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Antibacterial activity of *Cymbopogon citratus* essential oil (lemongrass) against *Aeromonas caviae* complex

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Abstract

Phytotherapic agents such as *Cymbopogon citratus* have been used by the folk medicine to treat innumerous health issues. The essential oil of *Cymbopogon citratus* has been reported as a potential antimicrobial agent against several microorganisms. The genus *Aeromonas*, an opportunistic pathogen, is an important agent responsible for food infection and for infectious complications in both humans and animals. As an antibiotic resistance is an important concern for the therapy management of infections caused by *Aeromonas spp*. and other antibiotic resistant bacteria, the search for new antimicrobial agents is a strategy to developing alternatives to solve this problem. In this way, the aim of this study was to investigate the *in vitro* antimicrobial activities of *C. citratus* essential oil alone and in combination with 3 different antibiotics (cefalexin, tetracycline and gentamicin) against *Aeromonas caviae* complex strain using minimum inhibitory concentrations (MIC), checkerboard test, and

time-kill assay. As results we found that the *C. citratus* essential oil has antimicrobial activity against *A. caviae* complex strain, by itself and when it was combined with the tested antibiotics, promoting a synergic and additive effect.

Keyword: Phytotherapic, lemon grass, synergism, citral.

Atividade antibacteriana do óleo essencial de *Cymbopogon citrates* (capim limão) contra *Aeromonas caviae* complex

Resumo

Agentes fitoterápicos tais como o *Cymbopogon citratus* têm sido utilizados pela medicina popular para tratar inúmeros problemas de saúde. O óleo essencial de *C. citratus* foi avaliado como um potencial agente antimicrobiano contra vários microrganismos. A bactéria pertencente ao gênero Aeromonas é um agente patogênico oportunista, responsável pela contaminação de alimentos e de complicações infecciosas em seres humanos e animais. A resistência aos antibióticos é uma preocupação importante na terapia de infecções causadas por Aeromonas spp. e outras bactérias resistentes a antibióticos. Sendo assim, a procura de novos agentes antimicrobianos é uma estratégia para o desenvolvimento de alternativas medicamentosas eficientes no combate dessas bactérias. Desta forma, o objetivo deste estudo foi investigar a atividade antimicrobiana *in vitro* do óleo essencial de *C. citratus* isoladamente e em combinação, contra *Aeromonas caviae* complexo. Como resultado verificou-se que o óleo essencial de *C. citratus* tem atividade antimicrobiana contra *A. caviae* complex, isoladamente e em combinação com os antibióticos testados.

Palavras-chave: Fitoterápico, capim limão, sinergismo, citral.

Introduction

Essential oils are sources of phytotherapeutic agents that have traditionally been used by native cultures to treat infectious diseases. Their use as antimicrobial and antifungal agents has been firmly established for decades (Burt 2004).

Among various species with medicinal properties, *Cymbopogon citratus*, a species popularly known as "lemon grass", shows an infinite number of applications and is popularly used by people in many countries. In Brazil, for example, the tea, infusion and extracts of *C. citratus*, which are prepared with fresh or dry leaves, are often used in the popular medicine as a restorative, digestive, anti-tussis, effective drug against colds, analgesic, anticardiopatic, anti-inflammatory of urinary ducts, diuretic, antispasmodic and antiallergic (Negrelle and Gomes 2007). In addition, the lemon grass is also used in the food as spice and in cosmetics industries in various countries (Oliveira et al. 1997). Besides that, previous studies have revealed interesting antimicrobial efficacy in essential oils obtained from *C. citratus* (Tyagi et. al., 2010; Singh et al, 2010).

As a consequence of innumerous applications of *C. citratus*, several studies have been done aiming at enlarging the knowledge about the chemical composition of lemon grass leaves, which are the parts used for medicinal purposes. These studies have revealed that, although the chemical composition of the essential oil and aqueous extracts of *C. citratus* varies according to the geographical origin, the isolated and identified substances from the leaves are mainly alkaloids, saponin, α-sitosterol, terpenes, alcohols, ketone, flavonoids, chlorogenic acid, caffeic acid, p-coumaric acid and sugars (Matouschek and Stahl 1991, Chisowa et al. 1998, Negrelle and Gomes 2007, Sousa et al, 2010). A major component of the lemongrass oil is citral which is a mixture of tautomers geranial (trans-citral) and neral (cis-citral) (Figure 1) (Stefanazzi et al, 2011; Habila et al, 2010; Katsukawa et al., 2010).

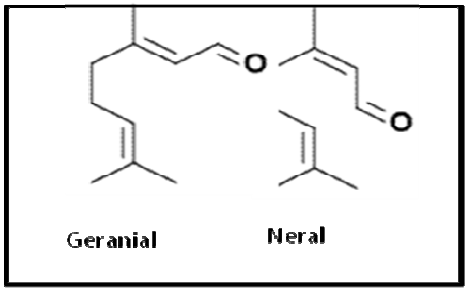


Figure 1- The chemical structures of Citral (geranial and neral), a major component of *C. citratus* essential oil.

Table 1 shows the chemical composition of *C. citratus* essential oil.

Table 1 - Major components of *C. citratus* essential oil analyzed by GC and GC-MS.

COMPONENT	**RATE (%)**
Citral	**57.46**
Geranial (*trans*-Citral)	31.43
Neral (*cis*- Citral))	26.03
Citral diethylacetal	**24.68**
Geranial diethylacetal	15.26
Neral diethylacetal	9.41
Limonene	**6.36**
Neryl or Geryl acetate	**2.05**
Myrcene	**1.21**
Methyl heptenone	**1.17**

Aeromonas spp. is a mesophilic and motile gram negative that is normal inhabitant of soil and freshwater, which have been isolated from a wide range of foods, such as fish and sea food, chicken and red meat, vegetables, raw and pasteurized milk, ice-cream and other dairy products. Since *Aeromonas* spp. multiply at 4°C, also under controlled atmosphere storage and under anaerobic

conditions, they represent a health hazard (Kirov et al.1993; Goni-Urriza et al., 2000).

Today, the genus *Aeromonas* is regarded not only as an important disease-causing pathogen of fish and other coldblooded species but also as the etiologic agent responsible for a variety of infectious complications in both immunocompetent and immunocompromised persons (Janda & Abbott, 2010).

The aim of this study was to investigate the *in vitro* antimicrobial activities of *C. citratus* essential oil alone and in combination with 3 different antibiotics (cefalexin, tetracycline and gentamicin) against *Aeromonas cavie* complex strain using minimum inhibitory concentrations (MIC), checkerboard test, and time-kill assay.

Methods

Chemicals and Strains

The *C. citratus* essential oil (CCEO) was obtained from Laslo Aromaterapia, Belo Horizonte (Brazil) and its quality parameters (appearance, color, purity, odor, density –20 °C, refraction index –20 °C) were described in a accompanying technical report. Growth media was purchased from Himedia, Mumbai, India.

Standard powder of tetracycline, cefalexin and gentamicin were obtained from Prati donaduzzi-Parana (Brazil), Novafarma-Goias (Brazil) and Cellofarm-Rio de Janeiro (Brazil), respectively. Stock solutions ware prepared and diluted according to Clinical and Laboratory Standards Institute (CLSI) standards and manufacturer's recommendations and stored at -20°C.The antibiotic discs were obtained from Laborclin, Paraná, Brazil.

Bacteria

Aeromonas caviae complex strain isolated from cheese in Paraiba State, Brazil and identified according to Janda & Abbott (2010) was used to evaluate the effect of *C. citratus* essential oil. The inocula were obtained from overnight

cultures grown on Nutrient Agar slants at 37 °C and diluted in sterile saline solution (NaCl 0.85% w/v) to provide a final concentration of approximately 10^6 count forming unit per mL (CFU/mL) adjusted according to the turbidity of 0.5 McFarland scale tube.

Agar disc diffusion assay

The disk diffusion (Kirby-Baurer) technique, which is recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2002), was used for antimicrobial test. It was performed using an 18 h culture at 37°C in 10 mL of Mueller Hinton Broth. The cultures were adjusted to approximately 10^5CFU/mL with sterile saline solution. Five hundred µL of the suspensions were spread over the plates containing Mueller-Hinton agar using a sterile cotton swab in order to get a uniform microbial growth on both control and test plates. Under aseptic conditions, empty sterilized discs (Whatman n°. 5, 6 mm in diameter) were impregnated with 2 µL of CCEO and placed on the agar surface. Standard discs containing gentamicin (10 µg/disc), cefalexin (30 µg/disc), penicillin (10UI) and tetracycline (30 µg/disc) was also used as reference control. The plates were left for 30 min at room temperature to allow the diffusion of oil, and then they were incubated at 37°C for 18 h. After the incubation period, the zone of inhibition was measured. Studies were performed in triplicate, and mean value was calculated. The means were analyzed by T test using Prism software package version 4.0 for windows. The results were expressed as mean ± SD. P values <0.05 were considered as significant.

Minimum inhibitory concentration

The Minimum Inhibitory Concentration (MIC) values of the essential oil were determined using the resazurin microdilution broth assay for microplates (Andrews, 2001; Sarker et al., 2007), with few modifications. Briefly, the essential oil was dissolved in dimethylsulfoxide (DMSO) with Tween 20 (0.5% v/v for easy diffusion) and the antibiotics was diluted in sterile distilled water,

then they were diluted in BHI broth (Himedia, Mumbai, India) to achieve concentrations ranging from 125 to 0.09 µg/mL. A final volume of 100 µg/mL was achieved in each well. One well with BHI broth and microorganism was used as control of the growth, and one inoculated well was free of the test essential oil or antibiotics to check the sterility of the media. MIC value was determined for CCEO, tetracycline (TET), cefalexin (CEF) and gentamicin (GEN) alone and for the combination between CCEO and antibiotics. The microplates were prepared in duplicate and incubated at 37 ◦C for 24 h. A volume of 30 µL of a homogenous aqueous solution of resazurin (0.01%) was added to each well to indicate viability of bacteria. The MIC endpoint was identified as the lowest concentration at which there was no visible growth indicated by the resazurin original blue color (Sarker et al., 2007).

Synergy testing by checkerboard assay

The checkerboard assay was made according to White et al. (1996).The combinations tested (in duplicate) were TET plus CCEO, GEN plus CCEO, and CEF plus CCEO. MICs of the antibiotics were determined in the presence of CCEO ranged from 62.5 to 0.48 µg/mL.

To evaluate the effect of the combinations, the fractional inhibitory concentration (FIC) was calculated for each antibiotic in each combination. The following formulas were used to calculate the FIC index: FIC of drug A 5 MIC of drug A in combination/MIC of drug A alone, FIC of drug B 5 MIC of drug B in combination/MIC of drug B alone, and FIC index 5 FIC of drug A 1 FIC of drug B. Synergy was defined as an FIC index of ≤ 0.5. Indifference or additivity was defined as an FIC index of >0.5 but of ≤ 4. Antagonism was defined as an FIC index of > 4 (Jayaraman et al., 2010).

Time-kill assay

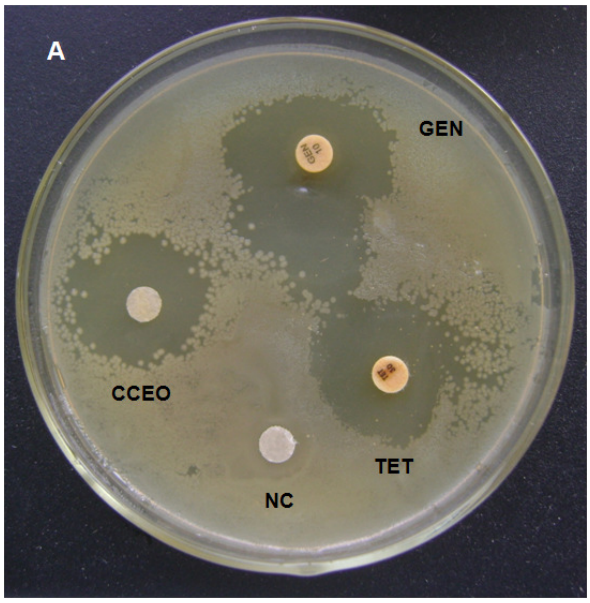
The time-kill assay was made in triplicate and according to Jayaraman et al. (2010). The assay were performed using 10 x MIC of the *C. citratus* essential oil against a single strain of *A. caviae* complex. Individual colonies

were isolated from the overnight growth plate and suspended in sterile BHI broth to approximate the density of 0.5 McFarland standards. The suspension was diluted 1:10 in BHI broth to obtain a standard inoculum of $1x10^6$ CFU/ml. A hundred µl of the diluted suspension was added to 0.9 mL of BHI broth. The tubes were incubated at 35°C for 24 h. From each tube 100 µl of sample was removed at 0, 4, 8 and 24 h, respectively and plated to count the viable cells. Growth control and sterility control were included for each assay. The killing rate was determined by plotting viable colony counts (CFU/mL) against time.

RESULTS

In vitro antimicrobial activity

This study explored the antimicrobial activity of the *C. citratus* essential oil and 4 antibiotics against *A. caviae complex* strain. In the disk diffusion technique, the *A. cavie* complex strain tested was resistant to both penicillin and tetracycline, but susceptible to cefalexin, and gentamicin. According to the results shown in Figure 2 and 3, the *C. citratus* essential oil exhibited a potent inhibitory effect with diameter of inhibition zones ranging from 12 to 23 mm, while it was 23 to 30 mm to gentamicin, 18 to 26 mm to cefalexin and 0 to 8 mm to tetracycline. According to these results gentamicin has the bigger diameter of inhibition zone, followed by cefalexin and by *C. citratus* essential oil. The difference between the diameter of inhibition zone of cefalexin and *C. citratus* essential oil are not statistically significant.



CCEO – *C. citratus* essential oil; GEN – Gentamicin; TET - Tetracycline; NC – Negative control.

Figure 2 - Inhibitory effect of the essential oil of *C. citratus* against *A. caviae* complex.

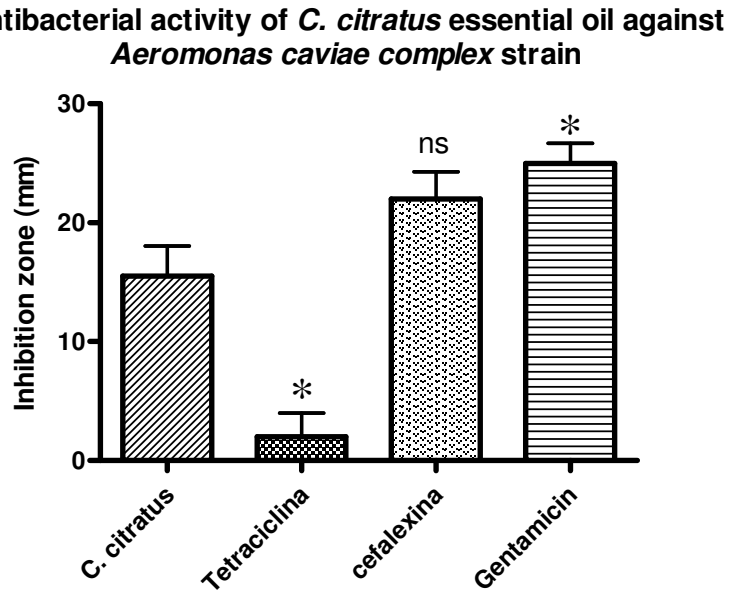


Figure 3 - Antimicrobial activity of the CCEO and 4 antibiotics against *A. caviae* complex strain in the disk diffusion technique.

Minimum inhibitory concentration (MIC)

The C. *citratus* essential oil show a substantial antibacterial activity against the *A. caviae* complex strain (MIC = 3.90 µg/mL), compared to tetracyclin (MIC = 0.97µg/mL), cefalexin (MIC = 31.25 µg/mL) and gentamicin (MIC = 0.39 µg/mL) (Table 2).

Synergy testing

The combination effects of antibiotics and *C. citratus* essential oil are summarized in Table 2. Our results show that the combinations of gentamicin plus *C. citratus* essential oil and cefalexicin plus *C. citratus* essential oil have synergistic mode of interactions. Tetracycline plus *C. citratus* essential oil show additivity against *A. caviae* complex strain under investigation. It is worth mentioning that none of the antibiotic plus *C. citratus* essential oil showed antagonism.

Table 2 - MIC values of antibiotics and CCEO against *Aeromonas caviae* complex strain.

	MIC[a] alone (µg/mL)	**Combined MIC (µg/mL)**	**FIC[c]**
CCEO[b]	3.90	-	-
Gentamicin	0.39	0.19	0.5
Tetracycline	0.97	3.90	4.0
Cefalexin	31.25	15.62	0.5

[a] MIC- Minimal Inibitory Concentration; [b] CCEO – *Cymbopogon citratus* essential oil; [c]FIC - Fractional inhibitory concentration

Time-kill assay

Treatment of *A. caviae* complex strain with *C. citratus* essential oil at 10 times the MIC demonstrated a decrease of 10.65 $\log_{10}$ CFU/mL of the initial inocula at 4 hour, with a maximum of reduction in the colony count at 8 hour,

when the colony count were zero. The time-kill curves are shown in the figure 4. The time-kill assay

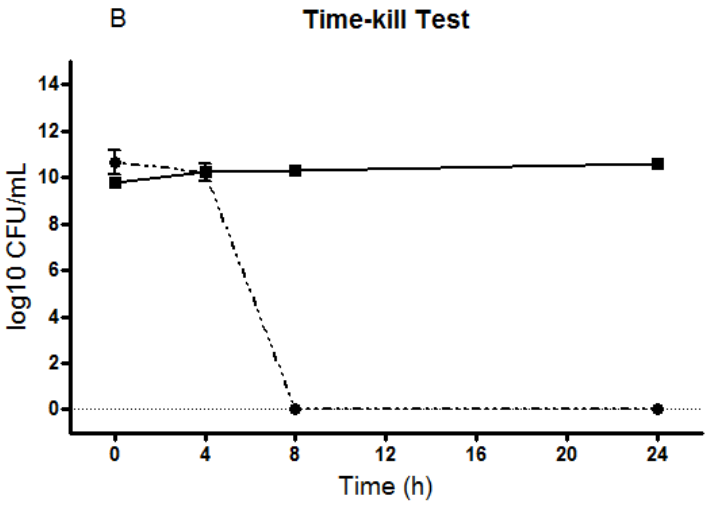


Figure 4 - The time-kill curve of *A. caviae* complex.

DISCUSSION

Aeromonas spp. shows a variable antimicrobial susceptibility according to its species and geographic areas of distribution. In our study *A. caviae* complex strain was resistant to penicillin and tetracycline and susceptible to cefalexin, gentamicin and essential oil of *C. citratus*. In a study from Brazil, Surek et al. (2010) demonstrate that all strains of *Aeromonas* ware resistant to ampicillin, 73.7 % were resistant to cefalotin and 15.78 % were resistant to tetracycline. Liu et al. (2008) reported that 39.3 % of *Aeromonas* were resistant to tetracycline. Obi et al (2007) found a high level of resistance to amoxicillin and ampicillin, followed by cefuroxime (79%), chloramphenicol (74%), and erythromycin (65%).

As we can observe *Aeromonas* has presenting an elevation antibiotic resistance, what can difficulty an effective management of the infection, especially in children and elderly people. In this way, several studies have

been made exploring the antibacterial potential of natural products with interesting results. In a study from South Africa, from eight different extracts of plants, three exhibited a significant activity against *Aeromonas* spp. isolates (*P. angolensis*, *S. cordatum*, and *Z. milneana)* (Obi et al., 2007). The antibacterial potential of the essential oil of basil on the growth of *A. hydrophila* was demonstrated by Wan et al (1998). Das et al (1999) demonstrated that *Azadirachta indica* is a potential antibacterial agent against *A. hydrophila*.

The antimicrobial activity of *C. citratus* essential oil has been demonstrated by several authors. Tyagi & Malik (2010) reported a strong antifungal activity of the *C. citratus* essential oil against *Candida albicans* and *Pseudomonas fluorescens*. Singh et al. (2010) reported that the *C. citratus* essential oil completely inhibited aflatoxin B production and the growth of *Aspergillus flavus*. Betoni et al. (2006) found in his study that the *C. citratus* essential oil has *in vitro* anti-*Staphylococcus aureus* activities besides of high synergism rate with antimicrobial drugs. Ohno et al. (2003) demonstrated that the essential oil from *C. citratus* have a bactericidal effect against *Helicobacter pylori* without the development of acquired resistance, suggesting that this essential oil may have potential for be included in anti-*H. pylori* therapy.

Our results show that the *C. citratus* essential oil has activity against the growth of the *A. caviae* complex strain under investigation. In the disk diffusion technique, *C. citratus* essential oil exhibited a potent inhibitory effect with diameter of inhibition zones ranging from 12 to 23 mm, followed by cefalexin (18-26 mm) and by tetracycline (0-8 mm). Only gentamicin presented higher inhibition zone (23 -30 mm) than *C. citratus* essential oil.

In the microdilution method, the MIC of *C. citratus* essential oil against *A. caviae* complex was 3.90 µg/mL, lower than MIC for cefalexin (31.25 µg/mL), but higher than tetracyclin (0.97µg/mL) and gentamicin (0.39 µg/mL). The time-kill curves are shown in the figure 2 b. In the time-kill assay was possible to observe that *C. citratus* essential had a maximum bactericidal

activity after 8 hour of contact with 10 times the MIC, when any growing was observed.

Citral is a major component of *C. citratus* essential oil (Stefanazzi et al, 2011; Habila et al, 2010; Katsukawa et al., 2010). Several studies have been reporting the antimicrobial activity of citral against innumerous microorganism, such as *Escherichia coli*, *Enterobacter sakasakii* and *Saccharomyces cerevisiae* (Espina et al., 2010; Arroyo et al., 2010; Belletti et al., 2010). As we know that the major component of *C. citratus* essential oil is citral, it is possible that the antimicrobial activity from the essential oil against *A. caviae* complex is due to this component.

Besides of antimicrobial activity of *C. citratus* essential oil alone, against *A. caviae* complex we also explored its effects in association with gentamicin, tetracycline and cefalexin. Our results show the reduction of the MIC for gentamicin and cefalexin when these antibiotics were associated with the essential oil (Table 2). As a result the *C. citratus* essential oil has synergistic interaction when it is combined with gentamicin and with cefalexin, and show additivity when it is associated with tetracycline against *A. caviae* complex.

The antibiotics under investigation in this study have different mechanism of action. Gentamicin and tetracycline inhibit the bacteria protein synthesis binding to the ribosome subunit 30S (Herman, 2007; Jayaraman et al., 2010). Cefalexin is responsible for inhibit enzymes involved in cell wall synthesis, which compromises the integrity of the bacterial cell wall (Draws & Bonomo, 2010). As the Gram negatives can develop antibiotic resistance by different mechanisms such as modifications in the permeability of membrane, hyperexpression of pump of efflux and beta lactamase production, association between antimicrobial drugs would be an interesting strategy to manage infections caused by *Aeromonas* multiresistant. The combination between gentamicin and *C. citratus* essential oil resulted in a synergistic interaction against the *A. caviae* complex strain under investigation. Thota *et al*. (2008) reported that the citral is a potent bacterial NorA efflux pump

inhibitors. Since, citral is a major component of *C. citratus* essential oil, there is a possibility that it may occur in the same way in combination with gentamicin and cefalexin.

Several studies suggest different antibiotic combinations for treatment of different kind of infections, but combinations of natural products and synthetic antibiotics are not reported in clinical practice. The results obtained in this investigation suggest that *C. citratus* essential oil could be considered to be used isolated or as adjuvant to antibiotic therapy against infection by *A. cavie* complex.

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