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Chemical composition and possible use as adjuvant of the antibiotic therapy of the essential oil of *Rosmarinus officinalis* L.

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ABSTRACT

Rosmarinus officinalis L. (rosemary) essential oil (REO) was tested for its antimicrobial activity alone and in combination with conventional antibiotics used against *Staphylococcus aureus*, *Escherichia coli*, and *Candida* strains. The essential oil was chemically described using GC–MS analysis and its antibiotic-resistance modifying activity was assayed using microdilution method. A significant modulatory effect on the drug resistance was verified when the REO was used in association with aminoglycoside antibiotics against *E. coli* and *S. aureus* multidrug resistant strains. With respect to the antifungal tested, there was a decrease on amphotericin B activity when this antibiotic was tested in association with the REO against *Candida krusei*. On the other hand, the combined use of the REO with benzoilmetronidazole was more effective than the antibiotic alone when tested against *C. krusei*. Rosemary essential oil could become a promising source of metabolites with antibiotic-resistance modifying activity for use against multi-drug-resistant microorganisms.

1. Introduction

Bacterial infectious diseases represent a serious risk to the world population once they have been responsible for high morbidity and mortality through the times. However, this problem has been much more serious in the last years with the increase in the prevalence of infections caused by multi-drug-resistant (MDR) strains (Nikaido, 2009). Emergence of MDR is a phenomenon occurring worldwide, due to the selective pressure exerted by extensive use of antibiotics and that has hindered the infectious illness therapy.

Therefore, the search for new antimicrobial agents or new compounds able to potentiate the antimicrobial activity of old antibiotics against resistant microorganisms has become an important area of research (Bolla et al., 2011; Gibbons, 2008). In this perspective, medicinal plants have presented as an important source of biomolecules actives against different microorganism groups (Bassolé and Juliani, 2012). The technologic prospection of medicinal plants and their pharmacological properties is of fundamental importance to the validation of its use in traditional medicine and development of bio-products of industrial interest as well as to its own conservation of the natural resource (Rose et al., 2012; Alves and Rosa, 2007).

Rosemary is a herb cultivated worldwide and used in folk medicine, cosmetics, and phytocosmetics (Celiktas et al., 2005). This medicinal plant shows hepatoprotective (Abdel-Wahhab et al., 2011), antiulcerogenic (Dias et al., 2000), and antioxidant effects (Beretta et al., 2011; Mata et al., 2007). Several studies have focused in the antimicrobial activity of the rosemary essential

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oil (REO) (Dimitrijević et al., 2007; Bozin et al., 2007). Previous researches have demonstrated the REO activity against several pathogenic microorganisms (Issabeagloo et al., 2012; Zaouali et al., 2010; Roldán et al., 2010; Gachkar et al., 2007; Djeddi et al., 2007).

The chemical analysis of the REO composition has showed the presence of terpenes and terpenoids, among them, the dominating constituents camphor, 1,8-cineole, α-pinene, camphene, α-terpineol and borneol (Atti-Santos et al., 2005; Touafek et al., 2004). It has been suggested that such hydrophobic compounds are able to disrupt the plasma membrane and the outer membrane of Gram-negative bacteria, causing changes in the membrane permeability and cell death (Hyldgaard et al., 2012; Wang et al., 2012).

The ability to potentiate the antibiotic activity by bioactive plant products has been investigated aiming to increase its effectiveness against MDR microorganisms (Matias et al., 2010; Souza et al., 2010; Aiyegoro and Okoh, 2009). The association of the REO with gentamycin, tetracycline, and sulfazotrim produced a synergistic effect against *Staphylococcus aureus*, and a similar effect was verified when the REO was tested in association with chloramphenicol, cefepime, and tetracycline against *Escherichia coli* (Zago et al., 2009). On the other hand, an antagonistic effect was verified when the REO was tested in association with ciprofloxacin against *S. aureus*, as well as, when tested in association with amphotericin B against *Candida albicans* (van Vuuren et al., 2009).

In the present study, the antimicrobial activity of the REO alone and its possible antibiotic-resistance modifying activity when combined with aminoglycoside antibiotics against multidrug-resistant *S. aureus* and *E. coli* strains, as well as when combined with poliene and nitroimidazole antibiotics against yeast strains was studied.

2. Materials and methods

2.1. Strains and drugs

The tests were performed with clinical *S. aureus* SA358 (Freitas et al., 1999) and *E. coli* EC27 (Coutinho et al., 2009) isolates, which are resistant to several aminoglycosides. Standard strains (*S. aureus* ATCC 25923 and *E. coli* ATCC 10536) were used as a positive control. Two yeast strains were utilized: *C. albicans* ICB12 and *Candida krusei* ATCC 6538. The standard strains were obtained through the National Institute of Quality Control in Health (INCQS) at the Oswaldo Cruz Foundation, Ministry of Health and the clinical isolates, from the Federal University of Paraiba, UFPB. The strains were maintained on Nutrient Agar (Himedia, India) slant at 4 °C and prior to the assay, the cells were grown overnight at 37 °C in Brain Heart Infusion (BHI, Himedia, India).

The REO was obtained *via* steam distillation as pure essential oil and was purchased from Ferquima, São Paulo, Brazil. Neomycin, gentamicin, amikacin, metronidazole, benzoilmetronidazole, nystatin, and anphotericin B were purchased from Sigma Chemical Corp., St. Louis, MO, USA. All drugs were dissolved in sterile water.

2.2. Gas chromatography/mass spectrometry (GC–MS)

Essential oil was analyzed after injection of 1 μL into a gas chromatograph (Thermo Scientific CG Ultra) with a flame ionization detector (FID) model ISQ and coupled with a mass spectrometer model ISQ (Thermo Scientific) equipped with a HP-5MS (Agilent, Palo Alto, CA) 95% dimethylpolisiloxane 5% phenyl capillary column (intern diameter = 0.25 mm, length 30 m, film thickness = 0.1 μm. Operating conditions were as follows: injector temperature, 250 °C; FID temperature, 270 °C; carrier gas (helium), flow rate of 1 mL min^{-1}. The oven temperature was initially 50 °C and then raised to 180 °C at a rate of 5 °C min^{-1} and then it was heated to 260 °C at a rate of 10 °C min^{-1}. The mass spectrometry conditions were the following: ionization voltage, 70 eV; mass range, 43–500 Da; ion source temperature, 200 °C. The retention time of alkanes (C_8–C_{20}) were used to calculate the retention indices for identified compounds and for reference standards. Compounds were preliminarily identified by use of NIST mass spectra library, as well as mass spectroscopy data from literatures (Adams, 2007; Pino et al., 2005).

2.3. Drug susceptibility test

The stock solution of the REO was prepared by dissolving 10 000 μg of the essential oil in 1 mL of dimethylsulfoxide (DMSO – MERCK), thus starting with an initial concentration of 10 000 μg/mL. The resulting solution was then diluted to 1024 μg/mL in sterile water. The minimal inhibitory concentration (MIC) of the REO was determined by the microdilution assay in BHI broth with suspensions of 10^5 CFU/mL and essential oil concentrations varying from 8 to 1024 μg/mL (Javadpour et al., 1996). The MIC was evidenced by adding 20 μL of an aqueous solution of resazurin 0.01% (w/v) into each cavity and incubation of the microtitulation plates in environment temperature during 1 h. A change in the medium color from blue to red was considered as positive for microbial growth. The MIC was defined as the lowest concentration of the essential oil at which no microbial growth was observed.

In the evaluation of the REO for modulatory effect on antibiotic activity, the MIC of the antibiotics were determined in the presence of the REO at sub-inhibitory concentrations (MIC/8) (Coutinho et al., 2010a), and then the plates were incubated at 37 °C for 24 h. Differences ranging only one point in the MIC were considered not significant. The bacterial strains were classified as sensible, intermediate and resistant according with criterions previously established (CLSI, 2005).

3. Results

Tables 1 and 2 show the antimicrobial activity data for the natural product tested against Gram-negative and Gram-positive bacteria as well as against two yeasts strains. The REO did not show antimicrobial activity at clinically relevant concentrations (1024 μg/mL) tested against *E. coli* (EC27 and ATCC 10536), *S. aureus* (SA358 and ATCC 25923) and *C. krusei* ATCC 6538 strains. On the other hand, REO was able to inhibit the *C. albicans* ICB12 growth at 512 μg/mL concentration.

In checking the antibiotic-resistance modifying activity of the REO, was verified that the essential oil changed the antibiotic-resistance phenotype of the strains when it was tested in association with aminoglycosides against MDR strains (Table 1), but no effect was observed on the tests with *S. aureus* ATCC 25923 and *E. coli* ATCC10536 (data not showed). In *E. coli*, the gentamicin and amikacin resistance phenotypes were changed to sensitive phenotype when the multidrug-resistant strain was exposed to the gentamicin-REO combination. For amikacin and neomycin there was an expressive reduction in the MIC values when the EC27 strain was tested with these antibiotics combined in the presence of the REO. In *S. aureus* there was a change from intermediate resistance phenotype to sensitive phenotype when the MDR strain was exposed to amikacin and gentamicin in association with the REO.

For *C. krusei*, the combined use of the REO with benzoilmetronidazol was more effective than the antibiotic alone, but the associated use of REO with amphotericin B caused a decrease in activity of this antibiotic. On the other hand, the essential oil did not interfere in the activity of all antifungal tested against *C. albicans*.

Table 1
MIC values of antibiotics in the absence and presence of a sub-inhibitory concentration of *Rosmarinus officinalis* L. essential oil for multi-drug-resistant *E. coli* and *S. aureus* strains.

Antibiotic/product	*E. coli* EC27		*S. aureus* SA358	
	Alone (μg/mL)	Antibiotic + REO (μg/mL)	Alone (μg/mL)	Antibiotic + REO (μg/mL)
Amikacin	≥5000 (R)	19.5 (R)	39.1 (I)	9.8 (S)
Neomycin	≥5000 (–)	19.5 (–)	19.5 (–)	19.5 (–)
Gentamicin	≥5000 (R)	≤1.2 (S)	5 (I)	≤1.2 (S)
REO	≥1024	–	≥1024	–

REO: rosemary essential oil; R: resistance phenotype; I: intermediate phenotype; S: sensitivity phenotype; (–) not determined.

Table 2
MIC values of antibiotics in the absence and presence of a sub-inhibitory concentration of *Rosmarinus officinalis* L. essential oil for yeast strains.

Antibiotic/product	*C. albicans* ICB12		*Candida krusei* ATCC 6538	
	Alone (μg/mL)	Antibiotic + REO (μg/mL)	Alone (μg/mL)	Antibiotic + REO (μg/mL)
Metronidazole	≥1024	≥1024	≥1024	≥1024
Benzoilmetronidazol	≥1024	≥1024	≥1024	8
Nystatin	≥1024	≥1024	≥1024	≥1024
Amphotericin B	≥1024	≥1024	32	≥1024
REO	512	–	≥1024	–

REO: rosemary essential oil; REO with 128 μg/mL.

The chemical analysis of the REO used in the present study is shown in Table 3. It was verified the presence of 1,8-cineole, camphor, α-pinene and β-pinene as the major compounds.

4. Discussion

Essential oils are volatile complex mixture of constituents obtained from aromatic plants which have been proposed for different technological applications, including as antimicrobial agents for food preservation and for medicinal purposes (Gómez-Estaca et al., 2010; Trajano et al., 2009; Luqman et al., 2007). However, the technological use of a specific essential oil must be supported by a sufficient number of scientific studies in order to determine its effectiveness as antimicrobial agent and to elucidate its action mechanism, as well as, to obtain information about its toxicity and its possible interactions with conventional antibiotics (Rios and Recio, 2005).

In this work we investigate the antimicrobial activity of the REO alone and associated with aminoglycosides against two Gram-negative and Gram-positive strains. We also tested the antimicrobial activity of the REO alone and combined with antifungals against two yeast strains associated with candidiasis in humans. The MIC value of the REO used in the present study was ≥1024 μg/mL when it was tested alone against *S. aureus*, *E. coli* and *C. krusei*. Although it was not possible to determine the MIC endpoints to the REO against these microorganisms, because of the low concentrations tested, our results are in agreement with other investigators who reported a moderate antibacterial activity against *S. aureus* and *E. coli* (Pintore et al., 2002; van Vuuren et al., 2009). In the case of *C. albicans* was verified an inhibitory effect of the REO at 512 μg/mL. This MIC end point was higher than that

Table 3
Chemical composition of *Rosmarinus officinalis* L. essential oil.

No	RT[a]	Compound	%Area	KI[b]	KI (Adams, 2007)	KI (Pino et al., 2005)
1	3.54	Butylacetate	1.33	811	807	–
2	5.58	Tricyclene	0.98	924	921	923
3	5.68	α-Thujene	2.23	929	924	931
4	5.86	α-Pinene	9.79	936	932	939
5	6.22	Camphene	5.14	951	946	953
6	6.93	β-Pinene	9.24	981	974	980
7	8.47	1,8-Cineole	30.87	1041	1026	1032
8	9.00	γ-Terpinen	0.44	1060	1054	1062
9	10.14	Linalool	1.46	1102	1095	1098
10	11.49	Camphor	10.13	1151	1141	1143
11	12.03	Isoborneol	4.66	1170	1155	–
12	12.30	Terpene-4-ol	1.80	1180	1174	1177
13	12.69	α-Terpineol	3.07	1194	1186	1189
14	15.27	Bornylacetic	3.45	1289	1287	–
15	17.64	α-Copaene	1.30	1379	1374	1376
16	18.82	Caryophyllene	6.76	1425	1417	1418
17	19.64	α-Humulene	2.00	1458	1452	1454
18	20.18	γ-Muurolene	1.19	1480	1478	1477
19	21.10	γ-Cadinene	0.65	1518	1513	–
20	21.32	δ-Cadinene, (+)-	1.15	1527	1522	1523
21	22.78	Caryophylene oxide	2.33	1589	1582	1581

[a] RT: retention time.
[b] KI: Kovats index.

obtained by Jiang et al. (2011) and lower than that detected by van Vuuren et al. (2009).

Many articles can be found in the scientific literature regarding the antibacterial and antifungal activities of the REO (van Vuuren et al., 2009; Jiang et al., 2011; Khosravi et al., 2013; Hussain et al., 2010, 2011; Prabuseenivasan et al., 2006). A comparative analysis between them shows that different MIC values have been obtained for a particular sample of REO. As an example, the MIC of the REO against *S. aureus* ranged of 30–12 800 μg/mL (Jiang et al., 2011; Prabuseenivasan et al., 2006). For *E. coli*, the results ranged between 300 and 6400 μg/mL (Jiang et al., 2011; Prabuseenivasan et al., 2006). These divergences could be assigned to several factors which can influence the REO composition, such as the geographical area of cultivation (Jordán et al., 2013a), the phenological stage of the plant (Jordán et al., 2013b) and the method of oil extraction (Hosni et al., 2013). The target microorganism and the method used to test the antibacterial activity can also to influence in the discrepancy of results (Hammer et al., 1999).

The main objective of this paper was to investigate if the REO was able to potentiate the antibacterial activity of aminoglycosides *in vitro* against *S. aureus* and *E. coli* resistant to these antibiotics, as well as, to potentiate the activity of different anti-fungal against two *Candida* species. The addition of REO in sub-inhibitory concentrations to the growth medium increased the activity of the aminoglycosides against *S. aureus* and *E. coli* MDR strains, but this effect did not observe for standard strains (*S. aureus* ATCC25923 and *E. coli* ATCC10536 possibly due the absence of any resistance mechanisms to aminoglycosides, showing a potentiating effect on antibiotics tested.

Aminoglycosides binds to the ribosomal subunit 30S leading to the synthesis of anomalous proteins that insert on plasma membrane changing its permeability (Mingeot-Leclercq et al., 1999). The main resistance mechanisms to the aminoglycosides occurring in both Gram-positive and Gram-negative bacteria are enzymatic inactivation and the presence of efflux proteins which are able to pump the antibiotic to extracellular medium (Morar and Wright, 2010). It has been suggested that plant products can to enhance the aminoglycoside activity against multi-drug resistant *E. coli*, possibly by interfering in efflux systems (Coutinho et al., 2010b). However, the intrinsic resistance of Gram-negative bacteria is due to both extrusion of the antibiotic and the presence of the outer membrane that acts as an impermeable barrier to various antimicrobial agents (Nikaido, 2009).

The chemical composition analyses of the REO studied in the present work showed the presence of several terpenes and terpenoids, according with previous studies (Hussain et al., 2010; Sacchetti et al., 2005). Due to its high lipophilicity, these compounds are able to interact with hydrophobic sites present in phospholipids and proteins presents on the plasma membrane and the outer membrane of Gram negative bacteria (Urzúa et al., 2008). This accumulation of hydrophobic components in the bacterial plasma membrane causes loss of their integrity and increases its permeability (Ojeda-Sana et al., 2013). In turn, these changes cause loss of cytoplasmic content, dissipation of the proton-motive force, lysis and cell death (Sikkema et al., 1995; Gustafson et al., 1998).

The decreases in the aminoglycosides MIC values when tested in combination with the REO against the MDR strains (EC27 and SA358) could be related to the damage caused by the oil components on the phospholipid bilayer of the cytoplasmic membrane and of the outer membrane, causing a higher mortality by direct osmotic lysis and by facilitating the entrance of the antibiotic into the bacterial cell (Rodrigues et al., 2009). We also cannot rule out is occurring inhibition of efflux proteins by essential oil components contributing to increase intracellular levels of antibiotic (Oluwatuyi et al., 2004; Bolla et al., 2011). However, further studies are needed to confirm these propositions.

For *C. krusei* strain, the REO was able to inhibit the amphotericin B activity, but did not affect the activity of the nystatin. Polyene antibiotics bind to the ergosterol molecules forming artificial channels which increase the permeability of the plasma membrane, however, nystatin binds to ergosterol with a lower affinity (Brajtburg et al., 1990). It is likely that the intercalating of the REO components could have interfered with the binding of amphotericin B to the ergosterol, decreasing its antimicrobial activity. On the other hand, the lack of interference in the activity of nystatin could be related with structural differences present between these two antibiotics.

Antibiotics nitroimidazoles such as metronidazole and benzoilmetronidazole docking in the DNA molecule, interfering with the replication and transcription processes, interrupting the production of structural proteins and enzymes necessary to cell metabolism, with *in vitro* activity against protozoa, helminthes and certain anaerobic bacteria (Grunberg and Titsworth, 1973). Our results showed that the REO has sensitized the *C. krusei* strain to the benzoilmetronidazole, which may have been a consequence of the increase in membrane permeability caused by oil components, allowing the entrance of this antibiotic in the fungal cell (Ahmad et al., 2011).

5. Conclusions

The present study provided evidence of the modifying-antibiotic resistance activity of the REO when tested *in vitro* in combination with aminoglycoside against multidrug-resistant *S. aureus* and *E. coli*. This REO also sensitized the *C. krusei* strain to benzoilmetronidazole. However, further studies are necessary to validate these results and to determinate if these effects are addictive or synergistic. Besides, it is also needed to elucidate the phytochemicals related with this activity, as well as, the mechanism behind it. The overall results provide baseline information for the possible use of the REO combined with aminoglycosides in the control of infections due to *S. aureus* and *E. coli*. In addition, our results indicate that combinations of the REO with amphotericin B against *C. krusei* should be avoided.

Competing interest

The authors have not competing interest to disclose.

Authors' contributions

H.M.B., I.S.L., M.F.B.M.B., C.C.A.T. and S.R.T. performed the antimicrobial assays; J.V.R., A.P.L.A., M.C.G.L. and R.W.G.O. performed the chemical characterization of the REO; E.O.L., H.D.M.C., A.M.G.L.C. and J.A.D.L. wrote the manuscript and supervised the work.

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