

Evaluation of Antioxidant and Antimicrobial Properties of the Angolan *Cymbopogon Citratus* Essential Oil With a View to Its Utilization as Food Biopreservative

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Abstract

It was studied the chemical composition, antioxidant, antibacterial and antifungal properties of the essential oil obtained from the *Cymbopogon citratus* of Angolan origin. Its major constituents analyzed by GC-MS were α -citral (40.55%), β -citral (28.26%), myrcene (10.50%) and geraniol (3.37%). The essential oil antioxidant capacity was statistically identical to that of synthetic antioxidants (DPPH IC₅₀ of 41.7 μ g/ml) and superior to that of extracts obtained from fresh leaves of the plant (DPPH IC₅₀ of 55.7 μ g/ml). The oil also demonstrated to possess high antibacterial activity even against multidrug resistant strains of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and *Klebsiella pneumoniae* and antifungal activity against *Candida albicans* and Non-*Candida albicans*, *Candida parapsilosis* and *Candida tropicalis*. The results of this study, in conjunction with already published data on the properties of other *Cymbopogon citratus* oils, provide evidence that it could have a potential application as food preservative.

Keywords: *Cymbopogon citratus*, essential oil, antioxidant, antibacterial, antifungal, biopreservative food

Abbreviations

AMC/AUG, amoxicillin/clavulamic acid; ATCC, American Type Culture Collection; BHT, butyl hydroxyl toluene; DMSO, dimethyl sulphoxide; DPPH, 2,2'-diphenyl-1-picrylhydrazyl; EO, essential oil; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; IC₅₀, half maximal inhibitory concentration; MET, methicillin; NCAC, non-*Candida albicans Candida*; P, penicillin G; VA, vancomycin.

1. Introduction

The use of essential oils (EOs) as additives in order to simultaneously tackle two issues related with food preservation, oxidation and microbial contamination, is an approach that gained adherents in recent years. This resulted from the realization that these natural products appeared to bring together the desired characteristics for that purpose, including, atoxicity at low concentrations, antioxidant activity, i.e. the ability to inhibit the process of food oxidation, antimicrobial properties and biodegradability. Hence it is not surprising the active research that is being conducted on the subject in order to uncover the properties of the thousands of EOs available (Lang & Buchbauer, 2012). Obviously, the higher the antioxidant activity demonstrated and the greater the spectrum of activity of the EO, the better will be its performance as preservative. It is also desirable that the addition of oil does

not change the organoleptic characteristics of the food. Interesting results have already been reported with EOs from various plants (Hyldgaard et al., 2012; Sessou et al., 2012). This paper describes the research done on the EO of *Cymbopogon citratus* (lemongrass) of Angolan origin. The EO of *C. citratus* is already in use by the pharmaceutical industry as a source of new phytochemical molecules for the development of new drugs. Previous studies on the leaf EO of *C. citratus* revealed, antityrosinase and antioxidant activities in human cells (Saeio et al., 2011), anti-inflammatory in rats (Gebnou et al., 2013), anti-carcinogenic effects and cholesterol reduction in mice (Costa et al., 2011; Bidinotto et al., 2012). Furthermore, many studies have reported the antimicrobial activity of lemongrass oil originating from different parts of the globe against a diverse range of microorganisms comprising Gram positive and Gram negative bacteria, yeast and fungi namely *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae* (Naik et al., 2010), *Salmonella choleraesuis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* (Falcão et al., 2012) and *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Alternaria alternata*, *Penicillium citrinum*, *Curvularia lunata* and *Trichoderma harzianum* (Mahanta et al., 2007). In the work referred to in this article the objective was to evaluate the antimicrobial activity of the *cymbopogon citratus* on multidrug resistant strains of *Staphylococcus (aureus and epidermidis)* *Escherichia coli*, *Klebsiella pneumoniae* and fungi (*Candida albicans*, *tropicalis* and *parapsilosis*). All these microorganisms may be food-borne and by this route represent a severe public health problem in large scale (Easa, 2010). Food-borne diseases caused by bacteria and yeast are of major concern worldwide even in developed countries were up to 30% of the population have been reported to suffer from them every year. The severity of the problem might become more pronounced due to the proliferation of antibiotic-resistant strains (McAdam et al., 2012). Among the predominant bacteria involved in food-borne diseases, *Staphylococcus aureus* is a leading cause of gastroenteritis resulting from the consumption of a food in which enterotoxigenic staphylococci have grown and produced toxins. These are considered a potential biological threat because of their stability at high temperatures (100°C for 1 h). While not regarded as highly lethal agents due to the low mortality associated with the illness, staphylococcal enterotoxins can incapacitate individuals for two weeks (Schelin et al., 2011). Moreover it is predictable that *S. aureus* will continue to develop new virulence characteristics and new patterns of synthetic antibiotic resistance (Fry et al., 2013). A similar drug resistance phenomenon is supposedly occurring with *S. epidermidis* (Widerström et al., 2012). *E. coli* is reported to cause bloody diarrhea and can sometimes cause kidney failure and even death. *K. pneumoniae* is second only to *E. coli* as a urinary tract pathogen and cases of enteroinvasive *Klebsiella pneumoniae* and *Escherichia coli* sepsis from contaminated hamburgers are documented (Sabota et al., 1998; Kim et al., 2005). As in the case of bacteria previously mentioned, *Klebsiella* infections are encountered far more often now than in the past probably due to the emergence of increasingly resistant strains (Saha et al., 2008). Apart from bacteria, fungal contamination of food is practically inevitable. *Candida* as many other yeasts and molds is undesirable in many foods mainly due to its food spoiling activity, nevertheless, dangerous levels of *candida* have been found in yoghurt (Montagna et al., 1998). *Candida* spp. is responsible for the most prevalent opportunistic fungal infections in humans and of particular significance in immunocompromised individuals (Muñoz et al., 2006; Rodloff et al., 2011).

Additionally, and as this is an important characteristic for a food preservative, the antioxidant capacity of the Angolan *Cymbopogon citratus* EO was evaluated and compared with that of synthetic antioxidants. The use of natural substances to replace the synthetic antioxidants in food industry can result in important health benefits, since the latter have been associated with genotoxic and carcinogenic effects (Kahl et al., 1993, Ndhlala et al., 2010).

2. Material and Methods

2.1 Plant Source

The fresh aerial parts of *C. citratus* were obtained from a local market in Benguela, (latitude 12°58' south; longitude 13°408' east), Angola. The plants were identified by a taxonomist (Dr. Pedro Catarino Pires, ISPB, Benguela) and deposited in the Instituto Superior de Saúde de Benguela, ISPB, Benguela. The fresh plant sample was submitted to distillation (4 h) using a Clevenger apparatus to obtain the EO for further analysis. The procedure followed was that described in the European Pharmacopoeia (2011). The yield in EO was of 1.3%, corresponding, on average, to 1.5 ± 0.4 ml of EO per 111 g of fresh plant sample. A total of six samples of fresh plant, purchased in different days were used to obtain the six samples of EO.

2.2 Isolation, Characterization and Identification of Constituents

The EO was analyzed by GC-MS carried out in a Hewlett-Packard 6890 gas chromatograph fitted with a HP1 fused silica column, interfaced with an Hewlett-Packard mass selective detector 5973 (Agilent Technologies) operated by HP Enhanced Chem-Station software, version A.03.00. Components were identified by their retention

indices on both SPB-1 and SupelcoWax-10 columns and from their mass spectra. Retention indices, calculated by linear interpolation relative to retention times of C8-C23 of n-alkanes, were compared with those of authentic standards included in our own laboratory database.

2.3 Total Phenolic Contents

The amount of total phenolic compounds was determined in the aerial parts of *C. citratus* according to Jang et al. (2007) using the Folin-Ciocalteu reagent. Each sample was extracted using methanol, for one hour. In brief, 2.5 g of a dried leaves sample was added to 100 ml of the solvent, under constant agitation for 1 hour. Afterwards, the solid was separated from the extract through vacuum filtration and a volume of 0.5 ml of each extract was added to 0.5 ml of Folin-Ciocalteu reagent. The mixture was left to rest for 3 min at room temperature before 1.0 ml of sodium carbonate 6% was added to it. After standing at room temperature for 120 min, absorbance readings were performed at 720 nm using a UV-Vis spectrophotometer (Beckman DU-64 spectrophotometer, Beckman Instruments Inc., Fullerton, CA). Total phenolics were quantified by means of a calibration curve obtained from measuring the absorbance of gallic acid standards. Results were expressed as milligrams of gallic acid equivalents per 100 g of fresh sample weight.

2.4 Antioxidant Activity

The antioxidant activity of *C. citratus* EO was evaluated by measuring the bleaching of the purple-colored ethanolic solution of 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH[•]). This methodology was performed using an ELX80 Microplate Reader (Bio-Tek) in the following way: the reaction mixture in each one of the 96-wells consisted of one of the different concentration of EO (20 µL) and 280 µL methanolic solution of DPPH radical (0.5 mM). After 30 minutes incubation, the changes in color (from deep-violet to light-yellow) were measured at 517 nm (Shimadzu, model UV-1800), according to the method previously reported (Brand-Williams et al., 1995). The butyl hydroxyl toluene (BHT) was used as a positive control while ethanol was the negative one. The percentage of inhibition (*I*%) of DPPH radical was computed through the equation:

$$I\% = \frac{A_{DPPH} - A_s}{A_{DPPH}} \times 100 \quad (1)$$

where, A_s represents the absorbance of the solution of the sample extracts containing DPPH[•], and A_{DPPH} is the absorbance of the DPPH[•] solution. All determinations were performed in triplicate. The concentration of extract sample required for 50% inhibition of the DPPH free radical (IC_{50}) was also calculated.

2.5 Antibacterial Activity

2.5.1 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: *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Candida albicans* (ATCC 10231), *Candida parapsilosis* (ATCC 2219) and *Candida tropicalis* (ATCC 750) and the antibiotic resistant strains isolated from Braga Hospital (Portugal, GPS: N 41°34' W 8°24'): *Staphylococcus aureus* 1 (*S. aureus* 1); *Staphylococcus aureus* 2 (*S. aureus* 2); *Staphylococcus epidermidis* 1 (*S. epidermidis* 1); *Staphylococcus epidermidis* 2 (*S. epidermidis* 2); *Escherichia coli* 830 (*E. coli* 830); *Escherichia coli* 986 (*E. coli* 986); *Klebsiella pneumoniae* 822 (*K. pneumoniae* 822).

2.5.2 Propagation and Maintenance of Microorganisms

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

2.5.3 In Vitro Testing

Empty sterilized discs were impregnated with 5µl of oil diluted with DMSO to obtain different concentrations (5%, 10%, 20%, 40%, 60%, 80% and 100%). DMSO was used as negative control. The inoculated plates were incubated at 37°C for 24 or 48 hours for bacterial and fungal assessment, respectively. Antibacterial activity was evaluated by measuring the zone of inhibition in millimeters after the incubation period. All experiments were done in triplicate and the results are presented as mean±standard deviation of three independent experiments.

2.5.3.1 In Vitro Antibacterial Activity

Antibacterial activity was determined by the disk diffusion method (Bauer et al., 1996). A positive control was done using the following antibiotics: methicillin (5 µg/disc); penicillin (10 µg/disc); amoxicillin/clavulanic acid

(augmentin) (30 µg/disc); vancomycin (5 µg/disc) in Gram positive strains and ciprofloxacin (5 µg/disc), nitrofurantoin (300 µg/disc), ceftazidime (30 µg/disc); gentamicin (10 µg/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 (2012).

2.5.3.2 *In Vitro* Antifungal Activity

Antifungal activity was also evaluated by the 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 0.5 g/ml of methylene blue. Yeast suspension was adjusted to a final concentration between 1 and 5 x 10⁶ cells/ml and incubated at 35°C during 48 hours. The antifungal fluconazole, at a concentration of 5µg/disc, was the positive control to *Candida* spp. Results were interpreted according to CLSI guidelines (2012).

2.6 Statistical Analysis

Data are reported as mean±standard deviation of nine measurements. Statistical analysis was performed using the statistical software 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

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 plants from other provenances (Sessou et al., 2012; Tajidin et al., 2012; Masamba et al., 2003). 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 |

^aRetention index relative to n-alkanes.

The preservation of food generally requires the use of various compounds to perform a range of functions namely antioxidants, antibacterial and antifungals. The ideal would be to find a single biopreservative that could perform all these functions simultaneously. In order to assess the antioxidant potential of the *C. citratus* EO, its free radical scavenging activity by the DPPH assay was measured (Figure 1).

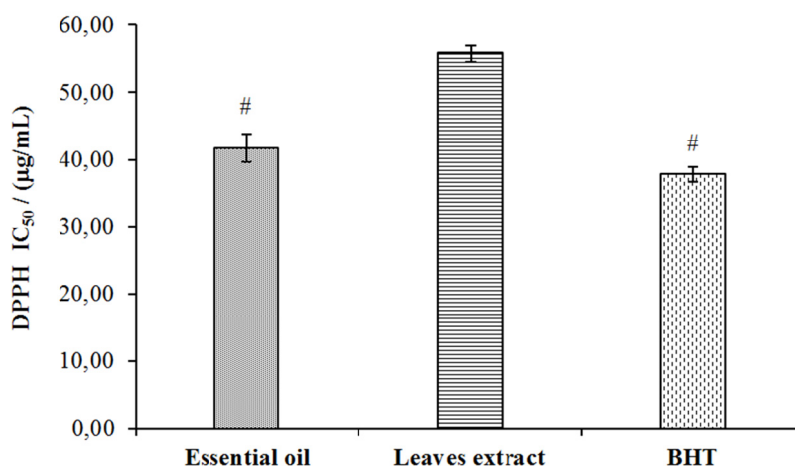


Figure 1. Free radical scavenging activities of the EO and methanolic extract obtained from the leaves of Angolan *C. citratus* versus BHT. #Statistically identical (95% significance)

Our results showed that no statistical significant differences exist between the free radical scavenging activities of the EO and butylated hydroxytoluene (BHT). The EO was able to reduce the stable free radical 2,2'-diphenyl-1-picrylhydrazyl to diphenylpicrylhydrazine with an IC₅₀ of 41.7 µg/ml, a value similar to that of the synthetic antioxidant (37.7 µg/ml). The dried leaves methanolic extract was found to be less efficient in radical scavenging with an IC₅₀ value of 55.7 µg/ml. The bioactive compounds found in vegetables do not all have the same antioxidant activity, in fact the phenolic compounds are known to have higher antioxidant activity than monoterpenoids (Gharib & Silva, 2013). The total phenolics contents found in the leaves were 578.32±0.60 mg/kg, nonetheless it is well known fact that for a complex extract, as the one in question, it is also necessary to take into account the synergistic or antagonistic effects among the various compounds present, which makes the antioxidant activity not only dependent of the concentration of each compound but also of the interaction between different compounds, antioxidants or not (Sanjust et al., 2008). Thus, an increase in the level of a compound does not mean a proportional increase of antioxidant activity of the matrix. We attribute the high antioxidant activity manifested by the EO to the synergic action of the monoterpenoid compounds such as α-citral, β-citral, and myrcene (Ruberto & Baratta, 2000). The chromatographic analysis (Table 1) reveals that these are abundant in the EO.

C. citratus EO was found to have greater antioxidant activity than methanolic extracts obtained from the leaves and similar to that exhibited by the synthetic antioxidant BHT. In this respect, however, the oil possesses an important advantage over BHT: due to their natural origin, the antioxidants obtained from plants do not induce the negative side effects provoked by synthetic ones, such as BHT, which has proved to cause hemorrhaging (Ndhala et al., 2010).

The antibacterial activity of the EO of the Angolan *C. citratus* is depicted in Figures 2 and 3.

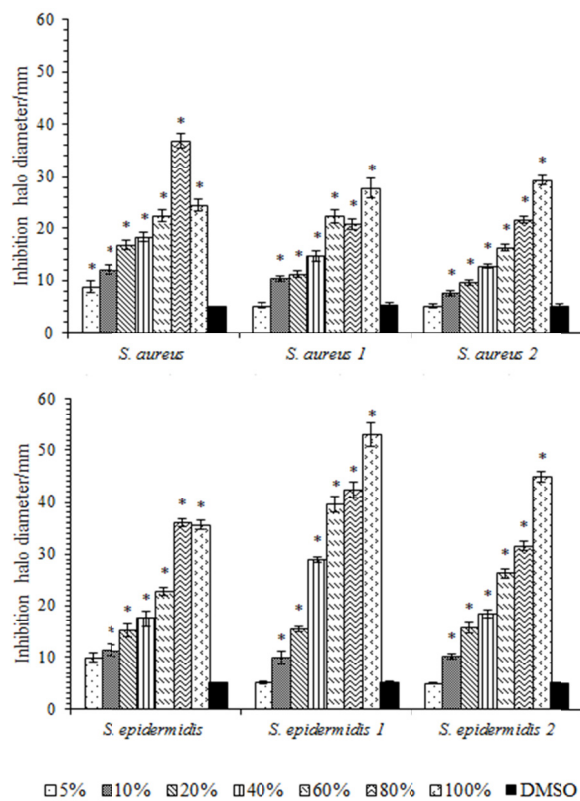


Figure 2. Antibacterial activity of different EO concentrations of *C. citratus* against Gram positive ATCC and multidrug resistant strains. *Statistically different (95% significance) relatively to negative control DMSO

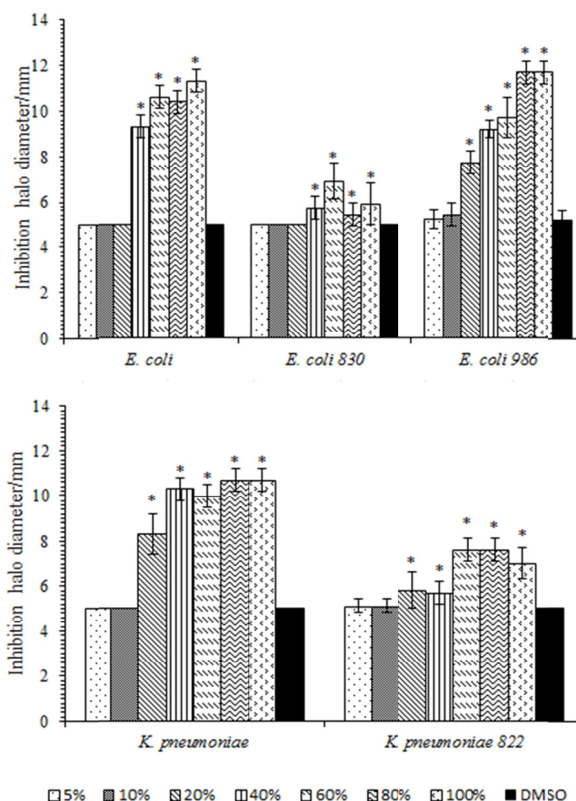


Figure 3. Antibacterial activity of different EO concentrations of *C. citratus* against Gram negative ATCC and multidrug resistant strains. *Statistically different (95% significance) relatively to negative control DMSO

It can be seen that hospital isolated *S. aureus* 1 and *S. aureus* 2 were sensitive to *C. citratus* EO at concentrations equal or superior to 60% while *S. aureus* ATCC 25923 growth was inhibited at even lower concentrations ($\geq 20\%$). *S. epidermidis* ATCC 12228 and the hospital isolated strains *S. epidermidis* 1 and *S. epidermidis* 2 had a similar pattern of sensitivity at EO concentrations equal or above 20%. These results are, in our view, relevant since they demonstrate the potential of the Angolan *C. citratus* EO as an antibacterial agent even against multidrug resistant strains. In order to better understand the effectiveness of the EO, the antibacterial activity of antibiotics in use against the strains studied was also evaluated. The results are summarized in Table 2.

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

| Bacteria (Gram +) | MET | P | AMC/AUG | VA | <i>C. citratus</i> EO concentration (%) |
|-------------------------|-----|---|---------|----|---|
| * <i>S. aureus</i> | S | S | S | - | $S \geq 20$ |
| <i>S. aureus</i> 1 | R | R | R | - | $S \geq 40$ |
| <i>S. aureus</i> 2 | R | R | R | - | $S \geq 60$ |
| * <i>S. epidermidis</i> | S | R | S | S | $S \geq 20$ |
| <i>S. epidermidis</i> 1 | R | R | R | R | $S \geq 20$ |
| <i>S. epidermidis</i> 2 | I | R | S | R | $S \geq 20$ |

Sensitivity values: R-Resistant; I-Intermediate; S-Sensitive. MET-Methicillin; P-Penicillin; AMC/AUG-Amoxicillin/Clavulamic acid; VA-Vancomycin (from CLSI guidelines).

Both hospitalar isolated strains of *S. aureus* presented an analogous pattern of resistance to methicillin, penicillin and amoxicillin-clavulamic acid, consistent with methicillin resistant profile. *S. aureus* (ATCC 25923) presented the expected sensitivity relatively to the tested antibiotics that usually have inhibitory effect on Gram positive bacteria (Table 2). *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.

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% (Figure 3). In the case of *E. coli* 986 and *K. pneumoniae* the inhibition effect of the EO started at inferior concentrations ($\geq 20\%$).

The results thereby suggest that the oil is more effective on Gram positive bacteria. The higher resistance manifested by the Gram negative bacteria may be associated to the constitution of its outer membrane which acts as a relatively impermeable barrier (Nikaido, 2003). Gram negative bacteria are inherently resistant to hydrophobic antibiotics, as their outer membrane limits the entry of these antibiotics into the cell (Poole, 2002). Thus we can hypothesize that the *C. citratus* EO is less effective against Gram negative bacteria due to the same reason (Saha et al., 2008).

In the last decades, fungi have emerged as major causes of human disease, with invasive candidiasis (IC) as a leading cause of mycosis-associated mortality in developed countries (Pfaller & Diekema, 2007). *Candida albicans* (*C. albicans*) is the most frequent species isolated, but other *Candida* are of special concern, due to abnormal virulence and reduced susceptibility to antifungal agents. *C. parapsilosis* and *C. tropicalis* are the most prevalent NCAC species with a crescent number of infections (Trofa et al., 2008; Kothavade et al., 2010). The *C. citratus* EO showed effectiveness in inhibiting the growth of all yeast strains studied as shown in Figure 4. This *Candida* also revealed sensitivity to fluconazol. No effect on yeast growth was detected when exposed to DMSO.

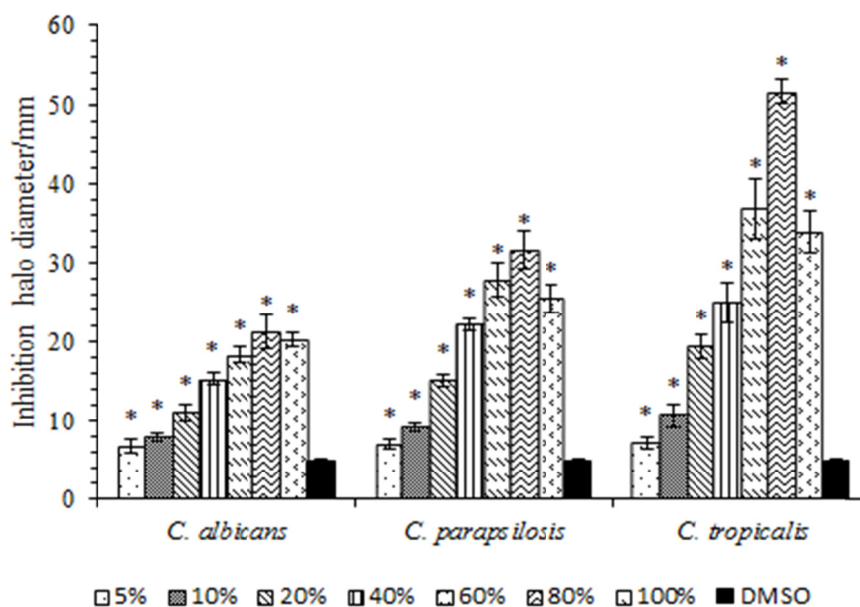


Figure 4. Antifungal activity of different *C. citratus* EO concentrations against *Candida* spp. * statistically different (95% significance) from negative control DMSO

Data on *C. albicans* is in accordance with other studies (Irkin et al., 2009; Tyagi et al., 2010) but, to the best of our knowledge, this is the first reported data on EO antifungal activity on the NCAC. The fact that EO has proved effective against NCAC at all concentrations tested is the more relevant as recent epidemiologic studies report that NCAC are currently estimated to be responsible for approximately 60% of fungaemia (Silva et al., 2009). These results put in evidence the antifungal activity of *C. citratus* EO, which should be considered in further investigations namely in order to assess the sensitivity of other NCAC.

4. Conclusions

The Angolan *C. citratus* EO was found to have greater antioxidant activity than methanolic extracts obtained from the leaves and similar to that exhibited by the synthetic antioxidant BHT. The EO exhibited high antibacterial and anti-yeast properties including against multidrug resistant strains of *S. aureus*, *S. epidermidis*, *E. coli*, *K. pneumonia* and NCAC spp *C. parapsilosis* and *C. tropicalis*. Taking into account the results of this study and the already reported antimicrobial activity of the *C. citratus* EO against many other pathogenic or spoilage microorganisms, it can be concluded that it meets several features that make it a good candidate for the biopreservation of food.

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