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Synergism of flavonoids with bacteriostatic action against *Staphylococcus aureus* ATCC 25 923 and *Escherichia coli* ATCC 25 922

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Key words: synergism, flavonoids, bacteriostatics, MIC

ABSTRACT: In our previous studies, bacteriostatic action of flavonoids against *Staphylococcus aureus* ATCC 25 923 and *Escherichia coli* ATCC 25 922 was demostrated. In the present work synergism of their combinations in order to improve the bacteriostatic action against the same microorganisms was determined. The experiences were made in nutritive broth, maintaining constant one drug concentration (20 μg/ml) and increasing the other one. A turbidimetric kinetic method was used and by means of a mechanism previously proposed, the minimal inhibitory concentrations (MIC´s) of each flavonoid combination were determined. The MIC´s for assayed combinations against *S. aureus* were: variable morin - constant rutin: 157.44 μg/ml and variable quercetin - constant morin: 29.9 μg/ml. The values obtained against *E. coli* were: variable morin - constant rutin: 78.5 μg/ml; variable quercetin - constant rutin: 47.4 μg/ml; variable quercetin - constant morin: 25 μg/ml; variable morin - constant quercetin: 27.4 μg/ml.

Introduction

Properties of flavonoid compounds in isolated form are widely known. The combinations of different flavonoids often improve considerably their biological activities, such as bactericidal and bacteriostatic action (Fukai *et al*., 1996; Fukai *et al*., 2002; Zeng *et al*., 1992), powerful antiviral activity (Dua *et al*., 2003; Wua *et al*., 2003), inhibition of colony formation in human leucemic cells (Fujita *et al*., 1997), clinical treatment of human ovary carcinoma (Kanasawa *et al*., 2003), amongst others. Polyphenolic compounds might inhibit carcinogenesis affecting molecular transformations in any of the initial, promotion or propagation stages (Yang *et al*., 2001). It has been suggested that tea-flavonoids have chemiprotector properties against cancer (Vaidyanathan and Wallet, 2001), action against cardiovascular disease (Hertog *et al*., 1997; Riemersma and Rice-Evans, 2001; Yochum and Kushi, 1999), vascular protection against arteriosclerosis (Zenhua and Yuan, 1991), espasmolitic action *in vitro* and antidiarrhoeic effect (Sadraei *et al*., 2003), hepatoprotector effect (Yoshikawa *et al*., 2003), antioxidative activity (Bergman *et al*., 2003; Hodgson *et al*., 2002; Mariken *et al*., 2003; Miura *et al*., 2003; Moridani *et al*., 2003), relaxant muscular activity (Hosseinzadeh *et al*., 2003) and other therapeutic applications. Synergism of bacteriostatic action using combination of flavonoid compounds against *Staphylococcus aureus* ATCC 25 923 and *Escherichia coli* ATCC 25 922 was determined, using a turbidimetric-kinetic method previously proposed (Pappano *et al*., 1990).

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Materials and Methods

Microorganisms assayed

S. aureus ATCC 25 923 and *E. coli* ATCC 25 922 (purchased in American Type Culture Collection) maintained by successive subcultures in trypticase soy agar (BBL) at 4ºC and by lyophilization.

Compounds: flavonoids of high purity were used: quercetin (3,5,7,3´,4´-pentahydroxyflavone), morin (3,5,7,2´,4´-pentahydroxyflavone) and rutin (glycosidic form of quercetin), were all obtained from Sigma (Fig. 1).

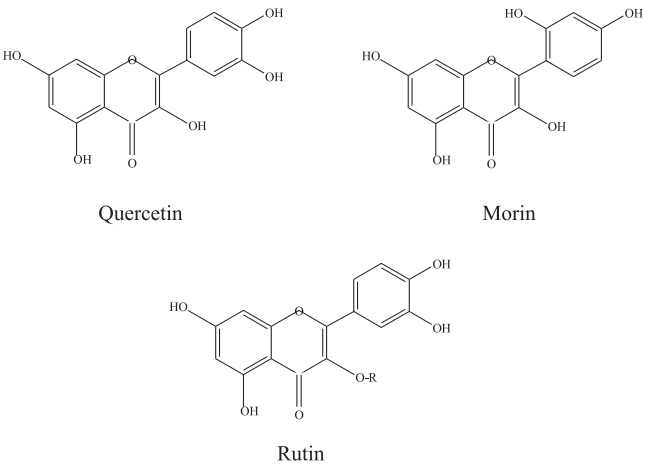

FIGURE 1. Structure of flavonoids.

Turbidimetric-kinetic method

A 24 h culture of *S. aureus* ATCC 25 923 or *E. coli* ATCC 25 922 in nutritive agar was transferred to 30 ml of Müller-Hinton broth (Oxoid) and incubated during 18 h at 35 ºC with permanent shake and it was used as inocule. Increasing quantities of one flavonoid (A) and a constant amount of another one (B) were added in erlenmeyers containing 100 ml culture media. There were other erlenmeyers which only contained increasing quantities of compound A. In addition, there was one erlenmeyer without flavonoid as control. Flavonoids A and B are structurally related. Each erlenmeyer was inoculated with 2 ml of inocule and then incubated in shaking Rosi 1000 culture chamber at 35ºC and 180 rpm. Aliquots were extracted at 20 min intervals during 5 h, and transmittance (T) was determined at 720 nm using a Shimadzu UV 160 A spectrophotometer. These T values were related to the number of cfu/ml (colony forming units/ml) N_t, by means of the following expressions (Pappano *et al*., 1994):

for *S. aureus* $\ln N_t = 27.4 - 10.3 \cdot T$ (1)

for *E. coli* $\ln N_t = 27.1 - 8.56 \cdot T$ (2)

Results

The combinations of assayed compounds were efficient against *S. aureus* ATCC 25 923 and *E. coli* ATCC 25 922. The number of cfu/ml as a function of times was obtained from expression 1 or 2, depending on the microorganism.

Considering the curve of microbial growth

$$\ln N_t = \mu \cdot t + \ln N_o \quad (3)$$

where t: time in min; N_o: cfu/ml at the t = 0; N_t: cfu/ml at the t; μ: specific growth rate in min^{-1}, the values of specific growth rates with increasing drug concentrations were obtained from the curve of ln N_t vs. t in the exponential phase.

According to previously exposed, results were satisfactory and agree with the mechanism of bacteriostatic inhibition before proposed (Pappano *et al*., 1990), where the specific growth rate (μ) varies according to the drug concentration in a linear relation:

$$\mu = \mu_T - k_i \cdot C \quad (4)$$

where: μ: specific growth rate (min^{-1}); μ_T: specific growth rate without drug (min^{-1}) (control); k_i: specific

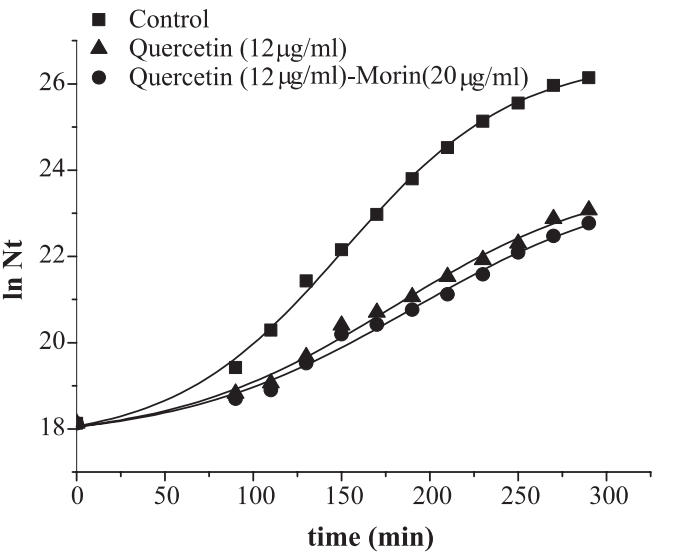

FIGURE 2. Growth of *Staphylococcus aureus* ATCC 25 923 in media containing quercetin and its combination with morin.

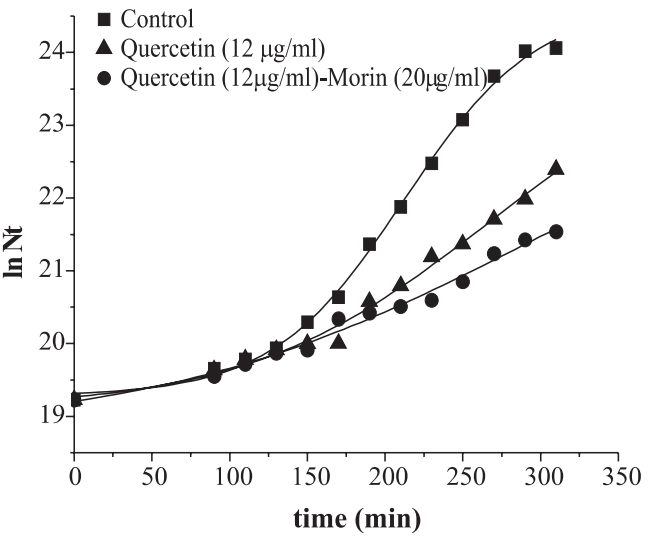

FIGURE 3. Growth of *Escherichia coli* ATCC 25 922 in media containing quercetin and its combination with morin.

inhibition rate (ml μg^{-1} min^{-1}) and C: drug concentration (μg/ml).

Figures 2 and 3 are the graphical representations of equation 3 for quercetin and quercetin plus morin against *S. aureus* and *E. coli*, respectively.

Figures 4 and 5 show the linear dependency of μ with C (Equation 4), for the assayed compounds and their combinations against both microorganisms. Minimal inhibitory concentrations values (MIC´s) were calculated by extrapolation at μ = 0 and are listed in Table 1.

Discussion

The results of the present work allow us to conclude that the noticeable diminution of MIC against *E. coli* in presence of a second flavonoid demonstrates synergism between studied compounds. This can be due to rutin which favors polar solutes entry, such as quercetin and morin, through structural membrane proteins named porines. Rutin binds to porines changing tridimentional conformation exposuring the hydrophilic character of the pore. This makes an easier passage of polar flavonoids by diffusion. Flavonoid may be as anions, in that case, their entry would be difficult through porines, formed by charged amino acids, opposing electrostatic repulsion to concentration gradient which impels its entry.

On the other hand, Gram positive bacteria exhibit teicoics, lipoteicoics and teicuronics acids into the cell wall and depending on its location and composition in the wall, these microorganisms present different resistance to bactericidal and bacteriostatic agents. In these cases, the transport defect through cytoplasmatic membrane is an intrinsic mechanism of resistance of some Gram positive and anaerobic bacteria. The penetration of these agents needs an active transport at cytoplasmatic membrane level and a force provided by electrical potential of electron chain. These mechanisms complicate entering agent to the action place, as probably happens in the case of flavonoids assayed against *S. aureus*, where synergism was practically null compared with observed against Gram negative bacterium *E. coli*.

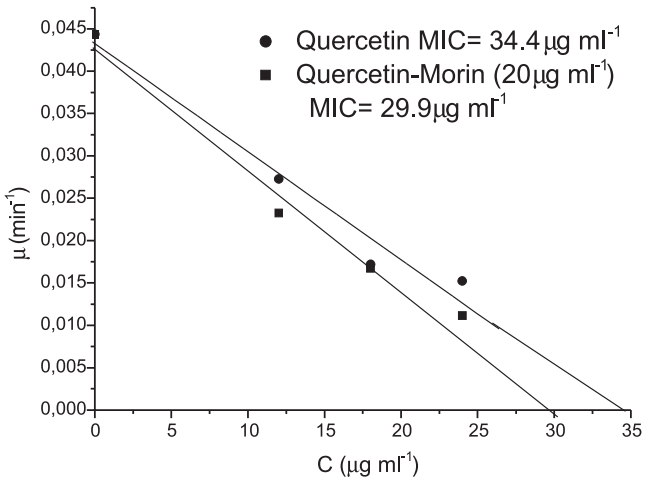

FIGURE 4. Graphical determination of MIC's (minimal inhibitory concentrations) by extrapolation at the abcis when μ = 0 for *Staphylococcus aureus* ATCC 25 923.

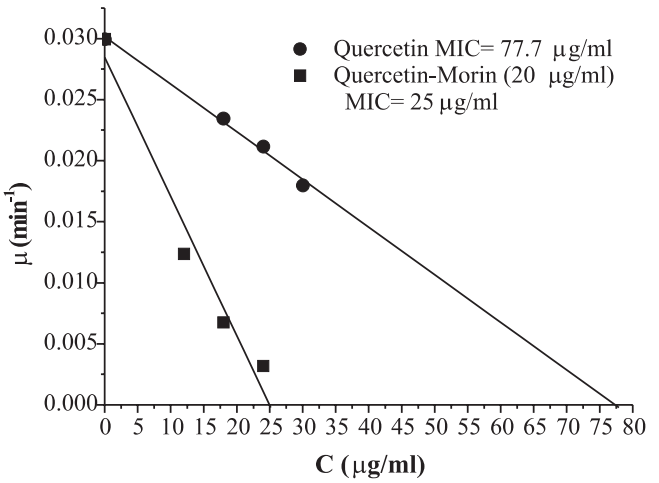

FIGURE 5. Graphical determination of MIC's (minimal inhibitory concentrations) by extrapolation at the abcis when μ = 0 for *Escherichia coli* ATCC 25 922.

TABLE 1.

Minimal inhibitory concentrations (MIC´s) for assayed compounds and their combinations against S.aureus ATCC 25 923 and E.coli ATCC 25 922.

COMPOUND	MIC (μg/ml)	
	S. aureus	*E. coli*
RUTIN	without activity	without activity
QUERCETIN	34.4	77.7
MORIN	157.4	109.5
$MORIN_V$ - $RUTIN_C$	157.4	78.5
$QUERCETIN_V$ – $RUTIN_C$	40.2	44.6
$QUERCETIN_V$ – $MORIN_C$	29.9	25.0
$MORIN_V$ – $QUERCETIN_C$	37.9	27.4

v: variable concentration; c: constant concentration.

Acknowledgements

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