Antimicrobial activity of quinoxaline 1,4-dioxide with 2- and 3-substituted derivatives

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A B S T R A C T

Quinoxaline is a chemical compound that presents a structure that is similar to quinolone antibiotics. The present work reports the study of the antimicrobial activity of quinoxaline *N*,*N*-dioxide and some derivatives against bacterial and yeast strains. The compounds studied were quinoxaline-1,4-dioxide (QNX), 2-methylquinoxaline-1,4-dioxide (2MQNX), 2-methyl-3-benzoylquinoxaline-1,4-dioxide (2M3BenzoylQNX), 2-methyl-3-benzylquinoxaline-1,4-dioxide (2M3BQNX), 2-amino-3-cyanoquinoxaline-1,4-dioxide (2A3CQNX), 3-methyl-2-quinoxalinecarboxamide-1,4-dioxide (3M2QNXC), 2-hydroxyphenazine-*N*,*N*-dioxide (2HF) and 3-methyl-*N*-(2-methylphenyl)quinoxalinecarboxamide-1,4-dioxide (3MN(2MF)QNXC). The prokaryotic strains used were *Staphylococcus aureus* ATCC 6538, *S. aureus* ATCC 6538P, *S. aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *E. coli* S3R9, *E. coli* S3R22, *E. coli* TEM-1 CTX-M9, *E. coli* TEM-1, *E. coli* AmpC Mox-2, *E. coli* CTX-M2 e *E. coli* CTX-M9. The *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* PYCC 4072 were used as eukaryotic strains. For the compounds that presented activity using the disk diffusion method, the minimum inhibitory concentration (MIC) was determined. The alterations of cellular viability were evaluated in a time-course assay. Death curves for bacteria and growth curves for *S. cerevisiae* PYCC 4072 were also accessed. The results obtained suggest potential new drugs for antimicrobial activity chemotherapy since the MIC's determined present low values and cellular viability tests show the complete elimination of the bacterial strain. Also, the cellular viability tests for the eukaryotic model, *S. cerevisiae*, indicate low toxicity for the compounds tested.

Keywords: Antimicrobial activity Quinoxaline *N*,*N*-dioxide derivatives Minimum inhibitory concentration Cellular viability

1. Introduction

Antimicrobial agents are largely used in treatment and prevention of microorganism infections. Among others, the misuse and, especially, the abusive use of this kind of drugs, in human health, veterinary and animal production, led to the development of drug-resistant and multidrug-resistant (MDR) microorganisms (Roe 2008; Moreno et al. 2008). In addition, the permanent contact with some antimicrobial drugs, besides the resistance development, allows the increase of allergies and respiratory complications which are affecting the human population worldwide (Santos et al. 2007; Vollaard and Clasener 1994; Butaye et al. 2001; Witte et al. 2008). Resistant bacteria are increasing and the interval between the appearances of new and multi-drug resistant species is happening in short periods of time (Alanis 2005). These conditions are becoming emergent public health issues in the sense that they compromise pharmacological activity and the efficacy of these antimicrobial agents (Fernandes et al. 2008, 2009) and thus the heath of the population.

Because MDR bacteria are increasing worldwide human kind deals with the urgent need of development of new drugs with enhanced antimicrobial activity able to fight pathogens with no adverse effects (Fernandes et al. 2013). It is also expected to develop drugs that can reverse the resistance observed overturning the actual bacterial profile. Some approaches have been developed in order to evaluate the bioactivity of numerous compound families against several strains of microorganisms (Gradelski et al. 2001; Moellering 2011).

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Quinoxaline is an organic heterocyclic compound that has been used as base of synthesis of bioactive derivatives and several investigation groups have demonstrated their potential in medical and pharmacological applications (Zanetti et al. 2005; Carta et al. 2002; Sanna et al. 1999). These studies point to chemotherapeutical interests regarding the anti-tumor, anti-bacterial, anti-fungal, and anti-viral including anti-HIV (De Clercq 1997; Waring et al. 2002; Haykal et al. 2008; Harakeh et al. 2004) applications of these compounds. Relevant bioactivity has been reported in *Mycobacterium* spp. strains (Zanetti et al. 2005). No studies were found reporting biological activity for the quinoxaline derivatives in the present study with the microbial strains used.

The quinoxaline derivatives with *N*-oxide and *N*,*N*-dioxide have particular interest since they present relevant anti-oxidant activity. Many compounds with nitrogen—oxygen bonds play important biological roles by releasing NO groups or by cellular deoxygenating (Burguete et al. 2011; Hossain et al. 2012).

The present study pretends to be a contribution to the characterization of antibacterial and antifungal activity some *N*,*N*-quinoxaline derivatives (Table 1).

The activity of these compounds was tested against bacteria and yeast in order to understand the biological activity in both eukaryotic and prokaryotic microbial models. In the present work *Saccharomyces cerevisiae* and *Candida albicans* were used as representative models of eukaryotic microorganisms. Likewise, several strains of *Staphylococcus aureus* and *Escherichia coli* were used as prokaryotic representative models of Gram-positive and Gram-negative respectively.

2. Material and methods

2.1. Quinoxaline N,N-dioxide and quinoxaline derivatives

The compounds used in the present study were previously used by some of our collaborators and were gently provided by the Center of Investigation in Chemistry of the University of Porto. Synthesis, spectra and thermochemical properties were already studied for the quinoxaline derivatives used in the present study (Table 1) (Acree et al. 1997; Ribeiro da Silva et al. 2004; Gomes et al. 2005, 2007).

Stock solutions of the compounds were prepared in a 500 mL volume at a 500 μg/L final concentration. Since the compounds are thermally stable, the solutions were sterilized in an autoclave (AJC Uniclave 88) for 20 min at 120 °C. From these solutions, standards were prepared at the final concentrations 500, 100, 50, 20, and 5 μg/L.

2.2. Bacterial strains

The strains used in this study were stored deep frozen at −80 °C. The selected strains, in order to evaluate the susceptibility of a bacterial cell model to the proposed compounds, included *S. aureus* ATCC 6538, *S. aureus* ATCC 6538P, *S. aureus* ATCC 29213, *E. coli* ATCC 25922, *E. coli* S3R9 and *E. coli* S3R22 (a penicillin resistant strain and a multidrug resistant strain, respectively). It was included some *E. coli* strains harboring extended spectrum β-lactamases (ESBL) such as TEM-1, TEM-1 + CTX-M9, CTX-M2, CTX-M9 and the AmpC β-lactamase MOX-2.

2.3. Yeast strains

The strains used in this study were *S. cerevisiae* PYCC 4072 (UNL, Portugal) and *C. albicans* ATCC 10231 and were also stored deep-frozen at −80 °C.

2.4. Microorganisms culture and zone inhibition

In order to assess the potential microbial activity of the compounds presented, disk diffusion method was used with two purposes. The first one was to determine the sensibility of the strains to known antibiotics, cefoxitin (FOX) and ciprofloxacin (CIP). The second was to assess the inhibition zone for new compounds. Bacteria and yeast cells were sub-cultured in broth agar (tryptic soy broth – TSB) and incubated for 24 h at 37 °C. Freshly prepared bacterial cells were transferred into a saline solution (NaCl 0.9%, Carlo Erba Reactifs, France) and density was settled in the interval 0.09 and 0.10, corresponding to 0.5 McFarland ($1–2 \times 10^8$ CFU/mL). The density of the solutions was measured at 625 nm using a spectrophotometer (Thermo Scientific Genesys 20). Solutions were spread onto a Trypticase Soy Agar (TSA; Cultimed, Spain) nutrient plate in a laminar flow cabinet. Yeast strains were spread onto Yeast Extract Peptone Dextrose (YEPD; Oxoid, Basingstoke, UK) nutrient plate, in laminar flow cabinet. Blank sterile disks were immersed in the standard solutions, with the final concentration of 500, 100, 50, 20, and 5 μg/L for each compound. Plates were incubated for 24 h at 37 °C and zone inhibition diameters were measured in millimeters. Each one of these bacteria was tested with cefoxitin disks (FOX) 30 μg and ciprofloxacin (CIP) 5 μg. Cefoxitin is a β-lactam and ciprofloxacin is a quinolone that has a similar structure of quinoxaline. The compounds studied have no established reference values regarding the sensitive/resistant behavior for the quinoxaline derivatives, so microdilution method was employed in order to determine de minimum inhibitory concentration. Strains studied were classified (Table 2) as susceptible (S) or resistant (R) according to Clinical Laboratory Standard Institute (CLSI) guidelines, by disk diffusion, considering the values of CLSI to the β-lactam and quinolone used in the present study. All results were confirmed by replica.

2.5. Minimum inhibitory assays

The minimum inhibitory concentration (MIC) for each quinoxaline derivative was estimated using the microdilution method, according to the CLSI (Rex, 2009; Wilder 2005, 2006). The MIC's were determined for each strain/compound pairs that presented antimicrobial activity.

The microplates used consisted of 96 wells. The TSB was dispensed into several wells, 8 for each chemical compound. One of these 8 wells corresponded to the positive control (containing the culture medium and bacterial suspension) and another to the negative control (containing only the culture medium). The remaining wells were used to prepare volumetrically diluted in series from stock solution (1:1, 1:2, 1:4, 1:8, 1:16 and 1:32) for each compound and considering the concentrations that presented inhibition halo. Fresh prepared cultures of the microorganisms were suspended in a saline solution to a density of 0.100 at 625 nm. Each well was prepared to a final volume of 200 μL. The microplates were closed and incubated at 37 °C, for 16–20 h. The presence or absence of turbidity was verified and rechecked by inoculating a fraction of the wells in solid culture medium (Mueller-Hinton broth, Cultimed, Spain). The plates were incubated for 24 h at 37 °C. The results obtained in the wells and plates were compared. All results were confirmed by replica.

2.6. Cellular viability of bacteria

In order to evaluate the growing or death performance cellular viability was analyzed for all strain/quinoxaline derivative pairs that presented growth inhibition and for which MIC's were determined. Microbial suspensions were prepared at 0.5 McFarland density with TSB medium for bacterial strains. The solutions of the studied compounds were colored and the optical density (OD)

Table 1
Quinoxaline *N*,*N*-dioxide and quinoxaline derivatives.

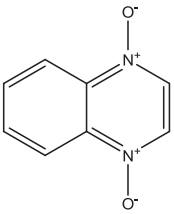
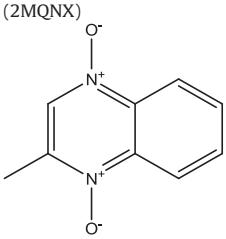
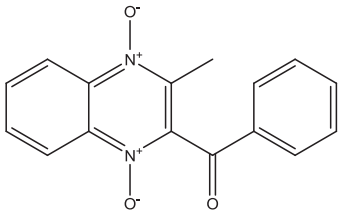
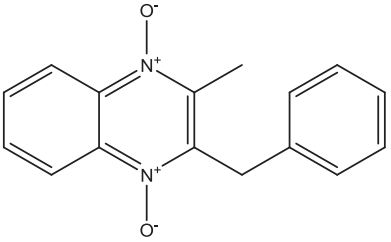
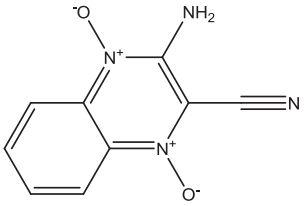
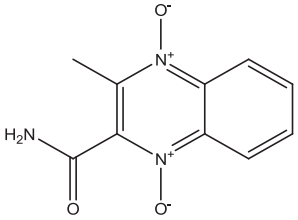
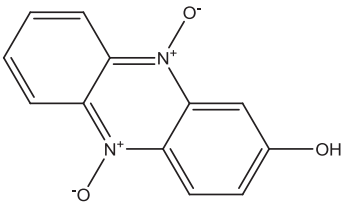
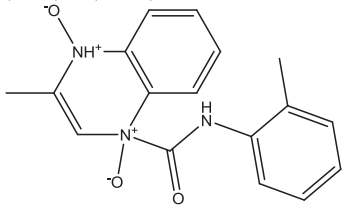
Quinoxaline-1,4-dioxide (QNX)	2-Methylquinoxaline-1,4-dioxide (2MQNX)	2-Methyl-3-benzoylquinoxaline-1,4-dioxide (2M3BenzoilQNX)
		
2-Methyl-3-benzylquinoxaline-1,4-dioxide (2M3BQNX)	2-Amino-3-cyanoquinoxaline-1,4-dioxide (2A3CQNX)	3-Methyl-2-quinoxalinecarboxamide-1,4-dioxide (3M2QNXC)
		
2-Hidroxiphenazine-*N*-dioxide (2HF)		3-Methyl-N-(2-methylphenyl) quinoxalinecarboxamide-1,4-dioxide (3MN(2MF)QNXC)
		

Table 2
Zone diameter interpretative Standards (*Enterobacteriaceae*) and equivalent minimal inhibitory concentration (MIC) according to CLSI document (Wilder 2005).

	Zone diameter interpretative standards (mm)			MIC (μg/mL)	
	R	I	S	R	S
Ciprofloxacin (CIP) (5 μg)	≤15	16–20	≥21	≥4	≤1
Cefoxitin (FOX) (30 μg)	≤14	15–17	≥18	≥32	≤8

R – resistant; I – intermediate; S – susceptible.

measurements lack accuracy. In alternative to the construction of cellular viability and death curves by platting was observed in a time-course assay. For 24 h, the presence of viable cells was tested and CFU/mL was determined. The time intervals were divided as presented in Table 3. A standard tube containing the studied strain and growing media was used as control and a test tube containing also the quinoxaline derivative in the MIC concentration determined previously was used as working solution. For CFU/mL counting, optimum dilution was determined by serial dilutions with 10^{-1} factor, in saline solution, adding 1/10 of strain media and 9/10 of saline solution (Table 3). All results were confirmed by replica.

2.7. Cellular viability for eukaryotic model

The aim of this method was to evaluate the cellular viability effects of compounds that presented antibacterial activity. The procedure was the same as described for bacteria, but the final concentration of compounds was 500 μg/L, corresponding to the highest MIC determined previously. Along 24 h, CFU/mL was determined if viable cells were present. The time intervals were divided as presented in Table 3. All results were confirmed by replica.

3. Results and discussion

3.1. Disk diffusion method

The quinoxaline derivative compounds studied have no reference values for the disk diffusion method, according to CLSI. Nevertheless, CLSI guidelines were used for known antibiotics. For the results obtained by this method, the absence of inhibition zone for the compounds tested was considered an indicative of no antimicrobial activity against that strain. Table 4 shows

Table 3
Dilution factor and time intervals for UFC/mL determinations.

	Optimum dilution factor	Time for counting (min)							
Bacteria	10^{-5}	0	30	60	90	120	180	1440	
Yeast	10^{-3}	0	30	60	90	120	180	210	1440

Table 4
Disk diffusion diameters for the quinoxaline derivatives.

Strain	Compound (500 μg/L)							
	QNX	2MQNX	2M3BenzoilQNX	2M3BQNX	2A3CQNX	3M2QNXC	2HF	3MN(2MF)QNXC
	Inhibition halo/mm							
S. aureus ATCC 6538					0			
S. aureus ATCC 6538P								
S. aureus ATCC 29213								
E. coli ATCC 25922	0	0	0	0	**11**	**24**	0	0
E. coli S3R9	0	0	0	0	**16**	**26**	0	0
E. coli S3R22	0	0	0	0	**24**	**26**	0	0
E. coli TEM-1 CTX M9	0	0	0	0	0	**14**	0	0
E. coli TEM-1	**24**	**16**	0	0	**16**	**26**	**14**	0
E. coli AmpC MOX-2	0	0	0	0	0	**15**	0	0
E. coli CTX M2					0			
E. coli CTX M9								
S. cerevisiae PYCC 4072					0			
C. albicans ATCC 10231								

Table 5
Classification of each strain as susceptible (S) or resistant (R) according to CLSI in presence of cefoxitin disk (FOX) and ciprofloxacin disk (CIP) by disk diffusion method (Wilder 2005).

Strain	Antibiotic	
	Cefoxitin (30 μg)	Ciprofloxacin (5 μg)
S. aureus ATCC 6538	S	S
S. aureus ATCC 6538P	R	S
S. aureus ATCC 29213	S	S
E. coli ATCC 25922	S	S
E. coli S3R9	S	S
E. coli S3R22	S	R
E. coli TEM CTX-M9	S	R
E. coli TEM-1	S	S
E. coli AmpC MOX-2	R	S

S. aureus: S – sensitive (CIP: zone inhibition ≥21 mm; FOX: zone inhibition ≥22 mm); R – resistant (CIP: zone inhibition ≤15 mm; FOX: zone inhibition <21 mm).
E. coli: S – sensitive (CIP: zone inhibition ≥21 mm; FOX: zone inhibition ≥18 mm); R – resistant (CIP: zone inhibition <15 mm; FOX: zone inhibition <14 mm).

Table 6
Minimum inhibitory concentration (μg/L) results for each group bacterial strain/compound by microdilution method, CLSI (Wilder 2006).

Strain	Compound	Minimum inhibitory concentration (μg/L)
E. coli ATCC 25922	2A3CQNX	500
	3M2QNXC	350
E. coli S3R9	2A3CQNX	320
	3M2QNXC	125
E. coli S3R22	2A3CQNX	320
	3M2QNXC	200
E. coli TEM CTX-M9	3M2QNXC	125
E. coli TEM-1	QNX	256
	2MQNX	400
	2A3CQNX	100
	3M2QNXC	80
	2HF	329
E. coli AmpC MOX-2	3M2QNXC	100

antimicrobial activity results for each bacterial or yeast strain and each compound tested. All the compounds studied showed no antimicrobial activity in the Gram-positive prokaryotic strains and eukaryotic strains since no inhibition zone was observed. On the other hand, the compounds only presented antimicrobial activity in Gram-negative prokaryotic strains, except for *E. coli* CTX-M2 and *E. coli* CTX-M9. The compound 2A3CQNX presented activity in four Gram-negative strains (*E. coli* ATCC 25922, *E. coli* S3R9, *E. coli* S3R22 and *E. coli* TEM-1); whereas 3M2QNXC was the compound that presented activity in a greater number of strains studied, namely, *E. coli* ATCC 25922, *E. coli* S3R9, *E. coli* S3R22, *E. coli* TEM-1 and *E. coli* AmpC MOX-2. The other compounds, QNX, 2MQNX and 2HF only presented activity in *E. coli* TEM-1. Several studies refer selective antibacterial activity. Meanwhile, others reveal activity in both Gram-positive and Gram-negative cells (Sykes et al. 1982; Blondeau et al. 2000; Neu and Labthavikul 1982). The results presented in Table 5 seem to indicate that *E. coli* S3R22 and *E. coli* TEM CTX-M9 are resistant to ciprofloxacin (5 μg) and *E. coli* AmpC MOX-2 is resistant to cefoxitin (30 μg). The other strains, *S. aureus* and *E. coli*, seem to be sensitive to both antibiotics.

3.2. Minimum inhibitory concentration

For the compounds that presented activity using the disk diffusion method, minimum inhibitory concentration (MIC) was determined and presented in Table 6. These results can be compared with MIC values previously determined for cefoxitin and ciprofloxacin and presented in Table 2 (Rex 2009). The results are presented for each well, bacteria, growth medium and different concentrations of each compound. The results were validated with both negative (compound and growth medium that presented no turbidity) and positive controls (bacteria and growth medium that presented turbidity). The MIC values presented are smaller than those indicated by the CLSI to CIP and FOX, suggesting a more effective activity of quinoxaline derivatives with respect to those antibiotics.

3.3. Cellular viability and CFU variation for prokaryotic cells

Cellular viability was analyzed for all bacterial strain/quinoxaline derivative pairs that presented growth inhibition by disk diffusion method and for which MIC's were previously determined. Fig. 1 shows an example of CFU/mL variation along time for Gram-negative prokaryotic strain (*E. coli* TEM-1). The results obtained show that every strain, in absence of any compound, grows up in the first minutes (viable cells), as expected (Chang-Li et al. 1988; Zwietering et al. 1990; Sezonov et al. 2007) and then decreases the number of CFU/mL until 24 h. For each bacterial strain/quinoxaline derivative pairs, the number of viable cells decreases with time, as expected, and at 24 h there are no viable cells. For *E. coli* TEM-1/2MQNX group the Fig. 1 shows that there are no viable cells at 180 min. CFU/mL variation for other bacterial strains/quinoxaline derivative pairs presented a similar behavior to *E. coli* in presence and absence of compounds.

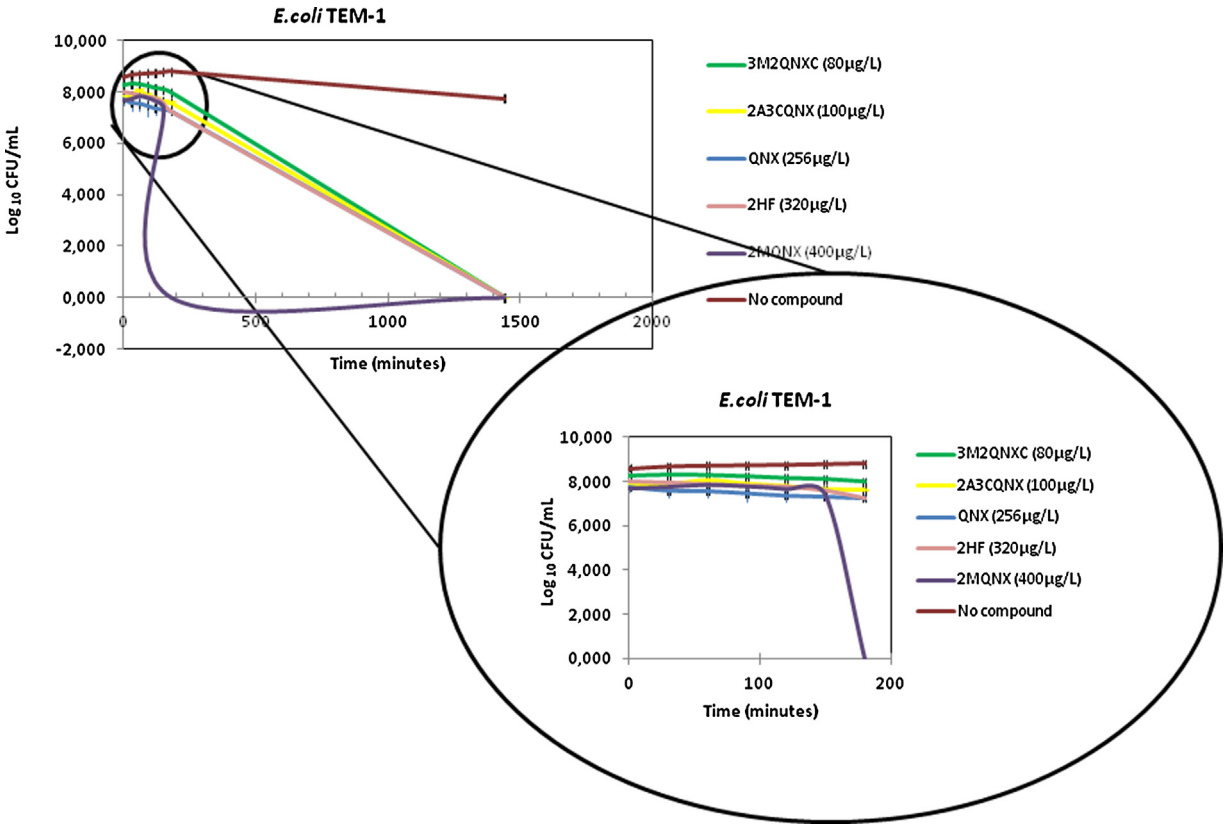


Fig. 1. Example of variation for Gram negative prokaryotic strains (*E. coli* TEM-1) in the presence of quinoxaline derivative at MIC determined.

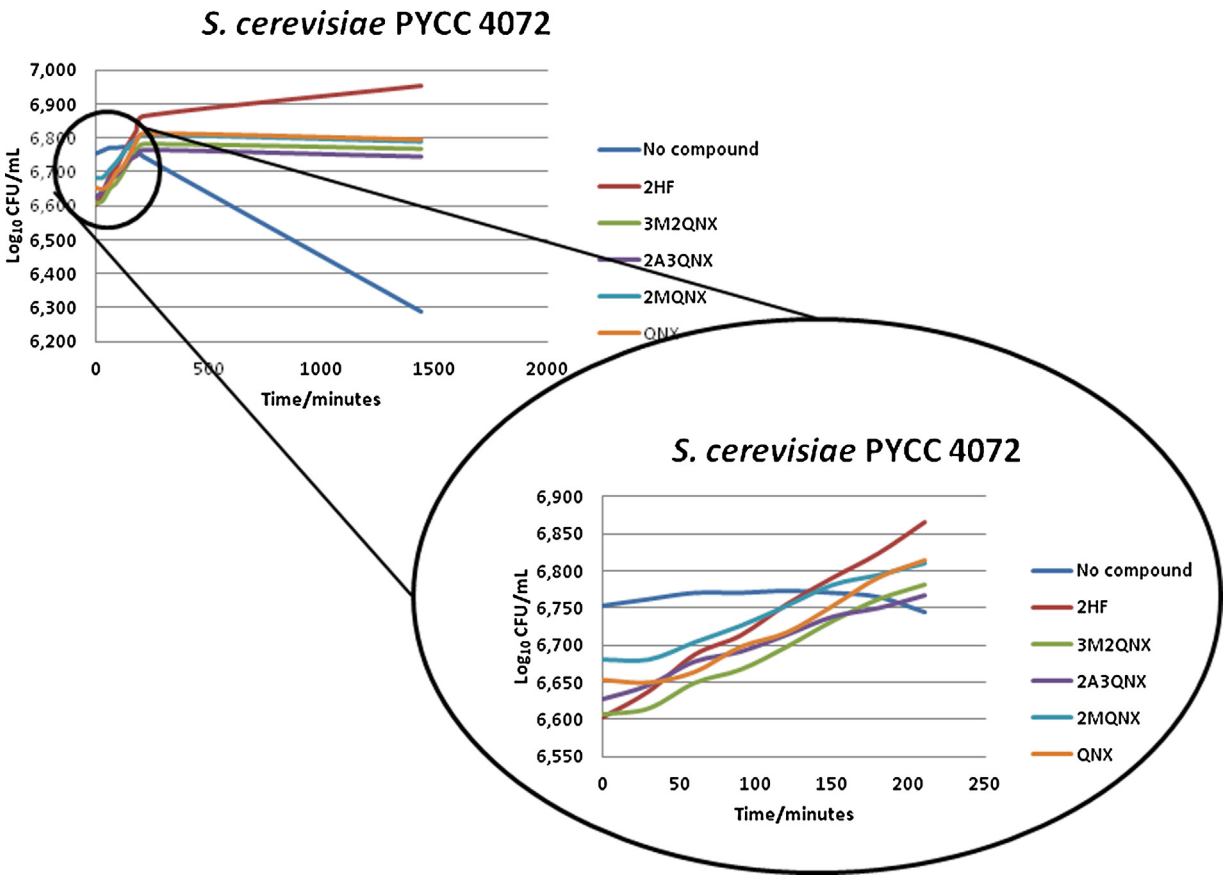


Fig. 2. Variation along time for *S. cerevisiae* in absence/presence of the compounds tested.

3.4. Cellular viability and CFU variation for eukaryotic cells

Both eukaryotic strains, *C. albicans* and *S. cerevisiae*, grow up in the first minutes. This is followed by a decrease of cellular viability (Barford 1990; Bochu et al. 2003). By exposing *S. cerevisiae* to the compounds that presented antibacterial activity (QNX, 2MQNX, 3M2QNXC, 2A3CQNX, 2HF), the number of viable cells calculated by CFU increases in the time-course. as observed in Fig. 2. These results seem to indicate that, in the presence of these quinoxaline derivatives, *S. cerevisiae* has a growth rate that increases. Surprisingly, all the quinoxaline derivatives studied showed no effect on growth or cell viability, when compared with the control. Actually, growth enhanced rather than declined. Naturally, we do not have at this point of the state of the art any explanation for these results, that we intend to clarify in further studies.

Nevertheless, it seems promising that compounds that may be potential antimicrobial agents used in humans or veterinary, may not be toxic in eukaryotic cells.

4. Conclusions

Quinoxaline derivative compounds studied in the present work presented selective antimicrobial activity between both eukaryotic and prokaryotic strains. Antimicrobial activity was observed only in the Gram-negative strains studied, except in *E. coli* CTX-M2 and *E. coli* CTX-M9. We believe that these two strains may encode some kind of mechanism that confers resistance to this group of compound. No antimicrobial activity was observed for Gram-positive prokaryotic strains, possibly due to the presence of peptidoglycan in Gram-positive cell wall, permeability properties of the membranes, or even due to metabolic dissimilarities between these two phylogenetic groups, that enables the entrance/activity of the compounds. This fact may also point to the need of these compounds to cross cellular walls to act. At the present time, there are no clues that might explain why some quinoxaline derivatives have a distinctive behavior between G+ and G− inhibition.

Moreover, we also have no data that supports why those quinoxaline derivatives which present antibacterial activity promote *S. cerevisiae* growth. The mode of action of these quinoxaline derivatives is not clear yet. We intend to develop further studies in order to contribute to this elucidation. The results showed in the present study, should guide these works. We now know that they seem not to be effective in yeast and in Gram positive strains. So, further studies on Gram negative response will be conducted. Since the mechanism of action of these compounds is not known it is difficult at this point of the state of the art to discuss structure activity relationship based on cell viability studies.

With exception to 2M3BenzoylQNX and 3M3BQNX, that presented no activity against the strains studied, all the compounds presented activity against both quinolone and/or β-lactams Gram-negative resistant strains. The MIC values determined were between 80 and 500 μg/L, and are smaller than the admitted values for approved drugs with antibiotic activity (CIP and FOX) and with similar chemical constitution (CIP) (Table 2).

The cellular viability for the eukaryotic strain and the death rate for the prokaryotic strains studied, at MIC concentrations, suggest these compounds as potential new drugs with selective antibacterial activity for Gram-negative bacteria.

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