



Chemical identification and antimicrobial potential of essential oil of *Piper rivinoides* kunth (BETIS-WHITE)

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ABSTRACT

The family Piperaceae is known for presenting in its species flavoring, healing and antimicrobial properties among others. The objective of the present study was: to study the chemical profile of the essential oil of *Piper rivinoides* (EOPR); to analyze its anti-bacterial and antifungal potential, as well as to evaluate the antifungal and antibiotic-modifying capacity. The chemical constituents were identified by gas chromatography with flame ionization detector (GC-FID), allowing the identification of 7 constituents of a total of 86.99%. E-Isoelemicin was identified as the main constituent of petroleum (40.81%). Clinically relevant MIC results were obtained against fungi in which the inhibitory concentration remained < 256 µg/mL, as for *Candida albicans* 4127 (217.6 µg/mL). The association of EOPR with an antifungal showed a high synergistic affinity against the strains of *C. tropicalis* 40042 and 4262. We concluded that no intrinsic EOPR activity was observed at any concentrations tested against bacteria. However, EOPR associated with Gentamicin acted synergistically against *S. aureus* 10 and *Escherichia coli* 06, but with Erythromycin there was a synergistic effect against *Escherichia coli* 06, and antagonism with norfloxacin.

1. Introduction

For a long time the use of medicinal plants in Brazil has been registered in rural and traditional communities in the form of baths and teas with leaves, stem barks, fruits, oils, roots, whole plants, sap and seeds (Macedo and Ferreira, 2004). The largest equatorial and tropical rainforest in the planet belongs to Brazil, with that the natural product wealth is enormous, bringing with it great plant genetic diversity that is readily available and is a current topic in Brazilian science among internationally recognised Brazilian chemists and pharmacologists (Bolzanida Silva et al., 2002).

Aromatic plants are well known, these being essential oil suppliers, which have been widely used as agents in cosmetics and medicines since antiquity (Angnes, 2005). Among the medicinal plants, aromatic plants constitute a prominent group due to the essential oils found in their different organs, such as leaves, stems, peels, resins, flowers and fruits, among others, presenting antimicrobial properties (Nunes et al., 2006). The *Piper rivinoides* species is rich in properties used for the treatment of infections, with its essential oil being composed of monoterpenes, sesquiterpenes, phenylpropanoids, aldehydes and ketones that present medicinal applications, such as insecticidal, bactericidal and fungicidal properties (Silva et al., 2014).

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Through chemical analysis and antimicrobial assays, new drugs which may be applied in future treatments can be discovered. The number of resistant microorganisms has increased dramatically and is now becoming one of the greatest threats to global health, affecting people of any age and anywhere in the world (WORLD HEALTH ORGANIZATION - WHO, 2015). Fungal infections in health care facilities have increased greatly in recent years with high morbidity and mortality rates. Many of these infections are acquired endogenously and others exogenously, the latter being from the hands of healthcare workers, therapeutic instruments and biomaterials, among other environmental sources, thus increasing morbidity and mortality rates in immunosuppressed patients (Sorendino et al., 2017; Nakamura et al., 2013).

According to (Basso et al., 2016), bacterial as well as fungal infections are among the leading causes of death. WHO data shows that 25% of worldwide deaths are caused by bacterial infections, due to the difficulty in identifying the microorganism for treatment. Thus, in addition to bacterial resistance being a natural phenomenon, the genetic capacity bacteria possess to adapt is enormous (Davies and Davies, 2010).

Due to the increasing number of antibiotic-resistant infections, new agents are needed to supplement current antibiotics. Extracts, oils and other plant origin products have been evaluated for their direct action or as resistance modifying agents. Chemical compounds, from synthetic or natural sources, have an indirect effect against many bacterial species; for example, phenothiazines revert the natural resistance of bacteria such as *Escherichia coli* causing plasmid elimination, increasing the activity of a specific antibiotic and inhibiting plasma membrane transport functions associated with antibiotics (Coutinho et al., 2010; Santos et al., 2007).

This study will address the chemical composition and antimicrobial potential of the *Piper rivinoides* essential oil against fungal and bacterial strains, both standard and clinical isolates, in addition to evaluating the oil's antibiotic and antifungal modifying action. Its potential to inhibit fungal virulence will also be evaluated by dimorphism.

2. Materials and methods

2.1. Plant material

The leaves were collected at the Biological Reserve of Bom Jesus, in the municipality of Guaraqueçaba - PR (years 2015/2016), located at 25° 13.644 'and W 48° 34.985', of altitude 7 Greenwich, Paraná, Southern Brazil. The collection of plant material in the reserve was made under license from the Environmental Institute of the State of Paraná by number 284/11. The vouchers of the copies were sent to the "Herbarium of the Municipal Botanical Museum (MBM)" with the number 396414. (UPCB). "

The sample of essential oil is part of the REBIFLORA project of the Network of Bioprospecting and Innovation in the Atlantic Forest, in partnership with the Department of Biological Chemistry of the Federal University of Parana. Brazil.

2.2. Essential oil insulation and method of analysis

The essential oil of *Piper cernuum* (EOPC) was extracted according to the method described in (Salazar et al., 2018).

2.3. Antifungal assays

The antifungal assays were performed as described in (Bezerra et al., 2018; Morais-Braga et al., 2016; Ernst et al., 1999).

2.4. Direct antibacterial test (MIC) and modulation of antibiotic activity

The determination of the antibacterial activity of MIC and

evaluation of the modulatory effect of antibiotics follows the methodology described in Salazar et al. (2018).

2.5. Statistical analysis

For the statistical analysis, the software Graphpad Prism, v. 6.0 was used. The data obtained were checked for their normal distribution and then analyzed by a two-way ANOVA ($P < 0.05$; $*P < 0.1$; $****P < 0.0001$), comparing the values for each concentration of the extract, point by point, with Bonferroni's post hoc test. The IC₅₀ values were obtained by non-linear regression with interpolation of standard curve unknowns obtained from fungal growth as a function of extract concentration and expressed in µg/mL. To analyze their values, multiple t-tests were used, one per line, whose statistical significance was determined by the Holm-Sidak method, with $\alpha \leq 0.05$. The results of the antibacterial assays were performed in triplicate and expressed as geometric mean. Statistical analysis was applied to two-way ANOVA followed by Bonferroni posttests.

3. Results

3.1. Chemical composition

The essential oil extracted from dry *Piper rivinoides* leaves by hydrodistillation using Clevenger was analyzed and identified the presence of 7 constituents, among which E-Isoelemicin was the major compound representing 40.81%, followed by δ -3-Carene with 16.88% (Table 1).

3.2. Fungal minimal inhibitory concentration (MIC) determination and antifungal modifying activity evaluation

With respect to the EOPR's intrinsic activity at concentrations less than 1000 mg/mL, the oil was effective against practically all strains except *Candida albicans* 40006 with a concentration of 1206.03 g/ml (Fig. 1). However, when comparing the antifungal and EOPR action against *Candida tropicalis* 40042, the OEPR was superior to the antifungal which obtained a MIC of 1421.6 µg/mL while the EOPR obtained a MIC of 782.9 µg/mL, until reaching an IC₅₀ (Fig. 3). As for the *Candida albicans* 4127 strain, the EOPR was so efficient as to eliminate 100% of bacterial growth at a concentration of 512 µg/ml (Fig. 2).

The modifying activity was evaluated by the combination of the triazole antifungal fluconazole with the EOPR, where the results from the association demonstrated excellent results. This synergistic biological activity was strongly identified against the *C. tropicalis* 40042 strain, where fluconazole obtained a MIC of 1421.6 µg/mL, which was reduced 5-fold to 56.3 µg/mL when associated with the EOPR. When treating the *C. tropicalis* 4262 strain, fluconazole obtained a MIC of

Table 1

Name of compounds present in the EOPR; refractive index- RI of each constituent; followed by the percentage of each identified compound and the total percentage of the sample.

Chemistry Composition				
Table 01. Constituents found in the <i>Piper rivinoides</i> species:				
EOPR	<i>Piper rivinoides</i>	Composition	Calculated RI	%
		Carene < δ -3 >	1013	16.88
		Caryophyllene < (E)- >	1425	4.5
		Germacrene D	1487	1.56
		Macrocarpene < β - >	1504	2.66
		Murolene < α - >	1507	12.45
		Carpacin < (Z)- >	1532	8.13
		Isoelemicin < (E)- >	1565	40.81
		TOTAL		86.99

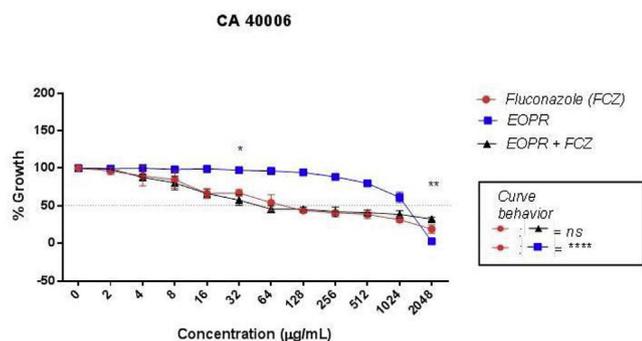


Fig. 1. Antifungal effect ($\mu\text{g/mL}$) of Piper rivinoides essential oil isolated and associated with fluconazole against *Candida albicans* strain 40006. NS- $p > 0.005$; * - $p < 0.005$; ** - $p < 0.01$; *** - $p < 0.001$; **** - $p < 0.0001$. Fluconazole IC50 85.14 $\mu\text{g/mL}$; EOPR 1206.03 $\mu\text{g/mL}$; Association 53.18 $\mu\text{g/mL}$.

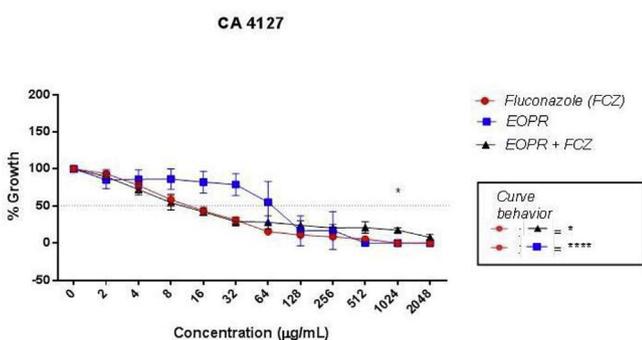


Fig. 2. Antifungal ($\mu\text{g/mL}$) effect of Piper rivinoides Essential Oil alone and in combination with Fluconazole against *Candida albicans* strain 4127. NS- $p > 0.005$; * - $p < 0.005$; ** - $p < 0.01$; *** - $p < 0.001$; **** - $p < 0.0001$. Fluconazole IC50 10.554 $\mu\text{g/mL}$; EOPR 68.5 $\mu\text{g/mL}$; Association 9.8 $\mu\text{g/mL}$.

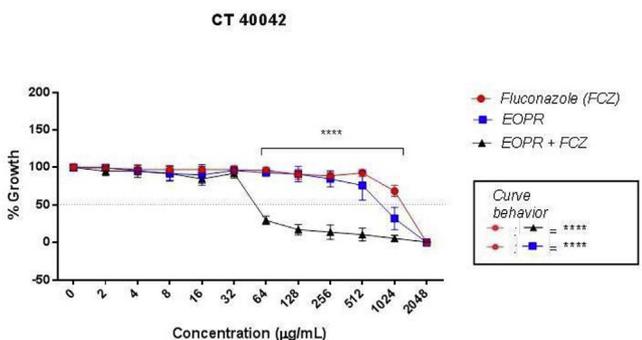


Fig. 3. Antifungal ($\mu\text{g/mL}$) effect of Piper rivinoides Essential Oil isolated and in combination with Fluconazole against *Candida tropicalis* 40042 strain. NS - $p > 0.005$; * - $p < 0.005$; ** - $p < 0.01$; *** - $p < 0.001$; **** - $p < 0.0001$. Fluconazole IC50 1421.6 $\mu\text{g/mL}$; EOPR 782.9 $\mu\text{g/mL}$; 56.3 $\mu\text{g/mL}$.

120.6 $\mu\text{g/mL}$, which when in association with the EOPR decreased to 63.2 $\mu\text{g/mL}$. Potentiation occurred against the other strains, however with low significance (see Fig. 4).

3.3. Fungal dimorphism effect

The positive EOPR result for a direct action against one of the fungal virulence factors can be observed in each graph, in comparison to the antifungal. From the results, the EOPC fungistatic effect against *Candida albicans* 40006 and *Candida albicans* 4127 at concentrations fungicide

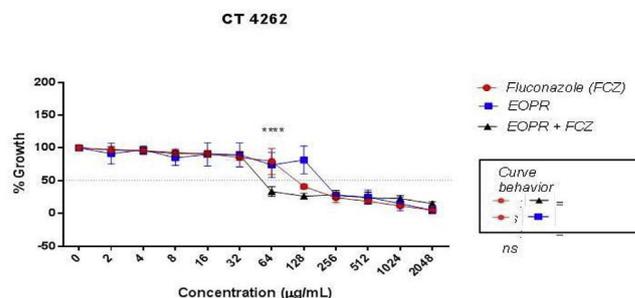


Fig. 4. Antifungal ($\mu\text{g/mL}$) effect of Piper rivinoides Essential Oil isolated and in combination with Fluconazole against *Candida tropicalis* 4262 strain. NS- $p < 0.005$ thus: ns - $p > 0.005$; * - $p < 0.005$; ** - $p < 0.01$; *** - $p < 0.001$; **** - $p < 0.0001$. Fluconazole IC50 120.6 $\mu\text{g/mL}$; EOPR 217.6 $\mu\text{g/mL}$; 63.2 $\mu\text{g/mL}$.

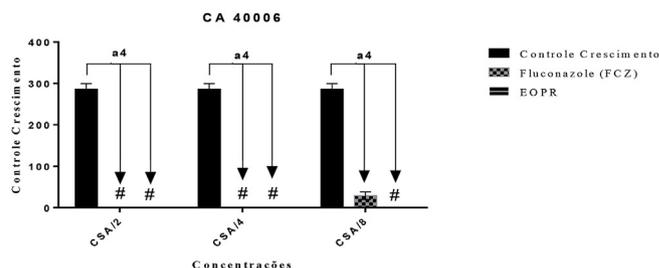


Fig. 5. Measurement of hyphae extension under the action of EOPR against strain *C. albicans* 40006. a4: $p < 0.0001$ vs growth control; no growth.

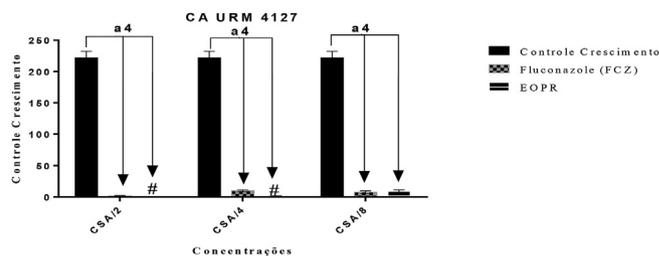


Fig. 6. Measurement of hyphae extension under the action of EOPR against strain *C. albicans* 4127. a4: $p < 0.0001$ vs growth control; no growth.

minimum CFM/2 (8192 $\mu\text{g/mL}$), CFM/4 (4092 $\mu\text{g/mL}$) and CFM/8 (2048 $\mu\text{g/mL}$) (Figs. 5; 6), not witnessing the presence of hyphae or pseudohyphae, results better than the antifungal, can be observed. The EOPC showed complete virulence factor inhibition against *Candida tropicalis* URM 40042 (Fig. 7) until reaching the MFC/8, where small hyphae were detected by field analysis, although this was still lower than the amount observed in the fluconazole assay. The oil was also more effective than the drug against *Candida tropicalis* URM 4262 (Fig. 8).

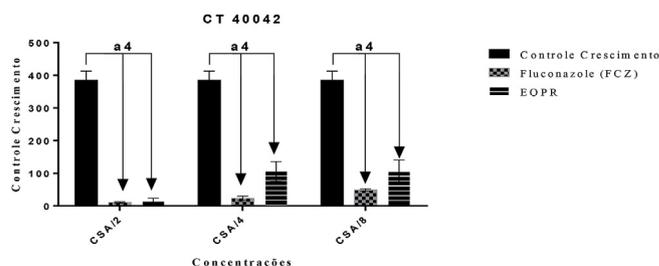


Fig. 7. Measurement of hyphae extension under the action of EOPR against strain *C. tropicalis* 40042. a4: $p < 0.0001$ vs growth control; no growth.

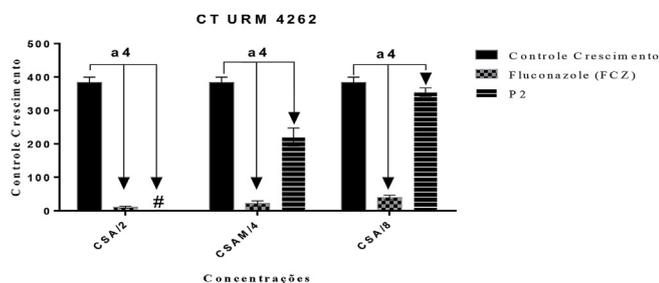


Fig. 8. Measurement of hyphae extension under the action of EOPR against strain *C. tropicalis* 4262. a4: $p < 0.0001$ vs growth control; no growth.

Table 2

Results of minimum inhibitory concentration CIM-OEPR

Substances	Experimental bacteria			
	S.A. ATCC	E.C. ATCC	S.A. 10	E.C. 06
EOPR	≥ 1024	≥ 1024	≥ 1024	≥ 1024

The table shows the values of Minimum Inhibitory Concentration for PRROs. No intrinsic effect was observed.

4. Bacterial assays

4.1. Minimum Inhibitory Concentration (MIC) determination

4.1.1. Antibacterial

The essential oil showed no intrinsic antibacterial activity against any of the tested strains (Table 2).

4.2. Antibiotic modifying activity evaluation

The EOPR when associated with gentamicin acted synergistically obtaining a MIC below $25 \mu\text{g/ml}$ against the *S. aureus* 10 strain, as shown in (Fig. 9). However, the EOPR did not present a synergistic nor an antagonistic effect when associated with Norfloxacin.

Results from the antibiotic modifying activity assays when in association with the EOPR were excellent, where Erythromycin and the EOPR presented synergism reducing the MIC against the *E. coli* 06 isolate (Fig. 10) 5-fold, and 2-fold when associated with Gentamicin (Fig. 10). As for the antibiotic Norfloxacin, an antagonism was observed with respect to *E. coli* 06 bacterial inhibition.

5. Discussion

5.1. Antifungal effects of EOPR

According to Guimarães and Monteiro (2006), *Piper rivinoides* is known to be widely used for medicinal purposes. Aromatic compounds

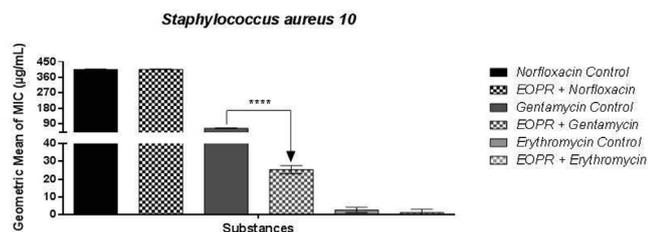


Fig. 9. Effect of antibiotic modifying activity against *S. aureus*: Data were analyzed using two-way ANOVA test using the geometric mean of triplicates as given center and the standard deviation. A post hoc Bonferroni test was then performed (where $p < 0.05$ and $p < 0.0001$ were considered significant and $p > 0.05$ not significant).

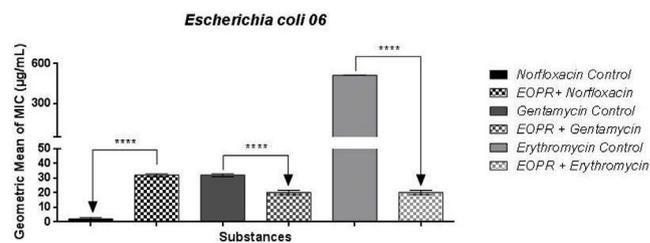


Fig. 10. Effect of antibiotic modifying activity against *E. coli*: Data were analyzed using two-way ANOVA test using the geometric mean of triplicates as given center and the standard deviation. A post hoc Bonferroni test was then performed (where $p < 0.05$ and $p < 0.0001$ were considered significant and $p > 0.05$ not significant).

present in essential oils originating from the secondary metabolism of plants, present complex chemical characteristics. According to (Silva et al., 2014) monoterpenes, sesquiterpenes, phenylpropanoids present medicinal applications such as insecticidal, bactericidal and fungicidal properties.

Thus, the intimate relationship between the chemical structure of a particular compound and the biological properties it presents is widely discussed by researchers. It is understood the EOPR action against fungal strains is due to the presence of compounds of the aliphatic side chain amino acid family, given by the major compound E-isoelemicin, a class known for its antioxidant value, in addition to the presence of the δ -3-Carene, a compound belonging to the monoterpene class representing 16.88% of the oil, which according to (Silva et al., 2014) shows bactericidal and fungicidal activities.

Broad spectrum antifungal agents have as their primary mechanism of action the inhibition of ergosterol synthesis, as well as being drugs capable of interfering with pyrimidine metabolism, which prevent the synthesis of DNA/RNA and consequently proteins in the fungal cell (Gannoum et al., 1999). The antifungal tested in association with the EOPR is a triazol-fluconazole capable of interfering with the activity of cytochrome P450, reducing ergosterol synthesis and causing membrane permeability alterations followed by loss of essential elements such as amino acids and potassium (WHO, 2015). When associating the antifungal with the EOPR, an interference with the action of ergosterol possibly occurred facilitating the antifungal's entrance to the cell's interior increasing its concentration and its action against all tested strains.

5.2. Antibacterial effects of EOPR

The compounds present in the EOPR function on changes in the permeability of the cell membrane, such as the ability to break or penetrate its lipid structure, mechanism of action against bacteria (Bertini et al., 2005). In a study, Burt (2004) evaluated the action of essential oils against microorganisms and identified cell membrane permeability alterations. Oils possess characteristics capable of preventing bacterial virulence, due to aromatic classes and their monoterpenic, sesquiterpene, phenylpropanoid and alcohol constituents. The combination of a natural product with a conventional antibiotic may increase its activity spectrum against several microorganisms, especially bacteria. The potentiation of an antibiotic's activity or inversion of antibacterial resistance classifies these compounds as antibiotic activity modifiers (Coutinho et al., 2009). Antibiotics may undergo changes in their chemical structure when associated with other substances, modifications which may potentiate their biological activity leading to effective results at low concentrations which are considered non-toxic, providing new forms of action against resistance mechanisms and bacterial virulence (Santos et al., 2007).

The antibiotics used present as mechanisms of action, inhibition of the gyrase, inactivation of the protein synthesis, binding to the 50S ribosomal subunits, as well as characteristics that promote membrane

rupture. Synergistic effects were observed when the antibiotics gentamicin and erythromycin were associated with EOFR, both with common characteristics, such as inhibition of protein synthesis, while erythromycin acts as a reversible ligand of the 50S ribosomal subunit.

Gentamicin acts by binding to the 30S ribosomal subunit, which causes errors in the order and type of amino acids inserted in the polypeptide chain, causing interruption of peptide chain elongation and bacterial death (Perianu, Rau and Vijan, 2019; Pratt et al., 2018).

As for the quinolone used, Norfloxacin, the EOFR did not alter its function against the first strain and was antagonistic against the second. Given Norfloxacin is the only antibiotic acting directly through gyrase inhibition, and the other antibiotics used herein have protein synthesis inhibition in common, changes to the structure of Norfloxacin inactivating its active site for the gyrase enzyme or possibly activating genes responsible for expressing efflux pumps may have occurred, inactivating and antagonizing its activity.

6. Conclusion

The positive results of the intrinsic fungal activity are due to monoterpene, sesquiterpene and phenylpropanoid compounds found in EOFR. The compounds caused fragility caused by EOFR on the membrane. The entry of fluconazole was facilitated in the cell, revealing its spectrum of action against the strains tested. The fungistatic action by EOFR against all fungal strains, the positive effect is due to the presence of lipophilic substances, forming a hydrophobic barrier that prevents the fungal dimorphism caused by the nanification in search of nutrients.

The oil had no intrinsic action against bacteria at the concentrations tested. This is not to say that EOFR does not have antibacterial action, since other strains and existing methods have not been tested. When EOFR was associated with antibiotics, synergistic effects were observed with gentamicin and erythromycin, where both antibiotics have common characteristics, such as inhibition of protein synthesis.

Although it did not have intrinsic activity, the oil caused damage to the membrane, where it altered the permeability and facilitated the entry of drugs, in order to potentiate the action of the antibiotics, presenting efficacy in low concentrations.

The only antibiotic used to act directly through inhibition of gyrase, norfloxacin was ineffective or antagonistic when associated with EOFR. Possibly this result is due to changes in norfloxacin structure that inactivated the drug, or the transporter bound to the present compounds, which led to their inactivation, as well as possibly activation of genes responsible for the expression of efflux pumps that led to inactivation and antagonism of their activity.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fct.2019.06.006>.

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