDI in the treatment of localized infections of Gram-positive and Gram-negative bacteria and fungi [16]. It has strong absorption in the visible region (550-700 nm) and, under visible radiation, produces triplet excited states (^{3}MB*) with relatively high quantum yield ($\Phi_T = 0.52$) [15]. ^{3}MB* participates in both type I and type II photosensitized oxidations [8].

It should be considered that the antibiotics inside the bacterial cell act on their target molecule and trigger oxidative stress secondary processes, due to the generation of ROS. Likewise, the PDI treatments also cause the bacterial cell death through ROS generation and the excited states of the sensitizers. Therefore, taking into account these facts, as well as the scavenging action of RSV, the following question arises: does RSV maintain its antioxidant capacity at concentrations equivalent to, or less than, its solubility in water? If it is the case, the daily oral consumption or the topical uses of RSV as an antioxidant could hinder bacterial killing by ROS produced either by the antibiotic or PDI treatment.

All in all, the present study aims to investigate the possible antagonistic action of RSV in the presence of antimicrobial agents able to produce ROS. Thus, the influence of RSV in the ROS mediated antimicrobial eradication is analyzed. The effect of RSV when is administrated to a *Staphylococcus aureus* culture together with: i) LVX that generates ROS inside the bacterial cell, and ii) MB, which eradicates microorganisms by ROS production and electron transfer mediated oxidation from MB excited states.

2. Materials and methods

2.1. Bacterial cultures

S. aureus ATCC 25923 was inoculated in 100 ml of Nutrient Broth (NB, Merck, Darmstadt, Germany) and grown overnight at 35 °C with shaking (250 rpm). The bacterial suspension was further adjusted with fresh NB to 1×10^6 bacteria/ml and used for the biological assays.

2.2. Antibiotic susceptibility testing for planktonic bacteria

First, the minimum inhibitory concentration (MIC) of LVX against planktonic *S. aureus* was assessed following the procedure described in CLSI guidelines [17]. The MIC was defined as the lowest concentration of antibiotic at which bacterial growth was not detected. As a second step, the killing of *S. aureus* planktonic cells was tested using different concentrations of LVX (1 to 128 x MIC) and constant concentration of RSV (15 □g/ml), and for comparative purposes assays using only LVX (control) were carried out.

2.3. Photodynamic antimicrobial assays

Photodynamic inactivation (PDI) assays against *S. aureus* planktonic cells were carried out to assess the antioxidant property of RSV as was described by Vecchio *et al.* [18] with minor modifications. Briefly, aliquots of 100 µl of bacterial suspensions (10^6 CFU ml^{-1}) containing RSV (30 □g/ml) were added to the wells of sterile 96-well microtiter plates containing 100 µl of serial two-fold dilutions of MB (Sigma Aldrich). The wells were incubated 15 min in the dark, and then exposed to visible radiation (8 watts white fluorescent tube, wavelength range 350-750 nm) for 40 min at 35 °C. Afterwards, the number of bacteria in the wells was determined using the plate count method. The plates were incubated at 35 °C for 24 h. The final concentration of RSV per well was 15 □g/ml. Control assays were performed by the

141 same procedure but without RSV in the bacterial suspensions. A triplicate series of
142 experiments and two replicates were carried out in each case.
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145

3. Results and Discussion

Gram-positive bacteria *S. aureus* were incubated in the presence of LVX and MB. Both antimicrobial agents induce cellular oxidative stress, and the drug-mediated ROS generation. Experiments in the presence and in the absence of RSV were performed, under identical conditions, to evaluate the possible antagonistic effect of this natural antioxidant.

3.1 Control experiments

In order to evaluate the effect of RSV in *S. aureus* viability, planktonic bacteria were grown in the absence and in the presence of RSV (15 □g/ml) for 24 h at 35 C. The RSV concentration employed in the biological assays was 2-fold lower than its solubility in water under physiological conditions. Bacterial growth was not inhibited in the presence of RSV compared with the control (RSV-free broth medium), showing no significant difference ($p>0.05$) in the number of viables bacterias (4.6 □□□2.7 x 10^9 and 5.2 □□□2.6 x 10^9 CFU/ml respectively). This result was expected since the antimicrobial action of RSV was found when concentrations several times higher than its solubility in water were employed (100 □g/ml -200 □g/ml) [11].

3.2 Levofloxacin

The minimum inhibitory concentration (MIC) of LVX on *S. aureus* culture was determined in the culture medium, and the value obtained was found to be 0.125 □g/ml. Then, the *S. aureus* viability was explored in the range of 1 to 128 x MIC in equivalent experiments, in the absence and in the presence of RSV (15 □g/ml) (Figure 2). In all assays, the bacterial viability was higher in the presence of RSV than in its absence, indicating that RSV prevents cells from death. At MIC concentration of LVX, the bacterial viability decreased 1000-fold in comparison with the initial inoculum when RSV was present in the culture media. But when

RSV is absent, that is RSV-free broth medium, the viability was 20000-fold lower compared with the initial inoculum (Figure 2). At concentrations of LVX higher than MIC (in the range of 1 to 8 x MIC), the cell viability was higher (at least 10-fold) in the presence of RSV than that found with the antibiotic alone. At higher antibiotic concentrations (16 x MIC) the difference in the bacterial viability for both treatments decreased, being no significant in case of 64 and 128 x MIC. However, for all concentrations tested, the remnant number of bacteria was higher when RSV was present. It is important to mention that, at 8 x MIC LVX with RSV joint administration, bactericidal action was similar than that obtain with LVX alone at 1 x MIC ($p>0.05$). This indicates that RSV co-administration would require the increase of the antibiotic concentration to preserve its bactericidal action. Our results are in good agreement with those presented in a recent work by Liu *et al.* [19]. These authors evaluated the interference of RSV in the activity of several antibiotics involving ROS as antimicrobial mechanism, against planktonic populations of *E. coli* and *S. aureus*. The bacterial killing was analyzed at short times (2 h) using fluoroquinolone antibiotics, concluding that the addition of RSV increased the viability of *S. aureus* planktonic cells. However, this increase in the bacterial survival produced by RSV could be overrated at the experimental conditions used by the authors, since the studies were carried out at short periods of time (2 h), at which the effect of RVS might be hidden by the delay in bacterial killing induced by the antioxidant.

Results answer our question: is RSV, as an antioxidant, able to hinder bacterial killing? They suggest that ROS are produced by LVX *in vitro*, although it is still controversial how the LVX acts. Thus, if bacteria are incubated with LVX and RSV, the availability of ROS, i.e. the amount of those species that cause damage to important macromolecules required for bacterial subsistence, decreases and consequently bacterial survival increases [10]. In this sense we consider that RSV inhibits ROS accumulation resulting from LVX action. Thus, at

196 higher RSV concentration, ROS accumulation are not enough to reach bactericidal action *per*
197 *se*. Along this line, Liu *et al.* [19] evaluated the *E. coli* viability after treatment with oxolinic
198 acid (quinolone antibiotic) with and without RSV. These authors concluded that RSV limits
199 quinolone-mediated accumulation of intracellular ROS, which is in agreement with our
200 results.
201
202 A possible explanation of the increase of the antibiotic concentration needed to preserve LVX
203 bactericidal action in the presence of RVS in the 64-128 x MIC range may be related to the
204 action of topoisomerases (gyrase and topoisomerase IV). It is well known that they are the
205 main targets of quinolone antibiotics, whose action mechanism involves the increasing of the
206 concentration of enzyme−DNA cleavage complexes, "poisoning topoisomerases" and
207 converting gyrase and topoisomerase IV into cellular toxins [20], leading to cell death.
208 Therefore, it is reasonable to hypothesize that at the highest antibiotic concentration (64-128 x
209 MIC) in presence of RVS that decreases ROS availability, cell death is mainly due to the
210 inhibition of gyrase/topoisomerase IV action by LVX and a higher concentration of LVX
211 under LVX + RVS is needed to achieved the effect of LVX alone.
212
213 *3.2 Methylene Blue*
214 The antimicrobial activity of MB against *S. aureus* was measured under visible radiation at
215 different concentrations (0-50 □g/ml). Control experiments (sample irradiated in the absence
216 of MB or RSV) indicated that the irradiation alone does not affect the bacterial viability
217 (Figure 3, growth control bar). On the other hand, irradiation experiments performed in the
218 presence of MB (6.25 to 50 □g/ml) indicate that bacteria viability decreases proportional to
219 MB concentration, and at 50 □g/ml the eradication of bacteria was observed (Figure 3). These
220 results are in agreement with those reported by Vecchio *et al*. [18]. In this way, PDI by MB

represents an alternative strategy to kill multi-resistant bacteria since ROS are able to oxidize biomolecules unspecifically and thereby kill cells, including persister cells (often refractory to conventional antibiotic treatments). Thus, it is important to highlight that under these conditions, bacterial eradication can be achieved.

Equivalent experiments were performed adding RSV (15 □g/ml). Co-administration of both MB and RSV, decreased MB efficacy, i.e. improved bacteria survival from 10 to 1000-fold in the whole range of MB concentration tested (Figure 3). At the lowest MB concentration tested (6.25 □g/ml), the minor difference in viability was observed. This fact might be attributed to an insufficient ROS production by the photosensitizer to enhance the bacterial killing. At higher MB concentrations, the protective effect of RSV on the viability of bacteria was more pronounced and was striking at the higher MB concentration (50 □g/ml) (Figure 3), e.g. RSV increases the bacterial viability from a negligible count to 1000 CFU ml^{-1} (1000- folds of increase). Taking into account that RSV is a good scavenger of ROS species, it may be assumed that most of the ROS generated by MB under visible irradiation have been eliminated from the media.

It is important to highlight that, in contrast to the behavior observed with the antibiotic treatment (Figure 2), the antagonist effect of RSV was exacerbated in PDI treatment at the maximum concentration of photosensitizer (Figure 3). This difference could be explained taking into account the main mechanism to kill bacteria involved in each case: in PDI treatment, ROS production is the main responsible of bacterial killing, while in the antibiotic therapy involves two collaborative mechanism, inhibition of the enzyme topoisomerase and ROS generation.

In view of our *in vitro* results, we could hypothesize that *in vivo* bacterial infection would be more difficult to control or eradicate in the presence of RSV because it affects the efficiency of the antibiotic treatment. Therefore, our results indicate that it would not be recommendable to supply an antioxidant such as RSV together with antibiotics formulations or during PDI treatment.

Conclusion

In the present study, the efficiency of planktonic *S. aureus* killing was clearly diminished by the presence of RSV during antibiotic treatment, using LVX, and PDI, using MB and visible radiation. In comparison, the decrease the of antimicrobial treatment efficiency was much greater during the photodynamic inactivation than during the administration of LVX. Considering that RSV is a well-known ROS scavenger, the antagonist effect may be due to the elimination of these species from the media. Taking this into account, the lowest decrease in the antimicrobial effect during treatment with LVX, can be explained considering the dual antimicrobial activity of this antibiotic. In spite of the deactivation of ROS by RSV, LVX still kills bacteria due to its activity related to DNA gyrase inhibition.

In summary, the co-administration of RSV, both in oral and topical forms, might significantly reduce the effectiveness of any antimicrobial treatment that acts through the production of ROS. Additionally, considering that the microbiocidal activity of host defenses (neutrophils, macrophages) is also mediated by ROS, and that the efficiency of the physiological response to microorganisms might be reduced when RSV is being consumed with cosmetic or antioxidant purposes, this undesirable side effect of RSV should be evaluated.

On the other hand, results also demonstrated that PDI by MB represents an alternative strategy to kill multi-resistant bacteria since ROS are able to oxidize biomolecules unspecifically and thereby can lead to bacterial eradication by killing persister cells.

Declarations

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Competing Interests: No

Ethical Approval: Not required

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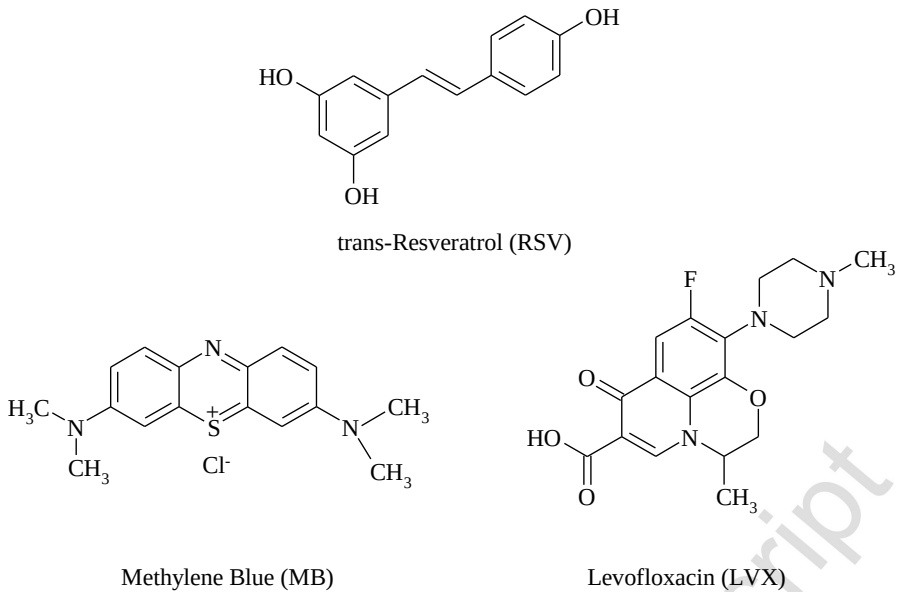

353 **Figure 1:** Molecular structure of RSV, MB and LVX

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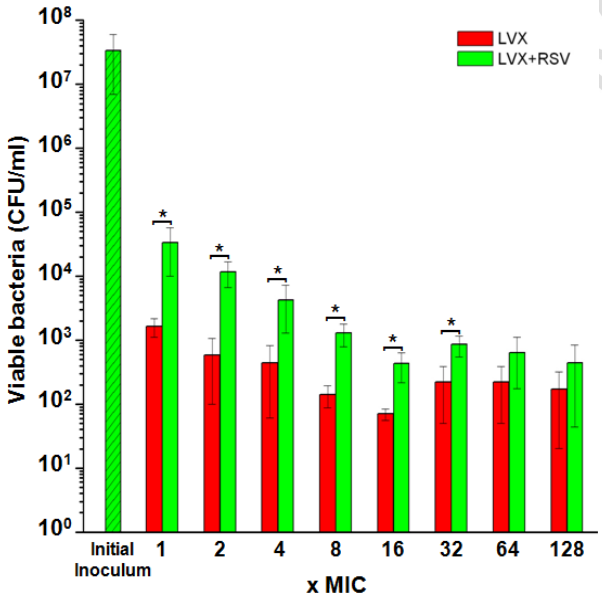

356 **Figure 2.** Planktonic cells of *S. aureus* exposed to levofloxacin. Quantification of viable
357 bacteria after the antibiotic treatment (1-128 x MIC, MIC = 0.125 μg/ml) with RSV (15
358 μg/ml, green bars), and without RSV (red bars). Initial inoculum refers to the number of
359 bacteria before the antimicrobial treatments. Bars labeled with an asterisk denote statistically
360 significant differences ($p < 0.05$).
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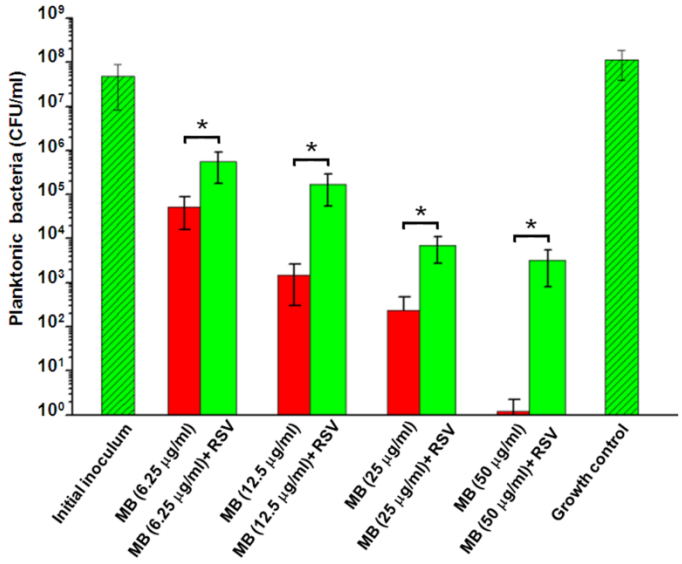

Figure 3. PDI of planktonic *S. aureus* cells using MB as photosensitizer. Planktonic bacteria were incubated with MB in the dark (15 min) and then irradiated (Vis) for 40 min in presence (solid green bars) and absence (solid red bars) of RSV (15 μg/ml). Growth control bars indicates the number of viable bacteria after dark incubation and then irradiated in absence of MB and RSV. Bars labeled with an asterisk denote statistically significant differences ($p < 0.05$).