



Antibacterial activity of the essential oil of *Citrus limon* against multidrug resistant *Acinetobacter* strains

Atividade antibacteriana do óleo essencial de *Citrus limon* contra cepas multidroga resistente *Acinetobacter*

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ABSTRACT

Acinetobacter species have gained importance in recent years due to their involvement in serious infections and antimicrobial resistance. New products with antibacterial activity have been studied including the essential oil (EO) of *Citrus limon*. The present study aimed to evaluate the effect of the essential oil of *C. limon* against multidrug resistant strains of *Acinetobacter* spp. isolated from clinical material. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined by the microplate bioassay, and a time kill study of *Acinetobacter* spp. treated with EO, was performed. The oil caused the growth inhibition in 16 (67%) of 24 strains tested, showing a MIC of 625 µg/mL and MBC of 1250 µg/mL. In a time kill study, the oil displayed a concentration-dependent antibacterial activity. These results suggest that EO of *C. limon* may suppress the growth of *Acinetobacter* species.

Keywords: Drug resistant, Bacteria, *Acinetobacter*; *Citrus limon*

RESUMO

Espécies do gênero *Acinetobacter* ganharam importância nos últimos anos devido ao seu envolvimento em infecções graves e sua resistência antimicrobiana. Novos produtos com atividade antibacteriana foram estudados, incluindo o óleo essencial (OE) de *Citrus limon*. O presente estudo teve como objetivo avaliar o efeito do óleo essencial de *C. limon* contra cepas multirresistentes de *Acinetobacter* spp. isoladas a partir de material clínico. A concentração inibitória mínima (CIM) e Concentração Bactericida Mínima (CBM) foram determinadas pelo bioensaio com microplacas, e um estudo de tempo de morte *Acinetobacter* spp. tratado com OE, foi realizado. O óleo causou a inibição do crescimento em 16 (67%) de 24 linhagens testadas, demonstrando uma CIM de 625 µg / mL e CBM de 1250 µg / mL. Em um estudo de tempo de morte, o óleo apresentou uma atividade antibacteriana dependente da concentração. Estes resultados sugerem que o óleo essencial de *C. limon* pode suprimir o crescimento das espécies de *Acinetobacter*.

Palavras-chave: Resistências às drogas, bactérias, *Acinetobacter* e *Citrus limon*

INTRODUCTION

Lemon has been valued as an important part of a healthy diet. It is well established that lemon fruit and its by-products constitute an interesting source of phenolic compounds (mainly flavonoids) and other nutrients and non-nutrient compounds (vitamins, minerals, dietary fiber, essential oils, organic acids and carotenoids), which are necessary for normal growth and the correct functioning of human physiological systems. Some of these known

compounds are, for example, essential oils mainly used as food flavoring, perfumes and pharmaceutical formulations due to their functional properties (Gonzales *et al.*, 2010). The essential oil of *Citrus limon* (Rutaceae) is rich in biologically active compounds, which are responsible for its antibacterial, antifungal, antiparasitic and antiviral activities (Prabuseenivasan *et al.*, 2006).

The volatile constituents are a mixture of monoterpene

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(limonene) and sesquiterpene hydrocarbons and their oxygenated derivatives including: aldehydes (citral), ketones, acids, alcohols (linalool) and esters (Fisher & Phillips, 2008)

Recent clinical attention has focused on the increasing frequency of *Acinetobacter* species pathogens responsible for hospital-acquired infections and as emerging pathogens that frequently cause infections in patients in intensive care units. These microorganisms can survive for long periods on dry surfaces and have the ability to tolerate desiccation, as well as showing resistance to several drugs, which may contribute to their persistence in the hospital environment (Bergogne-Bérézin & Towner, 1996; Roberts et al., 2001).

The mechanisms of multidrug resistance in *Acinetobacter* species, mainly expressed by *A. baumannii*, include the production of β -lactamases and aminoglycoside-modifying enzymes, diminished expression of outer membrane proteins, mutations in topoisomerases, and upregulation of efflux pumps play an important part in antibiotic resistance (Bonomo & Szabo, 2006).

With the increase of resistance to antibiotics, natural products could be an interesting alternative to find new substances with antimicrobial properties in combating this microorganism (Oliveira et al., 2007; Silva et al., 2007).

Therefore, many studies on biological activity have been performed with essential oils obtained from medicinal plants, such as *C. limon*, attempting to help overcome this problem. The present study aimed to evaluate the effect of the essential oil of *C. limon* against multidrug resistant strains of *Acinetobacter* spp. isolated from clinical material.

MATERIAL AND METHODS

Essential oil

The product tested was the essential oil of *Citrus limon* (lemon), acquired from FERQUIMA Industry/São Paulo.

Essential oil analysis

The essential oil constituents were analyzed using a Shimadzu GC17-A gas chromatographer coupled to mass spectrometer operated by electron ionization. Helium (1.6 mL/min) was used as carrier gas and the split relationship was adjusted to 1:5. Chromatographic separation was performed using a DB-5 capillary column (30 m x 0.25 mm (I.D.) x 0.25 μ m (d_i)). The temperature of the chromatographer oven was programmed from 60°C to 105°C (@ 5°C/min), 105°C to 190°C (10°C/min), 190°C to 280°C (20°C/min). The temperature of injector and detector were 260°C and 280 °C, respectively. The total time was 22 minutes and the injection volume was 1.0 μ L. Identification of individual components was based on their mass spectral fragmentation based on two computer library MS searches (Wiley 229), retention indices, and comparison with published data (Adams, 2007; Alencar et al., 1990).

The percentage compositions were obtained from electronic integration measurements using flame ionization detection (FID) also set at 250°C. n-Alkanes were used as reference points in the calculation of relative retention

indices. The concentration of the identified compounds was computed from the GC peak area without any correction factor. GC analyses were performed on a gas chromatographer Hewlett-Packard 5890 SERIES II equipped with a flame ionization detector (FID) and a J & W Scientific DB-5 fused silica capillary column (30 m x 0.25 mm x 0.25 μ m). GC oven temperature. Injector and detector temperatures were 270°C and 290°C, respectively. Hydrogen was used as carrier gas at a flow rate of 1.0 mL/min; split mode (1:10).

Microorganisms

Acinetobacter spp. strains used as test microorganisms were isolated from clinical material of different patients at different times (Table 1), using standard procedures (Koneman, 2008). An antibiotic resistance study was carried out according to CLSI 2011. Stock cultures were maintained on Muller-Hinton agar slants at 4 °C (\pm 1 °C). Overnight cultures inoculated on Muller-Hinton agar slants at 37 °C were used to prepare the bacterial inoculum to be used in the antimicrobial assays.

Inoculum

The inoculum consisted of 10⁸ colony forming units per mL (CFU/mL) prepared in sterile saline solution (0.85%) and standardized according to the 0.5 tube of the McFarland turbidity scale and adjusted to the desired bacterial density (Bauer et al., 1966; Cleland & Squires, 1991).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC was determined by the microplate bioassay. In a 96-well microplate was added Mueller Hinton broth (MHB) with bacterial inoculum prior to the assay (1:9 v/v) and the essential oil of *C. limon* concentrations from 10.0000 μ g/mL to 20 μ g/mL. The microplates were incubated at 35-37°C for 24 hours. Antibacterial activity was detected using a colorimetric method by adding of an aqueous solution of resazurin stain (0,01%) to each well at the end of the incubation period. The plates were further incubated for 24 hours at 35-37 °C. The MIC was determined as the lowest oil concentration that inhibited visible growth of microorganisms, as also indicated by the resazurin stain. To determine the MBC, 10 μ L of each of the wells without bacterial growth was placed on a sterile microplate containing MHB, the microplate was incubated at 37°C for 24 hours. Afterwards, resazurin was added and the microplate incubated for 24 hours. The MBC was determined as the lowest oil concentration that no visible microbial growth occurred, as also indicated by the resazurin method. These tests were performed in duplicate. Was performed the sterility control of medium (negative control) and the strain viability (positive control) (Eloff, 1998; Mann & Markham, 1998; Palomino et al., 2002).

Time kill

Time-kill was performed according to Klepser et al. (1998), with some modifications. Before testing, the microorganism was cultivated in Mueller Hinton Agar (MHA). Colonies derived from culture were suspended in

0.85% NaCl and turbidity adjusted to the range of 0.5 McFarland (1.5×10^8 CFU/mL). Concentrations of essential oil of *C. limon* tested were 0.5, 1, 2, times the MIC. These cultures were incubated at 37°C and at various time periods (0, 2, 4, 8, and 24 hours). An aliquot of 1 μ L of each dilution was removed and plated on MHA. The plates were incubated at 37°C for 24-48 hours and the numbers of colony forming units (CFU) were counted. The experiment was performed in duplicate. The minimum detection limit of this method is 1000 CFU/mL. The log₁₀ CFU/mL was plotted on a graph as function of time and used to compare the rate and extent of antibacterial activity in various concentrations of essential oil. It was considered bactericidal activity when there was a decrease greater than or equal to 3 log₁₀ CFU/mL of the initial inoculum, resulting in reduction of 99.9% or more CFU/mL in 24 hours compared with the initial inoculum. Activity lower than that described was considered bacteriostatic (Klepser *et al.*, 1998).

RESULTS and DISCUSSION

For more than 50 years, natural products have served us well in combating infectious bacteria and fungi. Microbial and plant secondary metabolites helped to double our life span during the 20th century, reduced pain and suffering, and revolutionized medicine. Essential oils are involved in many important processes related to plant survival, playing prominent role in its defense against microorganisms (Demain, 2009).

Phytochemicals and their relative percentages in the essential oil composition and retention times are shown in Table 1. As shown in Table 1, the GC-MS analysis resulted in the identification of ten components. Among phytochemicals, the neral was the major essential component of oil in *C. limon* fruit representing 29,4% of total constituents followed by limonene (21,5 %), geranial (19,9%), trans - nerolidol (7,7%) and citral (6,4%) in descending order of percentage.

Moufida & Marzouk, 2003 has been shown that the limonene is one of the major phytochemicals of essential oil of citrus fruits with relative concentration between 45–75%. The citral has been also shown to be present, in the form of stereoisomers neral and geranial in essential oil of lemon (Gonzales *et al.*, 2010). Citral and linalool have been highlighted as active compounds in citrus fruit oils (Caccioni *et al.*, 1998).

These variations in the composition of the essential oils have been occurred, because it can be influenced by climatic conditions, geographical position of the growing region, agro-technology of cultivation, extraction technique applied and the stage of plant maturity at harvest also depend on genetic factors (Castro, 2006).

Table 2 shows the sensitivity of *Acinetobacter* spp. strains to different antimicrobials and the essential oil of *C. limon*. The oil showed growth inhibition in 16 out of 24 strains used in antibiotic sensitivity testing, at 625 μ g/mL. The minimum bactericidal concentration (MBC) was 1250 μ g/mL for 16 (67%) of 24 strains.

Table 2. Origin and resistance profile of *Acinetobacter* strains and inhibitory activity of *Citrus limon* essential oil.

The results of the present study show that the essential oil of *C. limon* at 625 μ g/mL inhibits *Acinetobacter*

growth.

Products from *C. limon* have been studied for their pharmacological activities, including antibacterial and antifungal activities (Lima *et al.*, 2006; Burt, 2004), but assessment of EO of *C. limon* against multidrug resistance *Acinetobacter* species are not reported.

The results obtained in this work have been shown consistent with the results observed by Sá *et al.* (1996) in which the essential oil of *C. limon* inhibited the growth of strains tested. And it has been superior to those reported by Araújo (2003) wherein the reported essential oil was active on only 40% of the microorganisms tested. In the other hand, Prabuseenivasan *et al.* (2006) observed the antibacterial activity of *C. limon* essential oil against some bacteria, whose results were better than those obtained in the present study with (MIC of 312 μ g/mL).

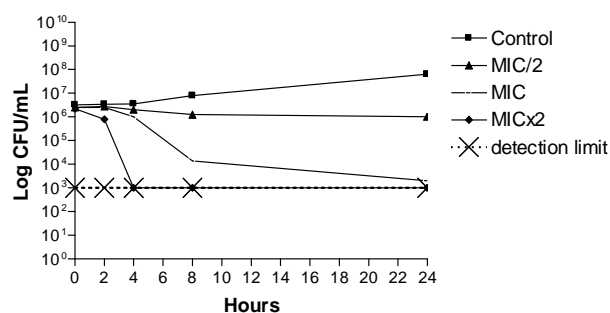


Figure 1. Time kill curves of the essential oil of *C. limon* against *Acinetobacter* spp., strain 004.

Figure 1 shows the time kill of the essential oil of *C. limon*. It is noted that 625 μ g/mL (MIC) up to 8 hours and MIC/2 up to 24 hours showed bacteriostatic activity, since there was a decrease of less than 3 log₁₀CFU/mL compared to initial inoculation. However, bactericidal activity was observed in 24 hours at MIC x 2, since there was a decrease greater than or equal to 3 log₁₀CFU/mL compared to initial inoculation.

Time kill curve showed that the higher the essential oil concentration, the greater the antibacterial activity from a bacteriostatic to bactericidal effect. Thus, *C. limon* had a concentration-dependent antibacterial activity. Higgins *et al.* (2000) in a comparative study of several quinolones against *Acinetobacter* strains found similar results for standard antibiotics.

The experiment conducted by Owen *et al.* (2007) using colistin against *A. baumannii* strains showed an extremely rapid concentration-dependent bactericidal activity against them. Other essential oils such as that of *Lippia javanicae* and *Melaleuca alternifoliade* also displayed concentration-dependent against the respective microorganisms tested (Viljoen *et al.*, 2005; May *et al.*, 2000).

The time kill characterization is very important because it has valuable therapeutic implications, such as adjusting the dose to make treatment more effective (Barros *et al.*, 2009). Thus, agents whose activity increases with increasing concentration can be optimized by the administration of large doses (Ernst *et al.*, 1996). As has been demonstrate in this work, the concentration of essential oil of *C. limon* implies the increase of its antimicrobial activity.

Table 1. Chemical composition (%) of the essential oil of *C. limon*

| Components | Retention time (min) | RI ^a sam. | RI ^b lit. | Relative percentage |
|-------------------|----------------------|----------------------|----------------------|---------------------|
| α -Pinene | 3,58 | 939 | 932 | 2,4 |
| β -Pinene | 4,23 | 981 | 974 | 4,8 |
| Limonene | 5,21 | 1031 | 1036 | 21,5 |
| Carenol | 8,09 | 1188 | 1190 | 4,2 |
| Myrtenal | 9,19 | 1193 | 1193 | 1,7 |
| Myrtenol | 9,51 | 1194 | 1196 | 2,0 |
| Neral | 10,13 | 1235 | 1235 | 29,4 |
| Citral | 10,39 | 1245 | 1240 | 6,4 |
| Geranial | 10,72 | 1270 | 1320 | 19,9 |
| Trans - Nerolidol | 13,08 | 1564 | 1565 | 7,7 |

a Calculated retention indices: n-alkanes were used as reference points in the calculation of relative retention indices.

b: Literature retention indices (literature values, Adams, 2007)

Table 2. Origin and resistance profile of *Acinetobacter* strains and inhibitory activity of *Citrus limon* essential oil

| Strain | Origin | PRP ^A | MIC (μ g/mL) | MBC (μ g/mL) | Positive control* | Negative control** |
|--------|--------------------|------------------|-------------------|-------------------|-------------------|--------------------|
| 002 | Tracheal aspirate | 4 | 625 | 1250 | + | - |
| 003 | Tracheal aspirate | 1 | 625 | 1250 | + | - |
| 004 | Urine | 1 | 1250 | 2500 | + | - |
| 005 | Tracheal aspirate | 1 | 1250 | 2500 | + | - |
| 006 | Tracheal aspirate | 5 | 625 | 1250 | + | - |
| 008 | Penile secretion | 1 | 625 | 1250 | + | - |
| 010 | Wound secretion | 1 | 625 | 1250 | + | - |
| 011 | Urethral secretion | 1 | 625 | 1250 | + | - |
| 013 | Urine | 1 | 625 | 1250 | + | - |
| 015 | Tracheal aspirate | 2 | 625 | 1250 | + | - |
| 019 | Tracheal aspirate | 2 | 625 | 1250 | + | - |
| 020 | Tracheal aspirate | 2 | 625 | 1250 | + | - |
| 021 | Wound secretion | 2 | 625 | 1250 | + | - |
| 022 | Tracheal aspirate | 2 | 625 | 1250 | + | - |
| 023 | Tracheal aspirate | 2 | 1250 | 2500 | + | - |
| 024 | Tracheal aspirate | 2 | 1250 | 2500 | + | - |
| 025 | Tracheal aspirate | 3 | 625 | 1250 | + | - |
| 026 | Catheter tip | 2 | 625 | 1250 | + | - |
| 030 | Catheter tip | 3 | 625 | 1250 | + | - |
| 032 | Tracheal aspirate | 3 | 625 | 1250 | + | - |
| 034 | Tracheal aspirate | 3 | 1250 | 2500 | + | - |
| 036 | Tracheal aspirate | 3 | 1250 | 2500 | + | - |
| 038 | Tracheal aspirate | 3 | 1250 | 2500 | + | - |
| 044 | Tracheal aspirate | 3 | 1250 | 2500 | + | - |

A: Phenotypic resistance profile: 1: CAM; CIP; LEV; CEP; CTZ; IMI; MER; PTZ. 2: AMI, CAM; CIP; LEV; CEP; CTZ; IMI; MER; PTZ, TIG. 3: CAM; CIP; LEV; CEP; CTZ; IMI; MER; PTZ, TIG. 4: CIP; LEV; CEP; CTZ; IMI; MER; PTZ. 5: AMI, CAM; CIP; LEV; CEP; CTZ; PTZ. **Abbreviations:** Amikacin (AMI); Ampicillin - Clavulanate (CAM); Ciprofloxacin (CIP); Levofloxacin (LEV); Cefepime (CEP); Ceftazidime (CTZ); Polymyxin B (POL); Imipenem (IMI); Meropenem (MER); Piperacillin - Tazobactam (PTZ); Tigecycline (TIG). * Microbial growth in broth without essential oil added. ** No microbial growth in broth.

CONCLUSIONS

The results obtained in this study have shown the high biological potential of essential oil of *Citrus limon*, particularly, the anti- *Acinetobacter* spp. multidrug resistant.

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