Antibacterial Efficacy of Dihydroxylated Chalcones in Binary and Ternary Combinations with Nalidixic Acid and Nalidix Acid–Rutin Against Escherichia coli *ATCC 25 922*

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ORIGINAL ARTICLE

Antibacterial Efficacy of Dihydroxylated Chalcones in Binary and Ternary Combinations with Nalidixic Acid and Nalidix Acid–Rutin Against *Escherichia coli* ATCC 25 922

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Abstract In order to determine the existence of synergism, the bacteriostatic action of flavonoids against *Escherichia coli* ATCC 25 922 between dihydroxylated chalcones and a clinically interesting conventional antibiotic, binary combinations of 2′,3-dihydroxychalcone, 2′,4-dihydroxychalcone and 2′,4′-dihydroxychalcone with nalidixic acid and its ternary combinations with rutin (inactive flavonoid) were assayed against this Gram negative bacterium. Using a kinetic-turbidimetric method, growth kinetics were monitored in broths containing variable amounts of dihydroxychalcone alone, combinations of dihydroxychalcone variable concentration–nalidixic acid constant concentration and dihydroxychalcone variable concentration–nalidixic acid constant concentration–rutin constant concentration, respectively. The minimum inhibitory concentrations of dihydroxychalcones alone and its binary and ternary combinations were evaluated. All chalcones, and their binary and ternary combinations showed antibacterial activity, being rutin an excellent synergizing for the dihydroxychalcone–nalidixic acid binary combination against *E. coli* ATCC 25 922. Thus, this synergistic effect is an important way that could lead to the development of new combination antibiotics against infections caused by *E. coli.*

Keywords Dihydroxylated chalcones · Nalidixic acid · *Escherichia coli* · Synergism

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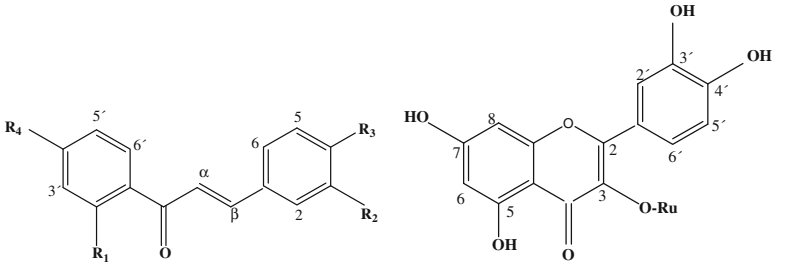
Introduction

In the past three decades bacteria have become resistant against antimicrobial agents as a result of chromosomal changes or the exchange of genetic material via plasmids and transposons. This involves the enzymatic inactivation of antibiotics and such genes are often transferred to other bacteria by a variety of mechanisms [1]. The members of the family *Enterobacteriaceae*, including *Escherichia coli*, which can cause diarrhea, urinary tract infection and sepsis, have become resistant to almost all previously used antibiotics [2–5].

The widespread use of these drugs in the community and in hospitals has increased this crisis. In order to limit bacterial resistance mechanisms must be adopted such as antibiotic control programs, improved hygiene and increased synthesis of agents with antimicrobial activity. The regular use of natural antibiotics is an alternative currently posed due to it does not generate resistance of microorganisms. The search for compounds derived from plants with antimicrobial action is intense [6–9], however, most research is conducted with plant extracts but not pure compounds [10–13]. The flavonoids family consists of structurally related compounds (chalcones, flavones, flavanones) and some of them are effective as antibacterial agents [14–17]. In our working group more than two decades of research on the antimicrobial action of pure flavonoids, natural or synthetic, against strains of *Staphylococcus aureus* and *E. coli* have been made [18–22]. Also binary combinations of an active and an inactive flavonoid against *E. coli* ATCC 25 922 were used to determine the existence of synergism [23, 24]. In the ongoing search for more effective antimicrobial agents combinations of active chalcones with a conventional antibiotic used against *S. aureus* strains were tested [25, 26].

The purpose of this study was to evaluate the antibacterial efficacy of binary combinations of dihydroxylated

Fig. 1 Structure of compounds



R_1=R_2=OH; R_3=R_4=H: 2´,3-dihydroxychalcone (I)

R_1=R_3=OH; R_2=R_4=H: 2´,4-dihydroxychalcone (II)

R_1=R_4=OH; R_2=R_3=H: 2´,4´-dihydroxychalcone (III)

Quercetin rutinoside (Rutin) (IV)

chalcones with a conventional antibiotic (nalidixic acid) and ternary (dihydroxylated chalcone nalidixic acid–rutin) against *E. coli* ATCC 25 922, using a kinetic-turbidimetric original method reported previously [19].

Materials and Methods

Compounds Used

Nalidixic acid (NA) was purchased from Sigma-Aldrich. 2′,3-dihydroxychalcone (**I**), 2′,4-dihydroxychalcone (**II**) and 2′,4′-dihydroxychalcone (**III**), were all synthesized in our laboratory by Claisen-Schmidt condensation [27] and identified by chromatographic and spectroscopic techniques (TLC, UV–Vis, IR, RMN). Rutin (Ru) (**IV**) (Sigma-Aldrich), was selected as synergist because it is inactive against the Gram negative bacteria as reported in previous studies [24]. Nalidixic acid and different chalcones and rutin solutions were prepared in absolute ethanol and diluted for antimicrobial assays. Figure 1 shows the structure of the compounds.

Bacterial Strain

Escherichia coli ATCC 25 922 strain, purchased from American Type Culture Collection and maintained by successive subcultures in trypticase soy agar BBL (Becton–Dickinson) at 4 °C and by lyophilization, was used.

Culture Media

Broth and agar nutritive and broth and agar Müller–Hinton (Oxoid) were used.

Kinetic-Turbidimetric Assays

In order to determine quantitatively the sensitivity of *E. coli* to dihydroxychalcones and its increase when used in binary combination with NA constant concentration and ternary combination with NA–Ru constant concentration, a previously developed kinetic-turbidimetric method was employed [19].

A 24 h culture of *E. coli* ATCC 25 922 in agar slant was transferred to 30 mL of Müller–Hinton broth and incubated at 35 °C for 18 h, with permanent stirring, in order to be used as inoculum. Kinetic experiments of microbial growth were performed in Erlenmeyer flasks containing 100 mL of Müller–Hinton broth with addition of increasing concentrations of antibiotic nalidix acid and 2 mL of previously prepared inoculum. Subsequently, Erlenmeyer flasks were incubated in a Rosi 1,000 culture chamber (35 °C, 180 rpm). Aliquots were extracted at 20 min intervals for 5 h and the transmittances were read at 720 nm. A flask without antibiotic was used as control. This first experiment enabled us to choose the optimal nalidixic acid to be used in the next trials (2 μg/mL).

For synergism determination, similar experiments in the presence of each dihydroxylated chalcone in increasing concentrations, alone or in combination with NA or in combination with NA–Ru, respectively, were performed.

In the kinetic-turbidimetric method previously developed [19], T (transmittance) values were related to the number of colony forming units/mL (CFU/mL)(N_t), by means of the following expression:

$$\ln N_t = 27.1 - 8.56 \times T \quad (1)$$

Results and Discussion

The number of CFU/mL at different times was obtained by the turbidimetric kinetic method (Eq. 1). The microbial growth can be expressed by the equation:

$$\ln N_t = \ln N_o + \mu \times t \quad (2)$$

where t is time in min, N_o is CFU/mL at t = 0, N_t is CFU/mL at t = t and μ is specific growth rate (in 1/min).

Growth rates values in media with increasing chalcone concentration and their combinations with constant concentration of nalidixic acid or combinations with constant

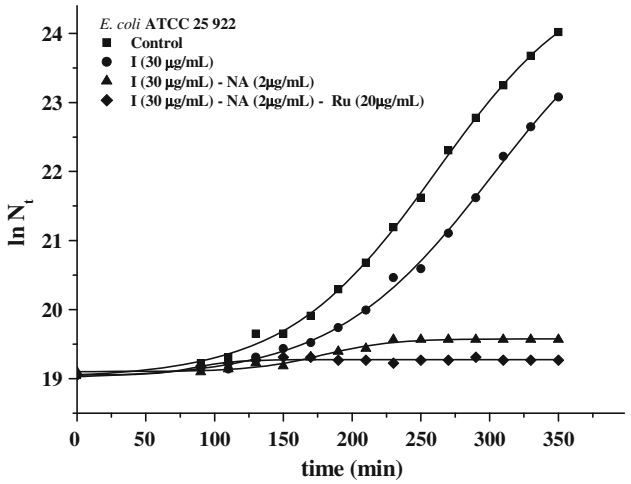


Fig. 2 Growth curves of *Escherichia coli*: *Filled square* control, *Filled circle* 2′,3-dihydroxychalcone (**I**), *Filled up pointing triangle* 2′,3-dihydroxychalcone (**I**)–nalidixic acid (2 μg/mL), *Filled diamond* 2′,3-dihydroxychalcone–nalidixic acid (2 μg/mL)–rutin (20 μg/mL)

concentration of nalidixic acid–rutin, respectively, were obtained from the exponential phase of ln N_t versus t plots.

Figure 2 shows, by way of example, the growth of *E. coli* alone (Eq. 2), in the presence of 2′,3-dihydroxychalcone isolated, in binary combination with NA (2 μg/mL), and ternary combination with NA–Ru (2–20 μg/mL).

Table 1 exhibits the specific growth rates of *E. coli* obtained in presence of chalcones and chalcones–nalidixic acid or chalcones–nalidixic acid–rutin combinations with the respective MIC values.

The specific growth rate values decreased when the experiments were made in presence of nalidixic acid constant or nalidixic acid–rutin constant concentrations. This fact is observed in Table 1 for all assayed combinations.

Table 1 Specific growth rates and minimal inhibitory concentration for the all systems assayed against *E. coli* ATCC 25 922

Chalcone Concentration (μg/mL)	μ (1/min)					MIC (μg/mL)
	0	10.0	20.0	30.0	40.0	
I	0.0310	0.0291	0.0273	0.0237	0.0218	121
I-NA	0.0310	0.0267	0.0219	0.0192	0.0146	76.5
I-NA–Ru	0.0240	0.0193	0.0141	0.0107	0.00481	52.5
II	0.0310	0.0266	0.0240	0.0172	0.0141	75.8
II-NA	0.0310	0.0230	0.0154	0.0103	0.00279	44.2
II-NA–Ru	0.0240	0.0114	0.00499	0.00	0.00	28.1
III	0.0310	0.0275	0.0229	0.0190	0.0150	74.8
III-NA	0.0132	0.0251	0.0213	0.0151	0.0101	57.5
III-NA–Ru	0.0186	0.0179	0.0138	0.00690	0.00130	41.5

I: 2′,3-dihydroxychalcone; **II**: 2′,4-dihydroxychalcone; **III**: 2′,4′-dihydroxychalcone

NA 2 μg/mL, *Ru* 20 μg/mL, *μ* specific growth rate, *MIC* minimal inhibitory concentration

Table 2 Minimal inhibitory concentration and percentual bacteriostatic efficiency of chalcones and their combinations with nalidixic acid and nalidixic acid–rutin against *E. coli* ATCC 25 922

Chalcone (Ch)	MIC Ch	MIC Ch–NA	MIC Ch–NA–Ru	PBE Ch	PBE Ch–NA	PBE Ch–NA–Ru
I	121.5	76.5	52.5	0.823	<1.31	<1.90
II	75.8	44.2	28.1	1.32	<2.26	<<3.56
III	74.8	57.5	41.5	1.34	<1.74	<<2.41

I: 2′,3-dihydroxychalcone; **II**: 2′,4-dihydroxychalcone, **III**: 2′,4′-dihydroxychalcone

MIC minimal inhibitory concentration, *PBE* percentual bacteriostatic efficiency

MIC values obtained indicate that all combinations tested showed synergism. While the combination 2′,3-diydroxychalcone–nalidixic acid–rutin showed greater synergistic effect, the combination 2′,4-dihydroxychalcone–nalidixic acid–rutin was more effective against *E. coli* ATCC 25 922 (MIC: 28.1 μg/mL).

The results were satisfactory and agreed to a bacteriostatic action mechanism previously proposed [19], where the specific growth rate (μ) varies with drug concentration in a linear relation leading to the following equation:

$$\mu = \mu_T - k_I \times C \quad (3)$$

where μ_T: specific growth rate without drug (1/min) (control), k_I: specific inhibition rate (mL/μg min) and C: drug concentration (μg/mL). The minimal inhibhitory concentration (MIC) was calculated by extrapolation at $\mu = 0$ from the graphical representation of Eq. 3.

In addition, percentual bacteriostatic efficiency values (PBE) [19] were obtained as:

$$PBE = 100/MIC \quad (4)$$

The PBE values show the synergism of all assayed binary and ternary combinations, and are informed in Table 2.

Conclusions

Chalcones activity againsts *E. coli* ATCC 25 922 was detected by kinetic-turbidimetric method. This inhibition was observed with chalcones, their binary combinations with commercial antiobiotic nalidixic acid, and in ternary combination with rutin, inactive flavonoid. The chalcones–nalidix acid–rutin combinations showed an interesting synergic action. We conclude that the inactive flavonoid (rutin) can improve the bacteriostatic efficacy against *E. coli* ATCC 25 922 of other flavonoids and its combination with a conventional antibiotic.

This can be explained by the composition of the outer membrane of Gram negative bacteria such as *E. coli*, which has proteins called porins that have to be associated together to form small holes in the membrane of approximately 1 nm diameter, that act as entry and exit channels of low molecular weight hydrophilic substances. There is a mechanism for opening and closing the pores, being the structure of the porin responsible for resistance to certain antibiotics. The flavonoid rutin favor opening the pores facilitating the entry of the active compounds, in this case the chalcones and nalidixic acid.

Due to increased drug resistance, the use of combinations of conventional antibiotics with flavonoids would be very important for the development of new strategies against the resistance constantly generated by microorganisms. Thus, the synergistic effect between dihydroxylated chalcones and nalidixic acid–rutin is an important way that could lead to the development of new combination antibiotics against infections caused by *E. coli.*

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