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Combinatorial Activity of Flavonoids with Antibiotics Against Drug-Resistant *Staphylococcus aureus*

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The use of resistance-modifying agents is a potential strategy that is used to prolong the effective life of antibiotics in the face of increasing antibiotic resistance. Since certain flavonoids are potent bacterial efflux pump inhibitors, we assessed morin, rutin, quercetin, hesperidin, and (+)-catechin for their combined activity with the antibiotics ciprofloxacin, tetracycline, erythromycin, oxacillin, and ampicillin against drug-resistant strains of *Staphylococcus aureus*, including methicillin-resistant *S. aureus*. Four established methods were used to determine the combined efficacy of each combination: microdilution checkerboard assays, time-kill determinations, the Etest, and dual disc-diffusion methods. The cytotoxicity of the flavonoids was additionally evaluated in a mouse fibroblast cell line. Quercetin and its isomer morin decreased by 3- to 16-fold the minimal inhibitory concentration of ciprofloxacin, tetracycline, and erythromycin against some *S. aureus* strains. Rutin, hesperidin, and (+)-catechin did not promote any potentiation of antibiotics. Despite the potential cytotoxicity of these phytochemicals at a high concentration (fibroblast IC_{50} of 41.8 and 67.5 mg/L, respectively), quercetin is commonly used as a supplement for several therapeutic purposes. All the methods, with exception of the time-kill assay, presented a high degree of congruence without any apparent strain specificity.

Introduction

The discovery of most of our current arsenal of anti- microbial products has been based on the well-established "one-compound-one-drug" paradigm.[33] However, the pharmaceutical industry's search for new antimicrobials has markedly slowed in recent years and strategies to mitigate resistance in existing drugs are urgently required, given the substantial increase in antibiotic resistance.

Antimicrobial combinations are frequently used in clinical practice to promote increased pharmacological action or to achieve synergistic activity to treat mixed bacterial infections in which the organisms are not susceptible to a common agent,[42,51] to overcome bacterial tolerance,[34] to prevent the emergence of drug resistance,[5,65] and to minimize toxicity.[25] Antibiotics may also be used in combination to increase efficacy. The use of agents that inhibit bacterial resistance mechanisms (resistance-modifying agents [RMAs]) can be highly effective. Several inhibitors of b- lactamases are used in combination with antibiotics: for example, clavulanic acid is used in combination with

amoxicillin (Augmentin®) and ticarcillin (Timentin®), while sulbactam is combined with ampicillin (Unasyn®) and tazobactam, used in combination with piperacillin (as Tazocin®).[2] There is considerable scope for expansion of the application of RMAs to increase the effectiveness of current antibiotics and to avoid the emergence of resistant variants during treatment.[23,46]

There has recently been a resurgence of interest in the use of drugs based on phytochemicals.[4] Plants can produce complex mixtures of structural different products that can interact simultaneously with several biological targets and which can lead to enhanced efficiency.[29]

There are several reports of phytochemical compounds, including flavonoids with limited antimicrobial activity that can enhance the effect of antibiotics.[2] Flavonoids are hydroxylated phenolics commonly found in leaves, fruits, and flowers. The existence, number, position, and degree of substitution of hydroxyl or methyl groups on the benzene ring provide much of the structural variation found in flavonoids[9] and they, consequently, have a diverse range of pharmacological properties, including anti-inflammatory, oestrogenic, anti-allergic, antifungal, antimicrobial, and antioxidant.[15,27,37] Importantly, several flavonoids were found to potentiate some antibiotics due to a possible activity as inhibitors of multidrug-resistant mechanisms, such as efflux pumps.[2,39]

Quercetin, rutin, hesperidin, and (+)-catechin were re- ported to have a synergistic activity when combined with oxacillin against vancomycin-intermediate *Staphylococcus aureus* (VISA).[7] The goal of this work was to evaluate whether these compounds would be able to potentiate other antibiotics against a range of clinical isolates of antibiotic-resistant *S. aureus,* including methicillin-resistant *S. aureus* (MRSA) strains. Morin, the isomer of quercetin, was also included in the tests. Ampicillin and oxacillin (b-lactams), ciprofloxacin (fluoroquinolone), erythromycin (macrolide), and tetracycline were selected as the resistance mechanisms for these antibiotics are already much disseminated. To obtain reliable conclusions about the antibiotic potentiation effects, four distinct methods were used (microdilution broth checkerboard, disc diffusion method, Etest, and time-kill assays). These tests measure distinct effects of antibiotic interactions against bacteria (growth inhibition or killing) and use different growth medium states (broth vs. agar).[10,18,64]

Materials and Methods

Bacterial strains and growth conditions

Test bacteria included MRSA (MJMC001, MJMC002, MJMC004) and MSSA (MJMC003, MJMC009, MJMC010).

All were low-passage clinical isolates, obtained from the Hospital Centre of Trás-os-Montes and Alto Douro, EPE (Vila Real, Portugal).

Additional test strains were *S. aureus* SA1199B, RN4220, and XU212, which over-express *norA, msrA*, and *tetK*, respectively, and were provided by S. Gibbons (University College London, London, United Kingdom)[23,24,47,62] and *S. aureus* XU212 (MRSA). *S. aureus* CECT 976, used in previous studies with phytochemicals,[1,57,60]

was included as a comparator. Bacteria were grown overnight at 37ºC and under agitation (150 rpm) in Mueller-Hinton (MH) broth (Merck).

Antibiotics and flavonoids

Ampicillin (AMP), ciprofloxacin (CIP), erythromycin (ERY), oxacillin (OXA), and tetracycline (TET) were pre- pared according to the manufacturer's recommendations. Morin (MOR), quercetin (QUERC), hesperidin (HESP), rutin (RUT), and (+)-catechin (CAT) (Fig. 1) were prepared in dimethyl sulfoxide (DMSO, 100%). All the compounds and reagents were obtained from Sigma.

Antibacterial susceptibility testing

Minimum inhibitory concentrations (MICs) of each agent were determined by broth microdilution testing according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[41] MICs were defined as the lowest concentration of the antimicrobial compound that inhibited bacterial growth. OD_{600} was measured to assess the growth of bacteria. Three independent experiments were performed for each compound, and eight replicates per each concentration were performed in each test. The highest concentration of DMSO remaining after dilution (10%, v/v) caused no growth inhibition (data not shown).

Antibiotic-flavonoid antibacterial effects

Antibiotic-flavonoid combinations were tested using the broth microdilution checkerboard, disc diffusion method, Etest, and time-kill assays. Three independent experiments were performed for each combination and test. Based on a new approach developed for interactions between compounds that have no antimicrobial activity but that can potentiate antimicrobials,[3] the combinations were classified as potentiation, additive, indifferent, or negative. Combinations were classified as additive if two or more methods presented that same interpretation of the results. Otherwise, results obtained between the limits defined for potentiation and negative interactions did not distinguish between additivity and indifference.

Checkerboard microdilution assay

Checkerboard assay was performed according to Bona- pace *et al.*[10] and Humphreys *et al.*[31] with some modifications. The concentration of each antibiotic tested ranged from 1/64 to 2 - MIC. The flavonoids were tested at several concentrations ranging from 10 to 1,500 mg/L. The anti- microbial solutions did not exceed 10% (v/v) of the volume used in each well (200 ml). Growth controls with DMSO at 10% (v/v) were assessed in wells not containing the chemicals. Negative controls were performed by adding fresh MH broth without bacteria to each combination. Incubation was performed for 24 hours at 37ºC, and readings were determined spectrophotometrically at 600 nm. For the combinations where one of the compounds has no antimicrobial activity, the fractional inhibitory concentration[36,53] might not be determinable. In those cases, potentiation was defined as an antibiotic MIC reduction of 4-fold dilutions, an additive interaction as an MIC reduction $\geq$2-fold and $\leq$ 4-fold dilutions, and a negative interaction as an MIC

increase of 4-fold dilutions.

Disc diffusion method

This method was a modification from the Kirby–Bauer method and was applied in similar studies.[1,57] The concentrations for the flavonoids were chosen according to the checkerboard results. Each flavonoid was added to MH agar (after autoclaved and cooled to ~30ºC), yielding the final concentration desired, and the medium was poured into 90 mm Petri dishes. The bacterial suspensions were adjusted to 0.5 McFarland standards and seeded over hardened MH agar Petri dishes. Sterile blank discs (6 mm diameter; Oxoid) were placed on the agar plate. A volume of 15 µl of each antibiotic prepared according to the CLSI guidelines (AMP, 10 µg/disc; CIP, 5 µg/disc; ERY, 15 µg/disc; TET, 30 µg/disc; and OXA, 1 µg/disc) was added to the blank discs.[14] As control, antibiotic discs were applied on simple MH agar plates (without flavonoid). After incubation at 37ºC, for 24 hours, each inhibition zone diameter (IZD) was recorded and analyzed according to CLSI guidelines.[14]

According to the scheme proposed in a previous study,[3] the combination between two bioactive agents was characterized as additive if $4 \leq$ (IZD combination - IZD most active agent) < 6 mm, and as potentiation if (IZD combination - IZD most active agent) ≥ 6 mm. The limits for negative interactions were not defined since no negative combinations were ever found.

Etest

This method was performed according to the manufacturer's instructions (AB Biodisk). The concentrations for the flavonoids were chosen according to the checkerboard results. The bacterial inoculum and the MH agar plates were prepared as described for the disc diffusion method, and antibiotic Etest strips (AB Biodisk) were applied in duplicate on the agar plates. As control, antibiotic strips were applied on simple MH agar plates (without flavonoid). After incubation for 24 hours at 37ºC, the MICs were read. For Etest, if the MIC of antibiotic was reduced by > 3-fold dilutions, the result was considered potentiation; a reduction of ≥ 2 but < 3 was considered an additive; and an increase of threefold dilutions of the MIC was classified as negative.[12,35,63]

Time-kill assay

This method was performed according to Bonapace *et al*.[10] with some modifications. The antibiotics were tested at MIC, 1/2 · MIC, and 1/4 · MIC. The concentration of each flavonoid was chosen according to the checkerboard results. The combinations were added to sterilized tubes and inoculated with each isolate in a total medium volume of 10 ml. The antimicrobial combinations did not exceed 10% (v/v) of the final volume. Controls were performed with each product separately. Colony forming units (CFU) counts were performed after 0, 4, 8, and 24 hours of the beginning of the incubation at 150 rpm and 37ºC. The limit of detection was 50 CFU/ml. Potentiation was considered as a ≥ 2- $\log_{10}$ decrease in CFU/ml between the combination and its most active constituent at the designated sample time; an additive interaction was defined as a $\log_{10}$ decrease ≥ 1 and <2 in CFU/ml; and a negative interaction was defined as a ≥ 2- $\log_{10}$

increase in CFU/ml.[18,40]

Cytotoxicity testing

The cytotoxicity of the flavonoids was evaluated ac- cording to ISO/EN 10993 (part 5)[32] guidelines using L929 cells (ATCC CCL 1), from an immortalized mouse lung fibroblast cell line. Cells were grown in 175 cm^2 culture flasks using Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 1% of penicillin and streptomycin solution (Sigma) and 10% fetal bovine serum (FBS; Biochrome). The flasks were incubated at 37ºC for 72 hours in a 95% air 5% CO_2 atmosphere with 100% humidity. Then, cells were trypsinised, plated in 96-well microtiter plates at a concentration of $1 \cdot 10^4$ cells per well, and left to adhere for 24 hours at 37ºC in a 95% air 5% CO_2 atmosphere with 100% humidity. The medium in each well was replaced by 200 ml of fresh DMEM with the flavonoids at several concentrations. The flavonoids did not exceed 2% (v/v) of the well final volume. Growth control was performed by adding fresh medium without any flavonoid and the negative control by adding DMSO at 2% (v/v). Each condition was performed in five wells and in triplicate. The plates were incubated for 72 hours (at 37ºC, in a 95% air 5% CO_2 atmosphere with 100% humidity). After incubation, the cell viability was assessed using the Cell Titer 96® One solution Cell proliferation Assay Kit (Promega). This assay involves the bioreduction of the substrate, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyl-methoxy-phenyl)-2(4-sulfo-phenyl)-2H tetrazolium] (MTS) into a brown formazan product by NADPH or NADP produced by dehydrogenase enzymes in metabolically active cells.[51] For the assay to occur, the medium with flavonoids was removed and replaced by 200 ml of a mixture of DMEM without FBS and MTS (1:5 ratio) and incubated for 3 hours (at 37ºC, in a 95% air 5% CO_2 atmosphere with 100% humidity). The optical density of each well was measured at 490 nm using a plate reader (Molecular Devices). From the dose-response curves obtained, the half maximal inhibitory concentration (IC_{50}) values were calculated by probit analysis, according to Sebaugh.[59]

Statistical analysis

For statistical analysis, the *in vitro* results were analyzed by Student's *t*-test using the statistical program SPSS version

19.0 (Statistical Package for the Social Sciences). Statistical calculations were based on a confidence level $\geq 95\%$ ($p < 0.05$) that was considered statistically significant.

Results

The classification of the *S. aureus* strains according to their resistance profile was done based on the comparison of the MICs/IZDs results of antibiotics and the susceptibility breakpoints of the CLSI guidelines (Table 1).[41] The agreement between the MICs obtained by microdilution technique and Etest (within – 1-log_2 dilutions) was 75% for TET, 88% for ERY, and only 38% for CIP. According to the classifications presented by Reynolds *et al*.[56] there was good agreement for ERY and poor agreements for TET and CIP. No MIC was detected for any flavonoid for concentrations lower than 1,500

mg/L (data not shown). Also, no IZD was obtained with the flavonoids for the same amount of com- pound (22.5 μg/disc).

Table 2 presents the results of the combinations of anti- biotics and flavonoids obtained by checkerboard, Etest, disc-diffusion method, and time-kill assay. Only the combinations that resulted in a significant modification in comparison with antibiotics alone for, at least, one method have been presented. The concentrations of the flavonoids for the combination assays were chosen according to the checkerboard results and were considered the minimal concentrations causing the best effects (for active compounds: MOR, QUERC, and RUT). For CAF and THEO that had no activity, the same concentration of the other compounds (500 mg/L) was used. Checkerboard and Etest methods revealed several MIC reductions of the antibiotics, especially in the presence of MOR and QUERC (at 500 mg/L). The same behavior was observed by disc-diffusion method, where the higher IZD increases were obtained, especially with MOR and QUERC. No MIC increase or IZD decrease was obtained for antibiotics with the addition of flavonoids ($p > 0.05$).

Figure 2 presents the $\log_{10}$ CFU/ml results versus time for each combination. Time-kill assays were performed only for *S. aureus* SA1199B, RN4220, and XU212 since this method was performed on combinations selected on the basis of their performance, assessed by the other three tests. No $\log_{10}$ CFU reduction was observed with the flavonoids alone for the concentrations tested when compared with the growth control ($p > 0.05$, data not shown). Log_{10} CFU/ml increases were obtained for *S. aureus* SA1199B when CIP (at 1/2 MIC) was combined with MOR and QUERC, compared with CIP alone (1.6 and 1.2 $\log_{10}$ increases of CFU/ml, respectively). The other methods provided potentiating effects for these combinations (Table 2). For *S. aureus* XU212, TET combined with MOR and QUERC caused a 1.8 and 1.7 $\log_{10}$ CFU/ml reduction, respectively, when compared with TET (at 1/4 MIC) and 1.2 and 2.6 $\log_{10}$ CFU/ml reduction, respectively, compared with TET (at 1/2 MIC). However, indifferent results were obtained for these combinations with the other three methods (Table 2). For *S. aureus* RN4220, time-kill assay and the other methods provided indifferent results.

The combinations tested with the four methods were classified as potentiation, additive, or indifferent as previously described (Table 2). No negative effects were observed. Considering the total agreement and minor disagreement cases (for checkerboard, Etest, and disc-diffusion method; Table 2), potentiation was found for 24 of 190 combinatorial cases (12.6%) and additive interactions only for 4 of 190 combinations (2.1%). The majority of the combinations (84.2%) were indifferent.

Potentiation was obtained for the combination of MOR with: CIP against SA1199B and MSSA strains (3/3), TET against CECT 976, MRSA (3/3), and MSSA strains (3/3), and ERY against MSSA (2/3); for the combination of QUERC with: CIP against CECT 976, SA1199B, and MSSA (1/3), TET against CECT 976, MRSA (3/3), and MSSA (2/3) strains, and ERY against CECT 976 and MSSA (1/3); and for the combination of RUT with TET against CECT 976. No additive or potentiating effects were obtained with CAT or HESP. Furthermore, no flavonoid potentiated the activity of either b-lactam (ampicillin and oxacillin).

Agreement between checkerboard, Etest, and disc-diffusion method was 85%. Agreement between time-kill assay and the other three methods for *S. aureus* SA1199B,

XU212, and RN4220 was 80%. However, time-kill assay usually failed to agree with the other methods for the most promising combinations (with MOR and QUERC) against *S. aureus* SA1199B and XU212. Agreement was observed in 108 of 120 cases (90%) between checkerboard and Etest, in 111 of 120 cases (92.5%) between Etest and disc-diffusion method, and in 174 of 190 cases (91.6%) between disc-diffusion method and checkerboard. Minor disagreements for checkerboard, Etest, and disc-diffusion method were obtained for 18 of 120 cases, respectively (15.0%). No major disagreements were obtained between these three methods.

Cytotoxicity tests of the flavonoids were performed using the MTS viability assay of L929 cells. The DMSO (2%) was not toxic for cells (data not shown). From the dose-response curves obtained, IC_{50} values were calculated by probit analysis. QUERC, MOR, and HESP presented low values of IC_{50} (41.8, 67.5, and 50.4 mg/L, respectively) after 72 hours of incubation with L929 cells. CAT had an IC_{50} of 302.5 mg/L, and RUT showed the highest value (1267.5 mg/L).

Discussion

In this study, various combinations of selected flavonoids and antibiotics were tested for their antibacterial potency using four different methods. This strategy maximized the likelihood of detecting potentiating combinations. Potentiating and additive interactions were found in 24 and 4 of 190 combinatorial cases (12.6% and 2.1%), respectively, and mainly for combinations involving MOR and QUERC. Both MOR and QUERC increased the activity of CIP and TET against *S. aureus* CECT 976; of CIP against SA1199B; and of TET against all MRSA and MSSA strains. In addition, some potentiation/additive interactions were found with MOR and QUERC combined with ERY. RUT only caused potentiation when combined with TET against *S. aureus* CECT 976. While some potentiating results were found on some *S. aureus* strains, this was not observed for others.

Experimentation on drug combinations can lead to opposite conclusions by different methodologies since they use different endpoints (inhibition or killing) and medium state (broth vs. agar).[10,18,64] There was a high degree of agreement between checkerboard, Etest, and disc-diffusion methods (85%) but generally lower agreement for time-kill assays, probably because this is a test for bactericidal activity, contrarily to the others. Other researchers have already developed some comparisons on the agreement between these methods, which varies a lot between studies.[3,13,38,44,48,64]

QUERC is the most abundant flavonol found in the human diet.[17] MOR is the isomer of QUERC and it has also been found to possess antibacterial activity. RUT is the glycosidic form of QUERC.[6] Reports on the antibacterial activity of these flavonoids are conflicting, especially regarding their MICs, probably owing to inter- and intra-assay variation in susceptibility testing or due to the difference in genetic variation of the strain.[7,16,30,54,55] Both MOR and QUERC (500 μg/disc) were reported to inhibit the growth of *aureus,* CAT (500 μg/disc) only had a slightly activity, and RUT (500 μg/disc) had no antimicrobial activity.[55] The concentration of 500 μg/disc is, however, much higher than the highest used in this study, 22.5 μg/disc. In another study, the MIC of

QUERC against *S. aureus* was found to be 80 mg/L.[54] QUERC (15 mg/L) was found to exert antibacterial activity against *S. aureus*, including MRSA, and to potentiate the activity of erythromycin, oxacillin, ampicillin, vancomycin, and gentamicin against MRSA.[30] Other investigations showed that 87.3% of the combinations be- tween eight flavonoids (including CAT, HESP, QUERC, and RUT) and OXA were synergistic against VISA.[7] However, in this study, no synergism was obtained with OXA against the tested *S. aureus* strains. Eumkeb *et al*.[20] also demonstrated that QUERC, galangin, and baicalein exhibited the potential to reverse bacterial resistance to b- lactam antibiotics against penicillin-resistant *S. aureus* apparently due to interaction with penicillinase, cytoplasmic membrane damage, inhibition of protein synthesis, and changes in the PBP2a.

Other studies with polyphenolic compounds also reported potentiating activities with some antibiotics against different bacterial strains. These studies were already reviewed elsewhere.[2,22] Besides, many of these compounds are re- ported to have common mechanisms of action by inhibition of efflux pumps, or PBP2a, or increasing the membrane permeability, their activity may significantly vary when interacting with other biomolecules. Also, considering that these studies used different bacterial strains, antibiotics, and methods, it can be difficult to compare results on the basis of a structure-activity relationship.

In this study, the only potentiation of an antibiotic against a strain resistant to that antibiotic was observed for SA1199B with the combination of MOR/QUERC and CIP. In the other combinations, potentiation occurred only with strains that were susceptible to the antibiotics. Therefore, with the exception of the first situation, which may be due to efflux pump inhibition, these activities are, in general, probably not related to RMA. In fact, several studies have examined the potential of QUERC to inhibit various bacterial enzymes, explaining why this molecule has such a diverse applicability (including anti-tumor therapy, neuro- protection, cardiovascular disease profylaxis, inflammation, diabetes mellitus, infection, etc).[21,26,58] However, it is clear that further research is necessary to understand MOR/ QUERC potentiation of CIP, TET, and ERY. One of the proposed mechanisms of action of QUERC was the inhibition of gyrases through two different mechanisms based either on interaction with DNA or with ATP binding site of gyrase.[52] Since CIP also functions by inhibiting DNA gyrase, the presence of a second compound with the same target could inhibit or potentiate its activity. In fact, in this study, potentiation of CIP by QUERC was found. To our knowledge, data on MOR as antimicrobial and RMA are scarce or even inexistent.

It has been suggested that flavonoids are likely to have limited toxicity, because they are widely distributed in edible plants and beverages and have previously been used in traditional medicine.[11] Indeed, in the United States, the daily dietary intake of mixed flavonoids is estimated to be in the range of 500–1,000 mg.[61] QUERC has also been used as a dietary supplement (250–500 mg thrice per day) for therapeutic purposes. However, in this study, QUERC presented low values of IC_{50} after 72 hours of incubation with L929 cells (41.8 mg/L). This value is in accordance with some other reports. Ngomuo and Jones[43] carried out the cytotoxic effects of QUERC and other compounds, and they

reported inhibition of cell growth by 50% after 48 hours of incubation for QUERC at 24 mg/L for Chinese hamster ovary cells, 36 mg/L for mouse fibroblast cells (3T3), and 21 mg/L for normal rat kidney cells. Pawlikowska-Pawlega and Gawron[50] reported that QUERC caused partial growth inhibition of mouse fibroblast cells (NCTC) when used at 10 mg/ L, and almost complete growth inhibition when applied at 50 mg/L. However, based on the information provided by Quercegen Pharmaceutics regarding toxicological safety studies (pers. comm.), human clinical studies corroborating epidemiological studies, human pharmacokinetic studies, as well as other information available to Food and Drug Ad- ministration (FDA), quercetin received generally regarded as safe (GRAS) status for its intended use as an ingredient in beverages, grain products and pastas, processed fruits and fruit juices, and soft candies at levels till 500 mg per serving. FDA has not, however, made its own determination regarding the GRAS status of the subject use of quercetin.

There are tens of reports in the literature regarding the potential benefits of QUERC for various applications, with various purported modes of action, while others refer to toxicity issues.[21,26,58] Although QUERC has tested positive for mutagenicity and genotoxicity *in vitro* in some reports, other *in vitro* studies suggest that QUERC is protective against genotoxicants and regarded as antimutagenic.[45] The implications of these conflicting findings in an assessment of human safety have not been established.[28] Therefore, the mechanisms underlying its biological effects remain obscure and understanding how QUERC causes either protection or cell death in the same model is a research priority.[49]

Considering the considerable number of studies reporting the potentially beneficial effects of QUERC on health, little is known about its bioavailability.[19] Currently, a number of studies have been carried out in both animals and humans. However, *in vivo* data on the disposition, absorption, bio- availability, and metabolism of QUERC are scarce and contradictory. The low bioavailability of flavonoids has been a concern. However, it can be improved by using food matrix components or particular delivery forms.[8] Harwood *et al*.[28] reviewed important studies about bioavailability, distribution, excretion of QUERC, and so on.

The clinical benefits and the effective synergism of these combinations have to be determined *in vivo* through care- fully designed pharmacokinetic studies in animals. De- termination of synergy or potentiation *in vitro* might not be reflected *in vivo* because of the potential failure to achieve synergistic levels of drugs in the desired tissue, the differences in plasma protein binding, and the drug metabolism.[33] The modification and optimization of the compounds based on the structure-activity relationship, in a way that the intrinsic pharmacological effect of each one is completely suppressed and only the potentiating effect with the antibiotics is present, is also a further step of research.

In conclusion, this study aimed at testing the combination of five selected flavonoids with common antibiotics searching for potentiating activities. MOR and QUERC were high lighted as antibiotic potentiators. However, the toxicity of these compounds may be a barrier. More information about pharmacokinetics of both compounds would be necessary. Their possible use in combination for surface coating can be a potential

immediate application. Despite measuring different end-points, the use of the four methods enabled a solid conclusion to be reached about the results and also permitted to overcome and circumvent the limitations of each method. Disc-diffusion method is raised as an excellent strategy, be- cause it is very easy to perform, has good agreement results with the other methods, and is low-priced.

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Disclosure Statement

No competing financial interests exist.

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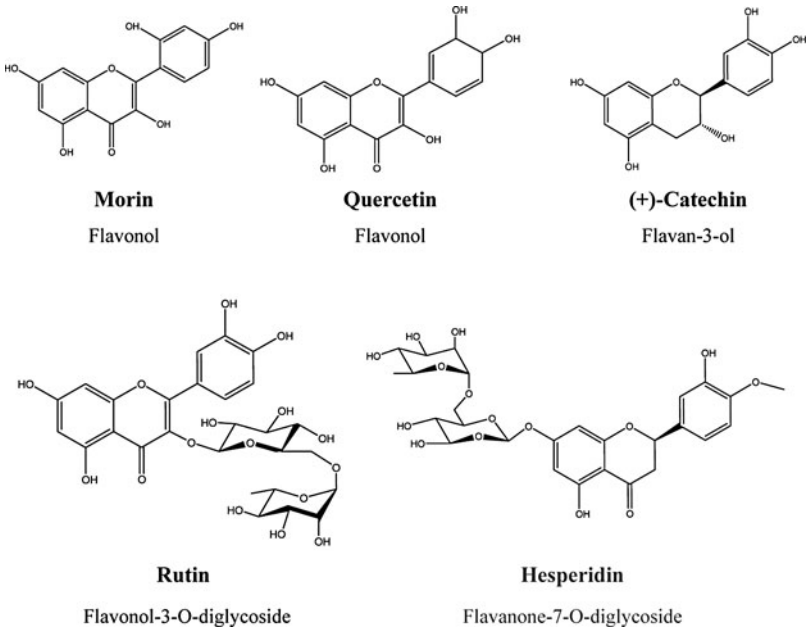

FIG. 1. Chemical structures of the flavonoids used in this study

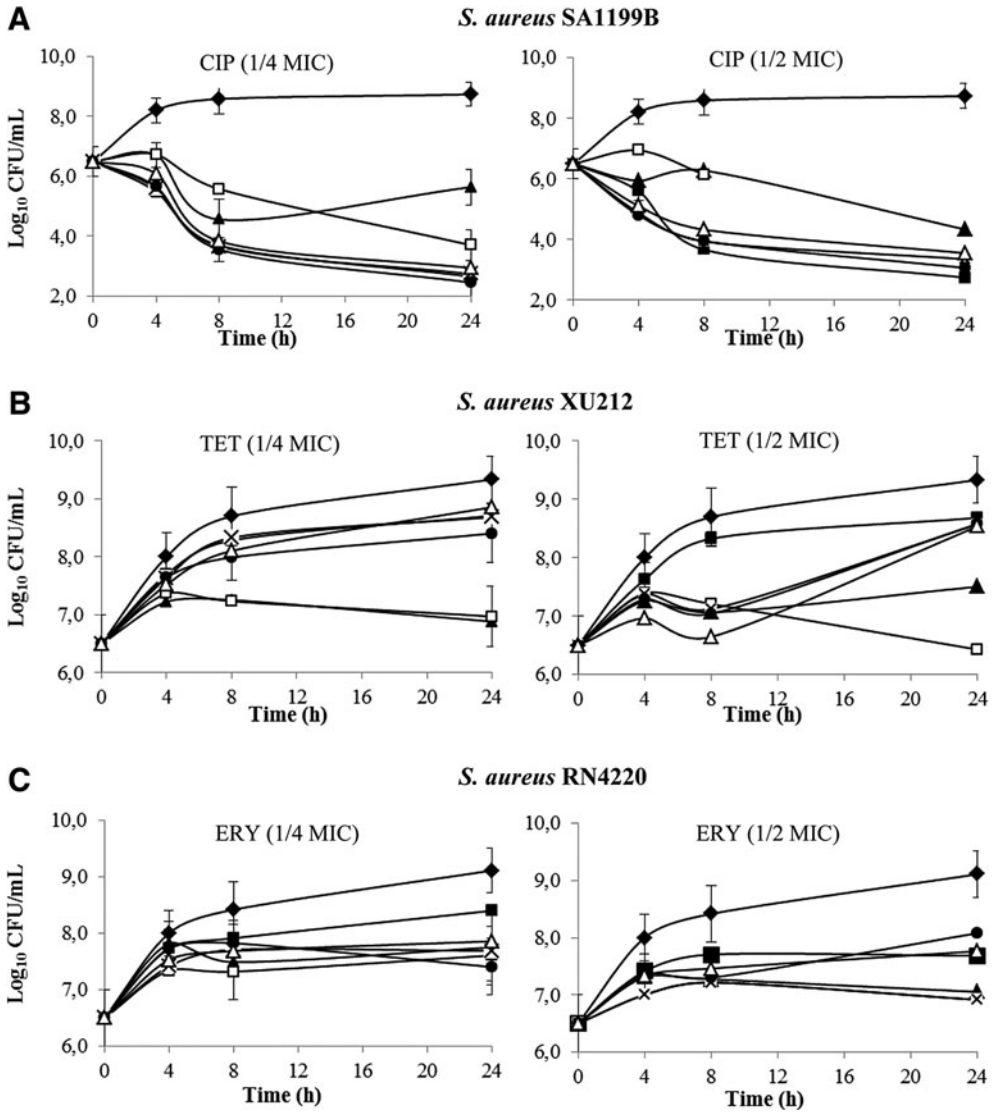

FIG. 2. Log10 CFU/ml obtained for 24 hours time- kill assay for: CIP (1/4 MIC and 1/2 MIC) + flavonoids (500 mg/L) against *Staphylococcus aureus* SA1199B (A); TET (1/4 MIC and 1/2 MIC) + flavonoids against *S. aureus* XU212 (B); and ERY (1/4 MIC and 1/2 MIC) + flavonoids against *S. aureus* RN4220 (C). Growth control (without antibiotic treatment) (A), antibiotic control (—), MOR (=), QUERC (⎓), RUT (·), HESP (C), CAT (=). The means – SD for at least three separate experiments are illustrated. Data are means and SD from at least three independent experiments. No significant CFU reduction was obtained with flavonoids alone ($p > 0.05$). CAT, (+)-catechin; CFU, colony-forming unit; CIP, ciprofloxacin; ERY, erythromycin; HESP, hesperidin; MICs, minimum inhibitory concentrations; MOR, morin; QUERC, quercetin; RUT, rutin; SD, standard deviation; TET, tetracycline.

Table 1. MICs Obtained by Checkerboard and Etest, and IZDs Obtained by Disc-Diffusion Method for the Antibiotics Alone Against the *Staphylococcus aureus* Strains Tested

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

Classification of the strains as susceptible, intermediate, or resistant according to CLSI guidelines.[41]

[a]Discrepancies between the categorizations were considered insignificant when an isolate found intermediate by one method was susceptible according to another, and no distinction was made in these cases. Time-Kill assays were performed only for *S. aureus* SA1199B, RN4220, and XU212 since this method was conducted on the basis of those that performed best in other tests; Etest was only directed for CIP, TET, and ERY which achieved good combinatorial results on the checkerboard and disc-diffusion method. Strains were classified as susceptible (S), intermediate (I), and resistant (R). Data are means and SD from at least three independent experiments.

AMP, ampicillin; CIP, ciprofloxacin; ERY, erythromycin; IZD, inhibition zone diameter; MICs, minimum inhibitory concentrations; MRSA, methicillin-resistant *S. aureus*; NI, no inhibition; NP, not performed; OXA, oxacillin; TET, tetracycline.

Table 2. MIC Fold Reductions (for Checkerboard and Etest) and IZD Increases (for Disc-Diffusion Method) and log10 CFU Reductions (for Time-Kill Method) Obtained with the Combination of Antibiotics with Flavonoids (at 500 mg/L)

S. aureus *isolates*	*Antib.*	*Flav.*	*Checkerboard*	*Etest*	*Disc diffusion*	*Time-kill*	*Final classification*
CECT 976	CIP	MOR	2.0 (A)	NC (I)	4.4 (A)	NP	A (m)
		QUERC	4.0 (P)	3.0 (P)	20.0 (P)	NP	P (T)
	TET	MOR	4.0 (P)	4.0 (P)	9.3 (P)	NP	P (T)
		QUERC	4.0 (P)	12.0 (P)	24.0 (P)	NP	P (T)
		RUT	NC (I)	3.0 (P)	7.0 (P)	NP	P (m)
	ERY	MOR	2.0 (I)	NC (I)	6.4 (P)	NP	I (m)
		QUERC	4.0 (P)	2.0 (I)	14.0 (P)	NP	P (m)
	AMP	MOR	1.5 (I)	NP	9.7 (P)	NP	—
SA1199B	CIP	MOR	NC (I)	3.0 (P)	13.7 (P)	−1.6 (I)	P (m)
		QUERC	4.0 (P)	3.0 (P)	18.9 (P)	−1.2 (I)	P (T)
XU212	TET	MOR	NC (I)	NC (I)	NC (I)	1.2 (I)	I (T)
		QUERC	NC (I)	NC (I)	NC (I)	2.3 (P)	I (T)
MRSA MJMC001	TET	MOR	4.0 (P)	3.0 (P)	6.0 (P)	NP	P (T)
		QUERC	8.0 (P)	8.0 (P)	6.1 (P)	NP	P (T)
MRSA MJMC002	TET	MOR	4.0 (P)	6.0 (P)	9.0 (P)	NP	P (T)
		QUERC	8.0 (P)	6.0 (P)	18.0 (P)	NP	P (T)
		RUT	NC (I)	NC (I)	8.3 (P)	NP	I (m)
MRSA MJMC004	TET	MOR	4.0 (P)	4.0 (P)	9.0 (P)	NP	P (T)
		QUERC	8.0 (P)	8.0 (P)	6.0 (P)	NP	P (T)
MSSA MJMC003	CIP	MOR	2.0 (I)	6.0 (P)	8.3 (P)	NP	P (m)
		QUERC	2.0 (I)	NC (I)	15.0 (P)	NP	I (m)
	TET	MOR	4.0 (P)	12.0 (P)	10.7 (P)	NP	P (T)
		QUERC	2.0 (A)	8.0 (P)	4.3 (A)	NP	A (m)
	ERY	MOR	2.0 (A)	2.0 (A)	15.0 (P)	NP	A (m)
		QUERC	NC (I)	NC (I)	5.0 (I)	NP	I (T)
MSSA MJMC009	CIP	MOR	NC (I)	6.0 (P)	9.7 (P)	NP	P (m)
		QUERC	NC (I)	NC (I)	6.3 (P)	NP	I (m)
	TET	MOR	8.0 (P)	8.0 (P)	6.0 (P)	NP	P (T)
		QUERC	4.0 (P)	3.0 (P)	11.7 (P)	NP	P (T)
	ERY	MOR	2.0 (I)	16.0 (P)	18.1 (P)	NP	P (m)
		QUERC	NC (I)	3.0 (P)	16.3 (P)	NP	P (m)
MSSA MJMC010	CIP	MOR	2.0 (I)	16.0 (P)	6.0 (P)	NP	P (m)
	TET	MOR	8.0 (P)	8.0 (P)	10.3 (P)	NP	P (T)
		QUERC	2.0 (I)	8.0 (P)	6.0 (P)	NP	P (m)
	ERY	MOR	2.0 (I)	3.0 (P)	10.1 (P)	NP	P (m)
		QUERC	2.0 (A)	2.0 (A)	13.0 (P)	NP	A (m)
	AMP	QUERC	NC (I)	NP	3.2 (I)	NP	I (—)

Only the combinations that were significantly different from the antibiotic control in, at least, one method were represented. An additive interaction was only accepted and distinguished from indifference when two or more methods indicated that same categorical result. Time-Kill assays were performed only for *S. aureus* SA1199B, RN4220, and XU212 since this method was conducted on the basis of those that performed best in other tests; Etest was only directed for CIP, TET, and ERY, which achieved good combinatorial results on the checkerboard and disc-diffusion method. Classifications of the combinations as potentiation (P), additive (A), or indifferent (I) are given in parentheses. The final classification considers the results of checkerboard, Etest, and disc diffusion method. Total agreement (T): All methods have the same interpretative category; minor disagreement (m): one method displays disagreement results. Data are means and SD from at least three independent experiments.

CAT, (+)-catechin; CFU, colony forming units; HESP, hesperidin; MOR, morin; NC, no change; QUERC, quercetin; RUT, rutin.