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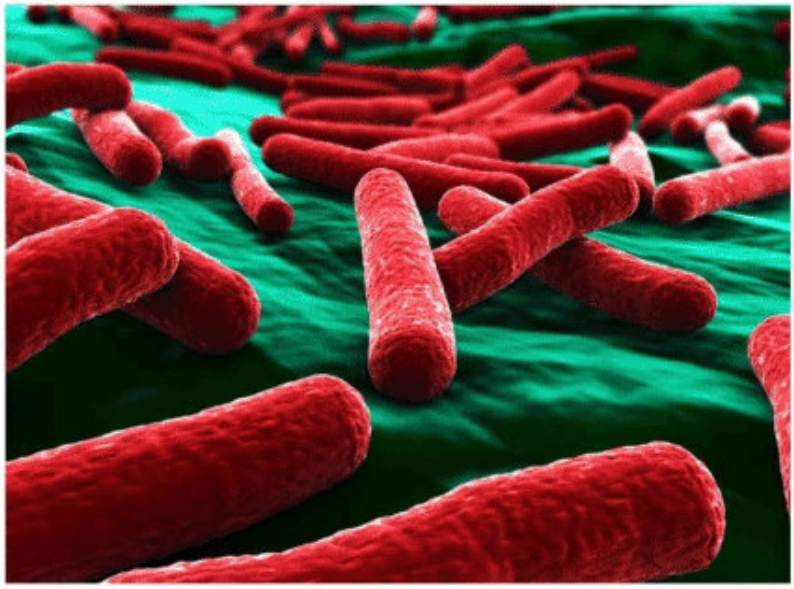
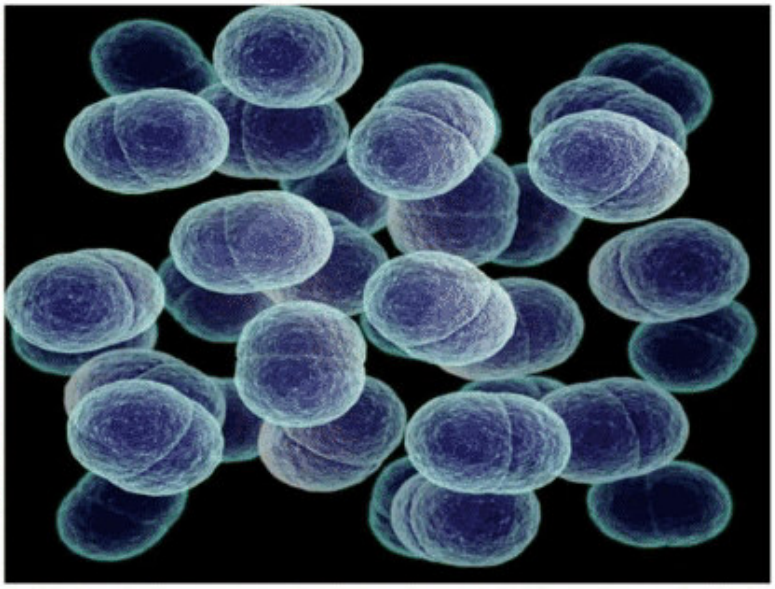
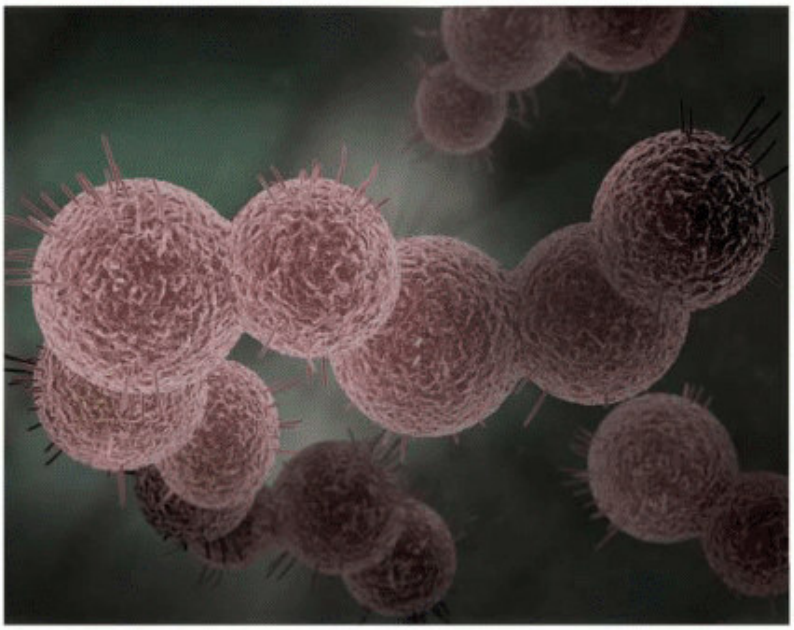


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Cymbopogon citratus EO antimicrobial activity against multi-drug resistant Gram-positive strains and non-*albicans*-*Candida* species

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We studied the antibacterial and antifungal activity of *Cymbopogon citratus*. These properties were studied on hospital isolated multidrug resistant strains *Staphylococcus aureus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli, Klebsiella pneumonia* and their respective ATCC control strains. Pathogenic *Candida albicans*, *Candida parapsilosis*, and *Candida tropicalis* were also tested. Results of the present investigation provide evidence that the EO of *Cymbopogon citratus* could have a potential application in the treatment and prevention of diseases caused by *Staphylococcus aureus* methicillin-resistant strains and by vancomicin-resistant *Staphylococcus epidermidis*. *Cymbopogon citratus* EO is also effective against *Candida albicans* as well as the emerging *Candida parapsilosis* and *Candida tropicalis* pointing to its usefulness as an antifungal agent.

Keywords Antioxidant*;* non-*albicans*-*Candida* species; Gram-positive bacteria; *Cymbopogon citratus* EO; multi-drug resistant bacteria.

1. Introduction

Plants have been explored comprehensively as potential sources of antioxidants and antimicrobials [1,2]. The essential oil (EO) of *C. citratus* is often applied in pharmaceutical industry to supply flavor and fragrance, and is also used as a source of new phytochemical molecules for the development of novel pharmaceutical products. Several previous studies on the leaf EO of *C. citratus* revealed antityrosinase and antioxidant activity in human cells [3], anti-inflammatory activity in rats [4], anti-carcinogenic effects in mice [5], anxiolytic-like effect in mice [6], and cholesterol reduction in mice [7]. Furthermore, numerous studies have reported the antimicrobial activity of lemongrass oil against a diverse range of microorganisms comprising Gram-positive and Gram-negative bacteria, yeast and fungi [8,9,10]. Antimicrobial substances other than antibiotics would be remarkably useful as an adjuvant in the treatment of multi-resistant strains [11].

The rapid adaptation of pathogenic bacteria to antimicrobial drugs in health care facilities, alongside delayed pharmaceutical drug development, has established the need of new molecular approaches. Treating inpatients at health institutions is becoming progressively defiant as new antibiotic resistant strains of bacteria and yeasts emerge. To overtake this problem it becomes an urgent issue to test new alternatives in antibiotherapy: either the production of new drugs or of adjuvants that enhance antimicrobial activity.

Multi-drug resistant microbial infections caused by Gram-positive bacteria such as *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis (S. epidermidis)* represent an exponentially increasing problem that affects communities worldwide. *S. aureus* is an opportunistic pathogen able to colonize the upper respiratory tract and skin surfaces in mammals [12]. *S. aureus* is a common pathogen present in community-acquired infections, most particularly skin, soft tissue and respiratory infections and after surgical procedures and methicillin resistant *S. aureus* nosocomial infections [13]. By analyzing the literature of *S. aureus* one can predict that these bacteria will continue to develop not only new virulence but also novel patterns of resistance [14].

S. epidermidis is present on the human skin as normal flora. In hospital environment this bacteria has become a problematic pathogen, implicated in health care-associated septicemia, including infections related to vascular catheters and prosthetic devices. Several studies have demonstrated that certain multidrug-resistant *S. epidermidis* genotypes become established as opportunistic pathogens in the health care setting as a novel ecological niche [15].

Gram-negative bacteria may be opportunistic or commonly pathogenic. They cause a wide variety of infections including low and high urinary tract infections, pneumonia and skin and soft tissue infections, among others. The avaliable data also refers the emergence of increasingly resistant strains of various Gram-negative bacteria [16].

Candida is a normal commensal of the skin, gastrointestinal and genitourinary tracts but it is also responsible for the most prevalent opportunistic fungal infection in humans, and this is of particular significance in patients that present risk factors such as immunosuppression while undergoing treatment for cancer [17], organ transplantation [18], receiving broad-spectrum antibiotics, suffering from acute renal failure, previous yeast colonization, neutropenia, parenteral nutrition, and central venous catheters [19]. *Candida albicans* (*C. albicans*) is the most frequent species isolated from clinical specimens, but other *Candida* species are of special concern, since some are highly virulent and are associated with treatment failure due to reduced susceptibility to antifungal agents. Many non-*albicans*-*Candida* (NCA) species, such as, *Candida parapsilosis* (*C. parapsilosis*) and *Candida tropicalis (C. tropicalis)* have recently emerged as important pathogens in immune suppressed individuals. This fact is highlighted by recent epidemiology studies reporting that NCA species are currently estimated to be responsible for approximately 60% of fungaemia [20].

We studied the antibacterial and antifungal activities of *C. citratus*. The fact that *C. citratus* EO has antimicrobial activity in bacteria and yeast prompted us to study the effect of this oil on multidrug resistant bacteria and yeasts.

2. Material and Methods

2.1. Plant source

The fresh aerial parts of *C. citratus* were obtained from a local market in Benguela, (latitude 12º 58' south; longitude 13º 408' east), Angola. The voucher specimen was deposited in the Instituto Superior of Saúde de Benguela, ISPB, Benguela, and plants were identified by a taxonomist (Dr. Pedro Catarino Pires, ISPB, Benguela). The fresh plant sample was submitted to distillation (4 h) using a Clevenger apparatus to obtain the EO for further analysis. The yield in EO was of 1.3%.

2.2. Antibacterial activity

2.2.1. Microorganisms

The tested microorganisms used in this study were obtained from the Culture Collections of the Centro de Investigação em Tecnologias da Saúde (CITS). The antimicrobial activity of *C. citratus* EO was evaluated using the following laboratory control strains: *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *E. coli* ATCC 25922, *Klebsiella. pneumonia* (*K. pneumonia*) ATCC 13883, *C. albicans* ATCC 10231, *C. parapsilosis* ATCC 2219 and *C. tropicalis* ATCC 750 and the antibiotic resistant strains isolated from Braga hospital: *S. aureus* 1; *S. aureus* 2; *S. epidermis* 1; *S. epidermis* 2; *E. coli* 830; *E. coli* 986; *K. pneumonia* 822,were a kind gift from Dr. Luisa Mesquita.

2.2.2. Propagation and maintenance of microorganism

The studied microorganisms were streaked on the nutrient agar slants and were then incubated overnight at (37±1 ºC). The cultures were kept under refrigerated conditions and were sub-cultured every fifteen days.

2.2.3. *In vitro* testing

Empty sterilized discs were impregnated with 5µl of oil diluted with DMSO to attain the different concentrations (5%, 10%, 20%, 40%, 60%, 80% and 100%), and DMSO was used as control. The inoculated plates were incubated at 37ºC for 24 to 48 hours. Antibacterial activity was evaluated by measuring the zone of inhibition in mm against the tested bacteria after the incubation period. All experiments were done in triplicate and the results are expressed as mean±standard deviation of three independent experiments.

2.2.3.1. *In vitro* antibacterial activity

Antibacterial activity was determined by disk diffusion. A positive control was done using the following antibiotics: methicillin (5µg/disc); penicillin (10µg/disc); amoxicillin/clavulanic acid (30µg/disc); vancomycin (5µg/disc) in Gram-positive strains and ciprofloxacin (5µg/disc), nitrofurantoin (300µg/disc), ceftazidime (30µg/disc); gentamicin (10µg/disc) in Gram-negative strains and a negative control was performed with DMSO for all tested strains. The antibacterial activity of all the tested antibiotics was interpreted according to the CLSI guidelines [21].

2.2.3.2. *In vitro* antifungal activity

Antifungal activity was also evaluated by disk diffusion method in accordance to CLSI guidelines M44-A2. *Candida* spp. were tested in agar Miller Hinton (DIFCO) supplemented with 2% of glucose and 0.5g/ml of methylene blue. Yeast suspension was adjusted to a final concentration between 1 to 5 x10^6 cells/ml and incubated at 35ºC for 48 hours. The antifungal fluconazol at concentration of 5µg per disc was the positive control for *Candida* spp. Results were interpreted based on CLSI guidelines [22].

2.3. Statistical analysis

Data are reported as mean±standard deviation of nine measurements. Statistical analysis was performed using the statistical package SPSS v 20.0 (SPSS for Windows; SPSS Inc., Chicago, IL). Mean comparison was made through an independent sample t-test. Levene's test was utilized to assess the equality of variances. One-way ANOVA was used to compare three or more groups, and post-hoc Dunnett's test was performed for simultaneous paired comparisons. P values less than 0.05 (95% confidence level) are reported as statistically significant.

3. Results and Discussion

3.1. *C. citratus* EO chemical composition

The results of the chromatographic analysis of the C. citratus EO presented in Table 1 reveal that the chemical composition of C. citratus EO obtained from plants grown in Angola is similar to that of *C. citratus* from other provenances [23, 24, 25]. Accordingly, the major constituents are α-citral (40.55%), β-citral (28.26%), myrcene (10.50%) and geraniol (3.37%). The volatile fraction of *C. citratus* EO revealed the presence of 10 different compounds accounting for 83.86% of total peak area.

Table 1 Angolan *C. citratus* EO main components as revealed by GC-MS.

Components	RI[a]	Peak area (%)
6-methyl-5-hepten-2-one	8.725	0.97
Myrcene	8.875	10.50
α-(Z)-Ocymene	10.533	0.22
β-(E)-Ocymene	10.917	0.27
Linalool	12.883	0.50
Citronelal	14.250	0.11
β-citral	18.025	28.26
Geraniol	18.458	2.37
α-citral	19.142	40.55
Linalool isobutyrate	22.967	0.11

[a]Retention index relative to n-alkanes.

3.2. Antibacterial activity of *C. citratus* EO

The antibacterial activity of *C. citratus* EO is depicted in Figures 1 and 2. Hospital isolated *S. aureus* 1 and *S. aureus* 2 were sensitive to *C. citratus* oil concentration equal or higher than 60%. *S. aureus* ATCC 25923 growth was inhibited at lower concentrations equal or superior to 20%. *S. epidermidis* ATCC 12228 and hospital isolated strain presented a similar pattern of sensitivity at concentrations equal or higher than 20%. Both hospital isolated strains of *S. aureus* present a similar pattern of resistance to methicillin, penicillin and amoxicillin-clavulamic acid consistent with methicillin resistant profile. *S. aureus* (ATCC 25923) presented the expected sensitivity to tested antibiotics that usually present inhibitory effect on Gram-positive bacteria (Table 2).

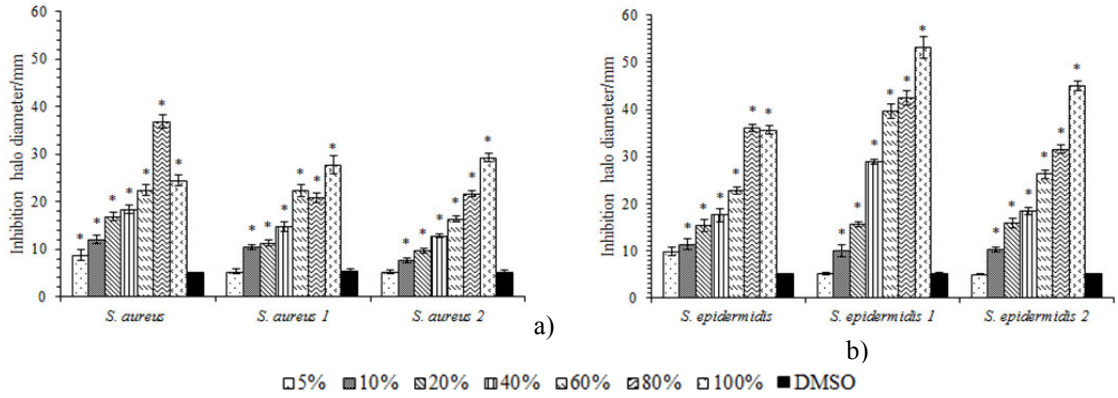

Fig. 1 Antimicrobial activity of different EO concentrations of *C. citratus* against ATCC and multidrug resistant Gram-positive hospital isolated strains. *Statistically different (95% significance) from negative control DMSO.

S. epidermidis 1 was resistant to methicillin, penicillin and amoxicillin-clavulamic acid and vancomycin consistent also with the methicillin resistant profile. *S. epidermidis 2* was sensitive to amoxicillin-clavulamic acid and resistant to

penicillin, methicillin and vancomycin. By comparison *S. epidermidis* ATCC 12228 was sensitive to all antibiotics tested for Gram-positive bacteria.

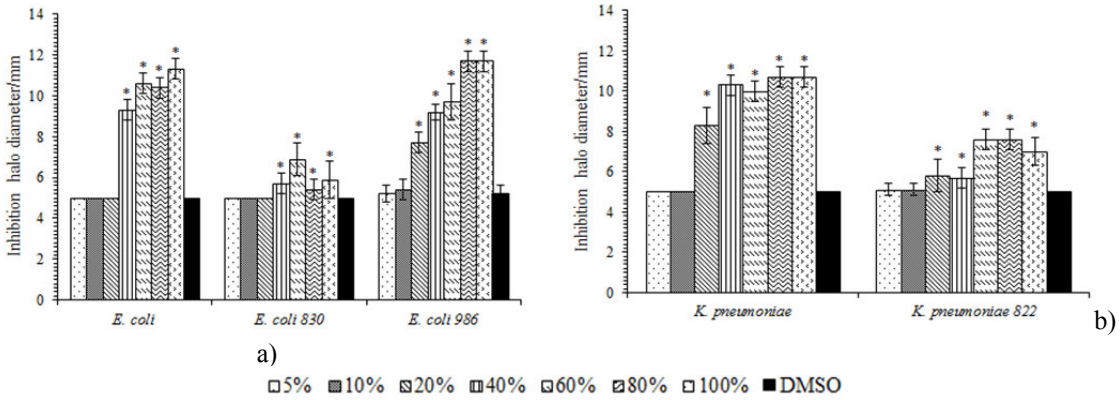

Fig. 2 Antibacterial activity of different EO concentrations of *C. citratus* against ATCC and multidrug Gram-negative resistant strains. *Statistically different (95% significance) relatively to negative control DMSO.

Table 2 Antibiotic sensitivity pattern of multi-drug resistant hospital isolated strains and their respective ATCC control strains.

Gram-positive Bacteria	MET	P	AMC /AUG	VA	*C. citratus* concentration (%)
**S. aureus*	S	S	S	-	S ≥ 20
S. aureus $_1$	R	R	R	-	S ≥ 40
S. aureus $_2$	R	R	R	-	S ≥ 60
**S. epidermidis*	S	R	S	S	S ≥ 20
S. epidermidis $_1$	R	R	R	R	S ≥ 20
S. epidermidis $_2$	I	R	S	R	S ≥ 20

Sensitivity values: R – Resistant; I – Intermediate; S – Sensitive. (-) not determined; MET-methilcillin; P-penicillin; AMC/AUG-amoxicillin/clavulamic acid; VA-vancomycin (from CLSI guidelines).

Gram-negative *E. coli* 830, *E. coli* 986 and *K. pneumoniae* 822 presented an intermediate resistance pattern with growth inhibition at *C. citratus* EO concentrations equal or superior to 40%. In *E. coli* 986 and *K. pneumonia* 822 this pattern was observed in concentrations equal or superior to 20%. This higher resistance pattern, comparatively with that of Gram-positive bacteria could be due to the constitution of the outer membrane of Gram-negative bacteria that acts as a relatively effective permeability barrier [26]. Gram-negative bacteria are inherently resistant to hydrophobic antibiotics, as their outer membrane limits the entry of these antibiotics into the cell [27]. In fact we can hypothesize that the *C. citratus* EO is less effective against Gram-negative bacteria because of the out membrane barrier that these bacteria present to hydrophobic molecules [16].

These results are in accordance to the data of Onawunmi et al. that uses components of the EO *C. citratus*, namely alpha-citral and beta-citral that individually presented antibacterial activity against Gram-negative [28].

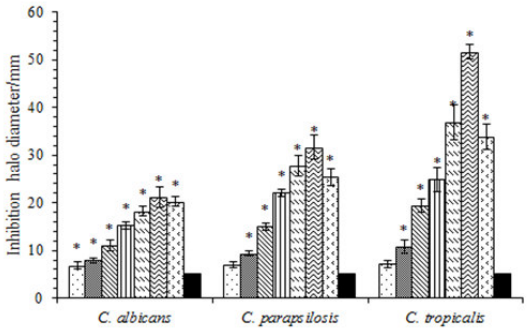

Fig. 3 Antifungal activity of different *C. citratus* EO concentrations against *Candida* spp. * statistically different (95% significance) from negative control DMSO.

3.3. Antifungal activity of *C. citratus* EO against *C. albicans* and NAC species

C. citratus EO showed effectiveness in inhibiting the growth of all yeast strains, as is shown in Figure 3. Presented data show the antifungal activity at different *C. citratus* EO concentrations against *Candida* spp. This sensitivity was also shown by these yeast to fluconazol. No effect in yeast growth was shown with DMSO.

NCA species exhibited sensitivity to all *C. citratus* concentrations tested. *C. albicans* was sensitive to *C. citratus* oil inhibition at all tested concentrations. This data is in accordance with other studies [29, 30]. *C. parapsilosis* has a pathogenic role in neonates, transplant recipients and patients receiving parenteral nutrition [31]. Our results show that this species is sensitive to all tested *C. citratus* concentrations. These data demonstrate high antifungal activity over *C. parapsilosis*, which is an innovative role for *C. citratus*.

C. tropicalis causes infection in neutropenic patients as occurs in malignancy [32]. *C. tropicalis* is the most prevalent of the NAC species, the number of infections of this specie is increasing higher as is its resistance to fluconazol [33]. Our results show that this species is sensitive to all *C. citratus* concentrations tested, being the sensitivity to 80% of this oil concentration higher in *C. tropicalis*. These data demonstrate antifungal activity over *C. tropicalis*, which is also an innovative role for *C. citratus* (Figure 3).

Our results prove the activity of *C. citratus* EO on *C. parapsilosis* and *C. tropicalis* and this suggests further studies in order to establish the sensitivity of other clinically relevant NCA species.

This evidence shows an antifungal role of *C. citratus* EO, which should be considered in further investigations. The aim should be establishing this extract as an antifungal agent with potential use in common *Candida* infections. Our data show a potential clinical role of this EO in topical therapeutics of NCA species. Published studies show that this EO is innocuous in mammals [34] and has shown lack of genotoxic or toxic effects in mice [7, 35]. Studies with the infusion of *C. citratus* in human volunteers have shown no toxic nor adverse effects [35].

4. Conclusions

This oil exhibited high antifungal and antibacterial properties. Although the *C. citratus* EO inhibition was higher in Gram-positive bacteria an effect in Gram-negative bacteria was also shown. At the tested concentrations of the EO we obtained an extensive *Candida* spp. inhibition. This effect was particularly pronounced in NCA species. *C. citratus* EO activity over Gram-positive multi-drug-resistant bacterial strains and over *C. albicans* and NAC species indicates a potential role in preventing spreading of hospital infections, namely by its use as an antiseptic for topical and for hand cleaning. Further studies are needed to establish the possibility of the systemic administration of this natural medicine.

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