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Science

ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS OF *SCHINUS TEREBINTHIFOLIUS* AND *EUGENIA UNIFLORA* UPON *CANDIDA TROPICALIS* STRAINS

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ABSTRACT The essential oils of herbal medicines are known to display pharmacologic actions, in the cell wall of fungus. This study was performed in order to identify new bioagents with microbicidal activity *in vitro*. We approached the effects of cultivated plant extracts upon a fluconazole-resistant (ATCC 750) and a fluconazole-sensitive (ATCC 18803) strains of *C. tropicalis* which were incubated with the essential oils of *Schinus terebinthifolius* and *Eugenia uniflora*, for 24 hours and analyzed by MTT method. Assessment of cytotoxicity on murine splenocytes was measured by [^{3}H] Thymidine incorporation and flow cytometry using rhodamine 123 probe, respectively. The structural changes were analyzed by scanning electron microscopy. Secondary metabolites present in the extracts were identified by gas chromatography. The concentrations of essential oils showed significantly (p <0.05) inhibited the viability of strains. GC-MS tests identified the presence of active substances recognized for its antimicrobial activity. Flow cytometric assays showed that the extracts-treated strains had changes in mitochondrial integrity as compared to the Carbonyl cyanide m-chlorophenylhydrazone (CCCP). Scanning electron microscopy showed that exposure to the essential oils caused remarkable structural changes with cell surface. These results suggest that the *S. terebinthifolius* and *E. uniflora* essential oils may be considered promising for antifungal purpose for candidiasis.

KEYWORDS : *Candida tropicalis,* Natural products, *Medicinal plants, Schinus terebinthifolius, Eugenia uniflora*

Introduction

Since the 1950s, scientific studies have shown activity of secondary metabolites, such as terpenes, counteracting the growth of different species of microorganisms that have different levels of sensitivity (Masquelier, 1959). Several antimicrobial agents have been discovered and synthesized by medicinal chemistry and fine chemistry developed during industrial revolution. Moreover, the frequent introduction of synthetic drugs by the pharmaceutical market contributed to the decline of traditional practices including the use of herbal medicine. This trend raises concerns about the increase in multidrug resistance, more easily developed for isolated compounds by fine chemistry. There are numerous resistance modifiers agents obtained from different animal, vegetal or microbial species and these natural products are active against many drug resistant microbes (Moloney, 2016). Natural products are able to overcome drug resistance in cancer cells (Wang et al., 2015), downmodulating p-glycoproteins (Noysang et al., 2014; Abdallah et al., 2015). Plant extracts can be combined for multitarget synergistic effects capable of further impairing resistance development (Chandra Shekar et al., 2015). The frequent and often irrational use of antimicrobial agents, promoted the selection of microbial drug resistance worldwide, resulting in treatment failures, increased mortality rates, and increased health care costs (Howard et al., 2003). In this scenario, the scientific community, encouraged by the World Health Organization, has stimulated interest in research on the use of natural products as antimicrobial agents. Considering the wide variety of natural products used as antimicrobial agents, essential oils stimulated the interest of researchers because of their wide spectrum of activity due to the diversified active metabolites composition (Chopra 2007; Low et al., 2011; Pinto et al., 2016). Scientific studies have clearly demonstrated the antimicrobial action of essential oils on various targets (Lu et al., 2012; Khan et al., 2014). The essential oils of medicines herbs are known to promote pharmacologic actions including antifungal and antibacterial properties of products such as lemon tea (Olorunnisola et al., 2014). *Schinus terebinthifolius* and *Eugenia uniflora* are species used commonly in folk medicine for the treatment of infectious diseases (Santin et al., 2009). Unlike the side effects often associated with the use of synthetic antimicrobial drugs, phytotherapic medicine can include a viable strategy, as the popularity of plant species was reached in view of the low costs and absence of adverse effects, thus suggesting industrial viability in the development of natural antifungals (Mukherjee et al., 2005; Ahmad et al., 2010). In the present study, we evaluated the phytochemical composition and *in vitro* antimicrobial activity of essential oils of *S. terebinthifolius* and *E. uniflora* on *Candida tropicalis* strains.

MATERIALS AND METHODS

Essential oils

The essential oils used in this study were extracted from *S. terebinthifolius* and *E. uniflora*. The plants were previously identified through voucher specimen in the Botanical Garden of Rio de Janeiro - RJ, by botanists of the RADAM Herbarium Brazil - HRB. More information regarding the origins and geographical distributions of the species mentioned by the informants can be found in the Species List of the Brazilian Flora (Almeida et al., 2014), available at http://floradobrasil.jbrj.gov.br; information about taxonomic revisions of the families and genera is available at http://www.tropicos.org.

Essential oil extraction

All the plant material was cut into small pieces and subjected to hydrodistillation using a Clevenger type apparatus (300 *g*, 3h). The obtained essential oils were filtered, weighted, and the % (w/w) oil yield calculated. Their percentage content was estimated based on the dry weights of the plant by means of calculation of the water content using a moisture analyzer before distillation. Afterwards, they were divided in 50 µL aliquots and stored in amber vials at -20 °C (Serafini et al., 2002).

Phytochemical approach

The samples were analyzed using a liquid chromatographer Agilent 1200 and a mass spectrometer iFunnel Agilent 6550 Q-TOF. The extracts were diluted in methanol and injected (1 µL) by the

autosampler via FIA with the infusion of samples in the electrospray source of the mass spectrometer. The LC mobile phase was methanol at a 0.1 mL/min flow rate. The electrospray ionization source operated in positive and negative mode. The ESI source operated at 290 °C of gas nebulization temperature, 3500 V capillary voltage, 320 V Nozzle Voltage, 14 mL/min drying gas flow rate, 45 psig nebulizer gas pressure, 350 °C auxiliary gas temperature and 12mL/min auxiliary gas flow. The time of flight analyzer operated at the range of 100 – 1000 m/z, 100 V Fragmentor, 750 V octapole voltage, and acquiring a spectrum/s obtained with high resolution, up to 5th decimal digit. The formulas were assigned to error of 1 ppm using the Agilent Mass Hunter Workstation software. The compounds were assigned using spectra database (De Rapper et al., 2013).

Strains and culture conditions

Strains of *Candida tropicalis* both resistant (ATCC 750) and sensitive (ATCC 18803) to fluconazole were obtained in the microbiology laboratory of the Federal University of Bahia - UFBA. These strains were previously maintained in cultures on agar plates potato and 4% dextrose for 48 hours at 37 °C and grown in Roswell Park Memorial Institute Medium (RPMI 1640 - Sigma) for the experiments (Cavalcanti et al., 2015).

In vitro susceptibility testing

The determination of the susceptibility was accomplished using the method of minimum inhibitory concentration (MIC_{50}), carried out in test tubes according to the Clinical and Laboratory Standards Institute (CSLI, formerly National Committee for Clinical Laboratory Standards - NCCLS). Assays were performed with $6x10^3$ of fungal suspension initial inoculum in 5 mL of RPMI medium supplemented with MOPS [3- (N-morpholino) propanesulfonic acid] in tubes. Yeast proliferation was quantified in triplicate in colorimetric method with methylene blue after 24 hours of incubation at 35 °C, optical density determined at 540 nm in a Versa Max microplate reader by Bio-Rad. The experiments were performed in at least three independent assays (De Rapper et al., 2013).

Evaluation of the cytotoxicity

The cytotoxicity of the compounds was evaluated in primary cultures of Swiss mice splenocytes. Cells ($3x10^6$ mL^{-1}) were cultured in 96 well plates for 24 hours prior to experiments. Tests were performed using the [^{3}H] thymidine incorporation method (Novafarma, Anapolis, GO, Brazil) and untreated controls received only 1 % DMSO (Sigma-Aldrich, São Paulo, Brazil). Experiments were reproduced as three independent assays, performed in triplicate as described previously (Pinto et al., 2016). The experimental protocols were reviewed and approved by the Animal Ethics Committee of CPqGM, under number L-IGM-012/09.

Cell viability

C. tropicalis strains of (ATCC 750 and ATCC18803) cultured on 96 well plates were treated with the essential oils of *S. terebinthifolius* and *E. uniflora* and incubated for 24 hours at concentrations of 0.01, 0.1,1,10,100, and 1000 mg mL^{-1}. The plates were then centrifuged at 1200 *g* for 10 minutes, the supernatants were removed and fresh RPMI 1640 medium devoid of phenol red, (Gibco/Life Technologies, Carlsbad, CA, Tewksbury, MA, USA) containing 0.5 mg mL^{-1} MTT (USB Corporation, Cleveland, OH, USA). The plates were incubated for further 4 h and centrifuged again. The supernatants were discarded, 150 µL of DMSO were added to solubilize the formazan precipitate generated. The absorbances were measured after 30 minutes in a spectrophotometer (SpectraMax 190 Microplate Reader, Molecular Devices, USA) at 570 nm. Three independent assays were conducted in triplicates accordance (Vannier-Santos et al., 2008).

Mitochondrial transmembrane potential

C. tropicalis strains were treated with different concentrations of the essential oil of *S. terebinthifolius* (0.3 and 13.0 mg mL^{-1}) and *E. uniflora* (0.4 and 0.6 mg mL^{-1}) for 24 hours and the transmembrane potentials were determined by flow cytometry by incorporation of rhodamine 123. After treatments, strains were incubated in 5 mg mL^{-1} rhodamine 123, in saline at 37°C in the dark for 15 minutes. Fluorescence was measured via at least 10,000 events analyzed by flow cytometry performed in triplicate in three independent assays (Pina-Vaz et al., 2014).

Scanning electron microscopy

Treated and untreated yeast were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Then, the samples were washed in the same buffer. After adhesion to poly-*L*-Lysine-treated coverslips, samples were post-fixed with 1% osmium tetroxide, 0.8% potassium ferrocyanide, in the same buffer and dehydrated in ethanol series (30-100%) for 10 minutes at each concentration. The samples were centrifuged and dried to the critical point CO_2 apparatus system (LEICA EM CPD030), mounted on metal stubs, coated with gold and observed under a scanning electron microscope JEOL JSM 6390LV 15 kV (Jesus et al., 2004).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.1 using one-way ANOVA and Dunnett post-test with significance level of $p<0.05$ considering three independent experiments. Values represent the mean ± standard deviation.

RESULTS

Taxonomic identification

The plant species approached were identified as *Schinus terebinthifolius*, also Called "Brazil pepper tree" or Aroeira and *Eugenia uniflora*, also called pitanga, Brazilian cherry, Suriname cherry, Cayenne cherry. Specimen sheets were registered under the numbers 482253 and 365891, respectively in the Rio de Janeiro Botanic Garden by botanists of the Radam Herbarium, Brazil.

Assessment of chemical constituents present in the essential oils of S. terebintifolius and E. uniflora - Pitanga by gas chromatography high - resolution GC -MS

The herbs are well known for having high variability of chemical components in its essential oils. The chromatograph peaks evidence derived substances of secondary metabolism of plants *S. terebintifolius* (Fig.1 A) and *E. uniflora* (Fig.1B) under study with biological activity already described above on the reduction of antimicrobial activity of several species. The expressed results show the mass (m/z) and abundance (%) of the compounds found in the reading for detection of compounds show more often, whether they are majority in testing for volatile substances and not-volatile.

FIGURE 1

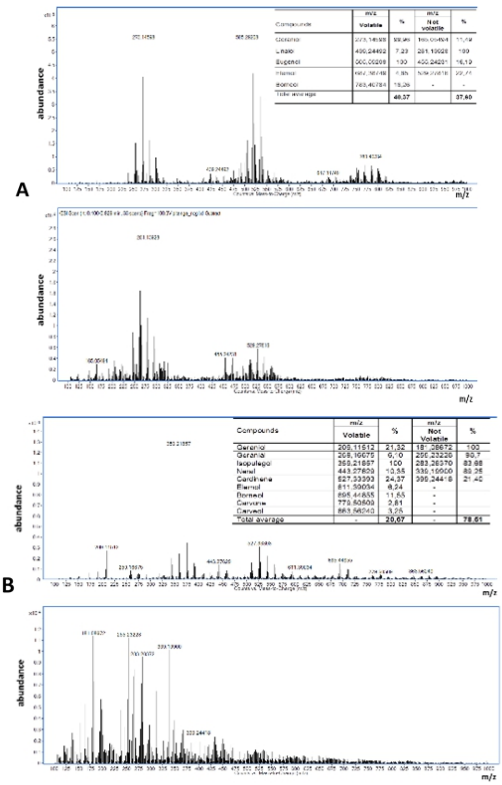


Fig. 1. The chemicals found most often in the peaks found in gas chromatography in the essential oils *S. terebintifolius* (Fig. 1 A) and *E. uniflora* (Fig. 1B) were evaluated by Gas Chromatography - GG.

Susceptibility testing

Susceptibility profiles of *C. tropicalis* strains (ATCC750 and ATCC18803) using the microdilution technique (Ferreira et al., 2014) are shown in Fig. 2. The strains used in this study showed different susceptibility to the essential oils from the tested species. The fluconazole-resistant and susceptible strains showed MIC_{50} values mL for the essential oil of *S. terebinthifolius* of 13.0 mg mL^{-1} and 0.3 mg mL^{-1}, respectively. *E. uniflora* essential oil produced MIC_{50} values 0.6 mg mL^{-1} and 0.4 mg mL^{-1} for the fluconazole-resistant and -sensitive strains, respectively.

FIGURE 2

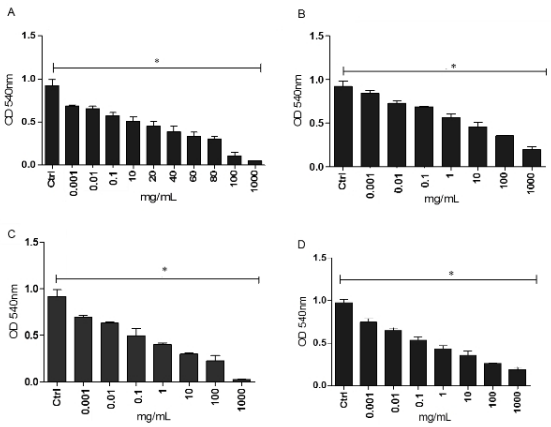


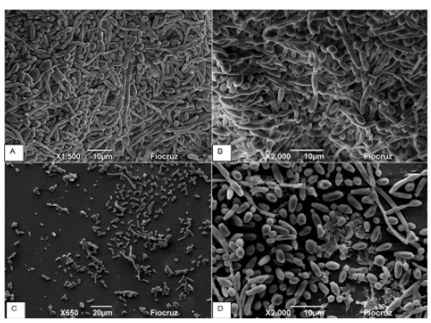
Fig 2. Dose-dependent effects of the essential oils of *Schinus terebinthifolius* (A, B) and *Eugenia uniflora* (C, D), upon *Candida tropicalis* accessed by colorimetric method with methylene blue. The fluconazole-resistant (ATCC 750) strain produced a MIC_{50} value for *S. terebinthifolius* of 13 mg mL^{-1}, whereas the fluconazole-sensitive one (ATCC 18803), 0.3 mg mL^{-1}. The antimicrobial action of E. *uniflora oil* on these strains was 0.6 mg mL^{-1} and 0.4 mg mL^{-1}, respectively. * $p <$ 0.05 One Way ANOVA - Tukey post-test).

Ultrastructural evaluation of essential oil activity *Schinus terebinthifolius* and *Eugenia uniflora* by Scanning Electron Microscopy (SEM)

The SEM images shown in Fig. 3 evaluates the three-dimensional structure and the surface topography of yeast treated with the concentration of 13.0 mg ml -1 and 0.3 mg ml -1 the essential oil of *S. terebinthifolius* in strains ATCC750 - RF and ATCC18803 - SSF respectively, for 24 h.

Fig. 3 shows the changes caused by the essential oil of *Schinus terebinthifolius*. The observed images are left corresponding to the resistant strain - RF, whereas the images are seen at right sensitive strains - SSF to fluconazole. In ATCC750 control group - RF (Fig. 3A) and ATCC18803 - SSF (Fig. 3B), it is possible to observe a large number of yeast showing its full surface, little pseudohyphae training and ovoid shape of the cells. In the IC_{50} concentrations of oil essence S. terebintifoulis 13.0 mg mL -1 (Fig. 3C) and 0.3 mg ml -1 (Fig. 3D) a reduction in the cellular volume, respectively. The figure (3E) show cracks on the cell wall of the strain ATCC 750 while (3G) shows loss of cytoplasmic contents showing the strain with cell wall stick together walls and scar apparent budding (arrows). In strain ATCC 18803 in Figure (3F) showed clear deterioration of the cellular structure while figure (3H) displays destruction strain, being possible to observe the budding scar the inner face (arrows).

FIGURE 3



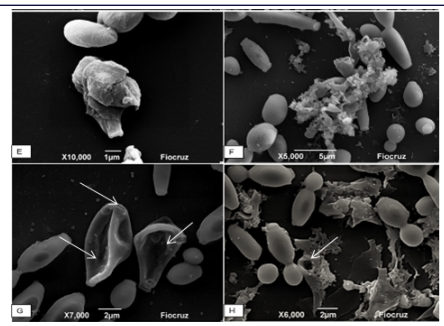


Fig. 3. Ultrastructural evaluation of cellular changes of *C. tropicalis* by scanning electron microscopy -MEV treated with concentrations of 13.0 mg mL- 1 and 0.3 mg essential oil mL -1 *S. terebinthifolius* the strain resistant (ATCC750) and sensitive to fluconazole (ATCC18803).

Fig. 4 shows the ultrastructural changes caused by the essential oil of *Eugenia uniflora*. In figure 3A - 3B and the control strains (ATCC750 - RF and ATCC18803 - SSF) of SEM boards, allows the visualization of yeast displaying its full surface, little amount of pseudohyphae and morphology unchanged format of the cells. In the IC_{50} concentrations of 0.6 mg ml-1 (Fig. 4 I) and 0.4 mg ml-1 (Fig. 4J) is known to decrease the exemplary strains when compared to the control group, showing consistency with the results obtained by MTT. At concentrations of 0.6 mg ml-1 the figure (4L) and 0.4mg ml-1 (4M) the ATCCs 750 and 18803 respectively expound the surface of the cells and the walls collapsed essential oil activity experienced. In figure (4N) ATCC 750 confirms the loss of *C. tropicalis* cytoplasm material exposing the apparent budding scars. As can be seen in Figure (4 O) ATCC 18803 into cells after disruption caused by bioagent tested with cellular debris and budding scar marked by the arrow.

FIGURE 4

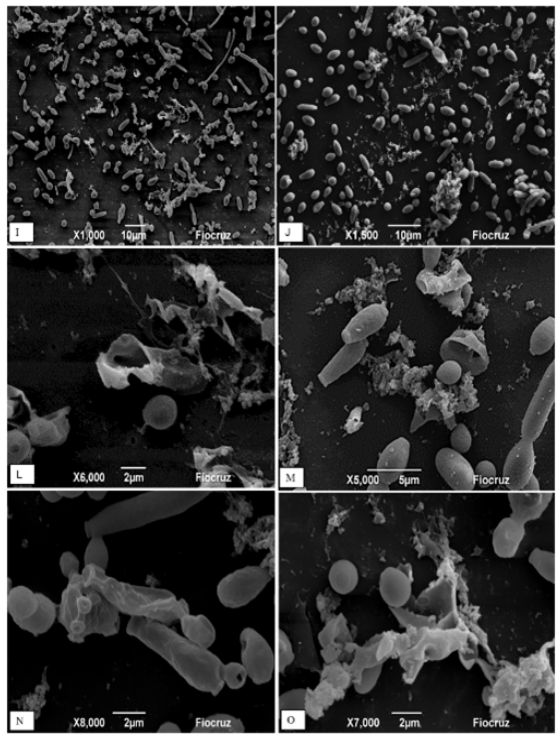


Fig. 4. Evaluation of ultrastructural cell of *C. tropicalis* by scanning electron microscopy - SEM, treated with concentrations of 0.6 mg mL-1 and 0.4 mg ml-1 essential oil of *E. uniflora* in the resistant strain (ATCC750) and sensitive to fluconazole (ATCC18803).

In order to verify whether the diminished cell proliferation was associated to loss of cellular viability, fungal cell redox potential, largely dependent on mitochondrial function, was accessed *C. tropicalis* cell function via MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-

diphenyltetrazolium bromide) assays. Both ATCC 750 and ATCC 18803 *C. tropicalis* strains displayed dose-dependent inhibition of metabolic activity by the testes extracts (Fig. 5).

FIGURE 5

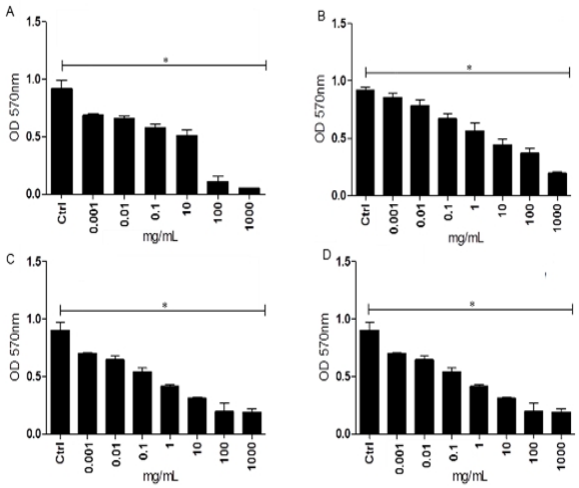


Fig. 5. Analysis of cell viability with MTT 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) in *C. tropicalis* ATCC 750 (A, C) and ATCC 18803 (B, D), cultured with the essential oils, *S. terebinthifolius* (A and B), *E. uniflora* (C and D). Yeasts (7.5 x 10^7 ml^{-1}) were incubated with 0.001, 0.01, 0.1, 1.0, 10, 100; 1,000 mg ml^{-1}) for 24 hours in RPMI medium at 35 °C. *p <0.05 One Way ANOVA - Tukey test.

This assay demonstrates a reduction in cell viability after 24h treatment with essential oils at higher concentrations. Under these conditions, it was demonstrated the effect of these bio-agents on the cell wall showing damage occurring.

Activity cytotoxic essential oils of *Schinus terebinthifolius* - Aroeira and *Eugenia uniflora* - Pitanga on splenocytes.

The Fig. 6 shows the IC_{50} values found in the cytotoxicity assay of the essential oils on normal murine cells. When examining the cytotoxic activity of essential oils on normal cells IC_{50} values were found for *S. terebinthifolius* (A) 0.01 mg ml-1 and *E. uniflora* (B) 9.8 mg ml-1 for splenocytes. These results demonstrate that the compounds do not exhibit this selectivity compared to the tested line. However, despite the essential oils have presented the cytotoxicity values found are those obtained for the IC_{50}, indicating the possibility of these oils were evaluated both in vitro and in a murine model, with possible application in pharmaceutical formulations. When examining the figure 6 it is possible to observe the cytotoxic activity with IC_{50} values of essential oils of *S. terebinthifolius* 0.01 mg ml-1 and the oil *E. uniflora* 9.8 mg ml- 1.

FIGURE 6

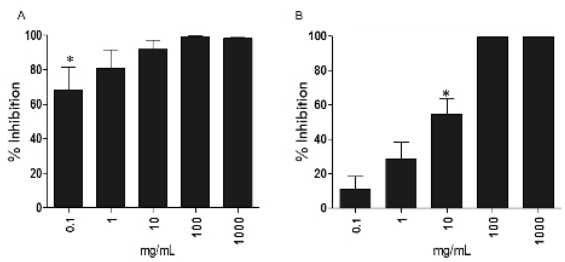


Fig. 6. Cytotoxicity evaluation of essential oils *S. terebinthifolius* (A) and *E. uniflora* (B) in murine splenocytes. Statistically significant representation when *p <0.05 One Way ANOVA - Tukey test).

Mitochondrial membrane potential and flow cytometry analysis

Our aim was to determine changes in membrane potential related to variations in mitochondrial metabolic activity that may be associated with conformational modifications affecting the internal mitochondrial membrane. Figure 7 shows a typical trace of the simultaneous measurement of fluorescence membrane potential of Rh123 cytometry in also is presented according to the states marked throughout the graph. The mitochondrial transmembrane potential was determined by retention of the dye rhodamine 123, analyzed in flow cytometer. The following picture shows mitochondrial depolarization of *C. tropicalis* ATCC 750 -RF treated essential oil concentrations, *S. terebinthifolius* (13.0 mg ml-1) and *E. uniflora* (0.6 mg ml-1) (A) for 24 hours. The results showed a significant difference in depolarization of mitochondrial membranes of *C. tropicalis* ATCC 18803-SSF when subjected to the values of the concentrations measured in IC_{50} essential oils, *S. terebinthifolius* (0.3 mg ml-1) and *E. uniflora* (0.4 mg ml-1) (B) relative to rhodamine after 24 hours of incubation bioagents.

FIGURE 7

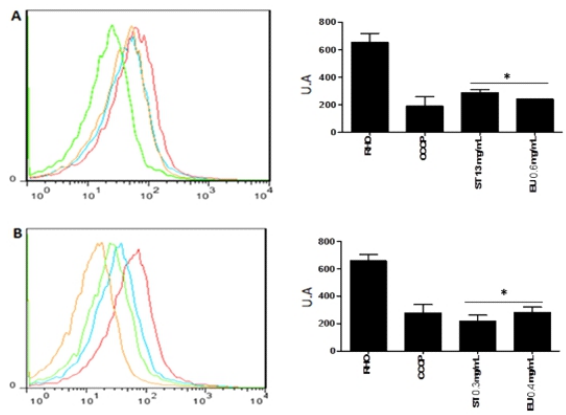


Fig. 7. Accumulation of rhodamine 123 in strains of *Candida tropicalis* treateds with essenciais oils. The data are expressed as arbitrary unit (AU) of three independent experiments. * p <0.05 when compared to the control group.

Numerous plant species make use of their secondary metabolism for adaptive defense mechanisms, evolving chemical structures with potential for biological assays, furnishing new biotechnological possibilities.

Discussion

The action of essential oils on Candida sp. is interesting since natural products can control oral infections (Chinsembu, 2016). The search for new biomolecules with antimicrobial activity is justified by the sharp increase in the resistance of pathogens to synthetic fungicides (Heisig, 2001). These new antifungals prototypes must act by different mechanisms of action of synthetic agents, marketed in order to avoid cross-resistance and reduce the side effects (Mcchesney, 1993).

The class of fungicides available in the pharmaceutical market is small when compared to the range of antibacterial. In addition to adverse side effects, such as gastrointestinal disturbances, skin reactions, hepatotoxicity and leukopenia worsening in immunocompromised, transplant, chemotherapy patients, the elderly and newborns. (Macchesney et al., 1993; Gupta, 1998; Carazo et al., 1999). The absence of regulatory norms related to pharmacovigilance allowed for years the indiscriminate use of these drugs, which made the population more susceptible to fungal infection. Enactment of the RCD 44/2010 regulated by the National Health Surveillance Agency - ANVISA, which deals with the requirement of prescription to acquire antimicrobial drugs, indicates the need for control in the proper clinical application of these drugs.

In order to investigate new molecules, it was determined antiproliferative activity and selective cytotoxicity, the essential oils of *Schinus terebinthifolius* - Aroeira and *Eugenia uniflora* - Pitanga were tested in resistant strains - RF (ATCC 750) and sensitive to fluconazole - SSF (ATCC18803), and normal mouse spleen cells. According to the results of this preliminary test, the IC_{50} values found for the essential oils in all strains were lower than 30 mg ml-1, was considered a favorable value for the development of an antifungal drug from plant extracts (Lee, 2011).

Using data obtained from the MTT assay, it was observed that the tested compounds could inhibit the proliferation of *C. tropicalis* strains, and this result was confirmed by exclusion test trypan blue dye. Using similar methodology, Xiang (2013) demonstrated the inhibitory effect induced by ethanolic extract of Brazilian propolis (100 µg / ml) in strains of *S. aureus* after 24 hours of treatment. In another study, Ahamad et al. (2014) noted the cytotoxic effect of hydroalcoholic extract of a type of Indian propolis on strains of *Candida* sp. The extract was capable of increasing cell death in nearly 50% after 24 hours of treatment in a dose-dependent concentration (2-20 µg / ml). The development of plant-derived drugs with high potential antifungal and minor side effects it is of great interest to the folk medicine, for now, there are few works that demonstrate the antimicrobial properties of the essential oils (Jeong et al. 2009 and Xiang, 2013). The pharmacodynamic effects of this secondary metabolites in medicinal herbs have been reported on the work of Zarrin et al. (2009) and discussed in the working Ahamad et al. (2015).

In our tests, essential oils were not selective for murine cells, in any case, it is arguable that traditional antifungal currently employed in treating these infestations have high toxicity being less selective (Ghosh, 2013).

The herbs are well known for having high variability of chemical components in its essential oils. The essential oils of *Schinus terebinthifolius* - Aroeira and *Eugenia uniflora* - Pitanga were evaluated by Gas Chromatography - GC, and the chemicals compounds often found in both oils belong to class of monoterpenes and sesquiterpenes. The monoterpenes and sesquiterpenes are natural compounds that represent the class of secondary metabolites. Studies have intensified in recent years due to their promising results in various pharmacological studies (Ferreira et al., 2010). The main interest in monoterpenes and sesquiterpenes stems from its ability to induce oxidative stress in cells (Benites, 2008; Ferreira et al., 2010.). The monoterpenes and sesquiterpenes have been thoroughly researched by its use in a wide range of biological and medical applications, among which stand out anti-tumor activity, antibacterial and antifungal (Ferreira et al*.,* 2010; Cavalcanti, 2015).

Considering that the secondary metabolism of medicinal plants is directly related to the native ecosystem, it is possible to understand quali-quantitative variables in the results observed in other studies. Ahamad et al. (2015) show quantitative differences of neral chemical constituents (32.9% versus 34.6% Bassolé (2011) versus 10.35% of the values found (VE) in our study), geraniol (6.1% Lou et al. (2008) versus 99.96% Low et al. (2011), linalool (27.0% Malele (2007) versus 7.23% and eugenol VE 1.5% Ahamad (2015) versus 100% EV). Notoriously aromatic plants have changes in their constituents, thus it is necessary that other experimental trials involving their biological activities are accompanied by molecular identification to document specific chemotypes, according to the native flora allowing proper discussion and promoting foundation for future scientific research. The essential oils of herbs have been known to damage cell membrane and functions structures may also bind to proteins and structural steroids and promote changes in cell membrane wall and leading to distortion and cell death (Khan et al., 2014).

To better characterize the pathway involved in the death process induced by the compounds tested in this study, we evaluated the effect of essential oils on the mitochondrial transmembrane potential. The results from this analysis show that the essential oils of *Schinus terebinthifolius* - Aroeira and *Eugenia uniflora* - Pitanga altered mitochondrial integrity of *Candida tropicalis* strains (ATCC 750 and ATCC 18803) treated for 24 hours, in accordance with the results found in IC_{50} and MTT experiments, suggesting also the participation of mitochondrial intrinsic pathway in cell death of the studied strains induced by the tested. Death by apoptosis seems to be a common mechanism of action associated with monoterpenes and sesquiterpenes. A study by Santos (2012) showed that the ethanol extract of *Eugenia uniflora* (100 mg ml-1) found in the Brazilian Northeast could inhibit a significant proliferation after 12 hours of treatment.

The SEM was used to evaluate the three dimensional structure and surface topography of the treated yeast with the concentration of 13.0 mg ml-1 the essential oil of *Schinus terebinthifolius* and 0.6 mg ml-1 of *Eugenia uniflora* the strain resistant to fluconazole (ATCC750) and the strain does not resist fluconazole (ATCC18803) 0.3 mg ml-1 and 0.4 mg ml-1 of the oils cited respectively, for 24 h. In the control groups, as well as finding multiple cells with a normal volume (Fig 3A and 3B), it was possible to observe a large number of yeasts showing its full surface, pseudohyphae formation and little ovoid shape of the cells. In the several concentrations tested in this experiment, it was observed that there was a reduction in cell volume, cracks in the wall strain was flexible wall, collapsed walls, a greater number of cells with leakage of cytoplasmic material besides the significant decrease in cell number when compared with the control showing degradation of the structure of the tested strains. These results are consistent with the findings of (Gomes Jr, 2014) in *Candida tropicalis* strains and those reported by (Melo, 2015) in strains of *Candida albicans* in their doctoral thesis work. These cited works were considered various mechanisms that may be involved in the infeasibility of yeast, also reporting evidence more pronounced by scanning electron microscopy and transmission that cell death may have been caused by necrosis, which is triggered by oxidative stress. Further studies are needed to elucidate the mechanisms involved in the death of *Candida tropicalis* strains.

Conclusion

Although both *Schinus terebinthifolius* and *Eugenia uniflora* had reported antimicrobial activity, this is the first study to examine Brazilian Northeast essential oils on *Candida sp*. with ultra-structural focus of the fungal cell with alteration of functional results. This work, therefore, will contribute for a better understanding of the pharmacological potential of the local flora and natural source of possibility product with an interest in biotechnology and applications for the pharmaceutical industry. However, other works developed with these plant species will allow a greater amount of scientific information on the potential of secondary metabolites with antifungal activity.

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References:

1. Abadallah HM, Al-Abd AM, El-Dine RS, El-Halawany AM 2015 P-glycoprotein inhibitors of natural origin as potential tumor chemo-sensitizers: A review. J Adv Res.; 6(1): 45-62.
2. Ahmad W, Ahmad WYW, Zakaria ZA, Yusof NZ 2014. Isolation of Pigment-Producing Bacteria and Characterization of the Extracted Pigments. Springer Briefs in Molecular Science, 25-44,
3. Ahmad A, Khan A, Manzoor N, Khan LA. 2015. In vitro synergy of eugenol and methyl eugenol with fluconazole against clinical Candida isolates. J. Med. Microbiology
4. Almeida M Z, Leda P H O, Silva M Q.O.R, Pinto A, Lisboa M, Guedes M L, Peixoto A 2014. Species with medicinal and mystical-religious uses in São Francisco do Conde, Bahia, Brazil: a contribution to the selection of species for introduction into the local Unified Health System Rev Bras Farmacogn 24: 171-184
5. Bassolé I H N 2011. Chemical composition and antimicrobial activity of Cymbopogon citratus and Cymbopogon giganteus essential oils alone and in combination. Phytomedicine.
6. Benites J 2008. Studies on quinones: Synthesis of furylquinone and hydroquinones with antiproliferative activity against human tumor cell lines. Bioorg. Med. Chem. n. 16 p. 862–868.
7. Carazo J L S, Losada L O, Sanjuan V P.1999. Tratamiento de las micosis superficiales. Rev. Iberoam. De Micol. v. 16, p. 26-30.
8. Cavalcanti I M F 2015. Antimicrobial activity of β-lapachone encapsulated into liposomes against meticillin-resistant Staphylococcus aureus and Cryptococcus neoformans clinical strains Journal of Global Antimicrobial Resistance.
9. Chandra S B R, Nagarajappa R, Suma S, Thakur R 2015. Herbal extracts in oral health care - A review of the current scenario and its future needs. Pharmacogn Rev.9(18): 87-92.
10. Chinsembu K C 2016. Plants and other natural products used in the management of oral infections and improvement of oral health. Acta Trop. 154:6-18.
11. Chopra I 2007. The increasing use of silver-based products as antimicrobial agents: a useful development or a cause for concern J. Antimicrob. Chemother. 59 (4), 587–590.
12. De Rapper S, Kamatou G, Viljoen A, Van V S 2013. The in vitro antimicrobial activity of Lavandula angustifolia essential oil in combination with other aroma therapeutic oils. Evid. Based Complement. Altern. Med. 852049, 10.
13. Ferreira V M, Balandrin L C, Calabricc M V 2014. Essential oil of Melaleuca alternifolia for the treatment of oral candidiasis induced in an immunosuppressed mouse model. BMC complementary and alternative medicine v. 14, p. 489.
14. Ghosh S 2013. Evaluation of the wound healing activity of methanol extract of Pedilanthus tithymaloides (L.) poit leaf and its isolated active constituents in topical formulation. Jornal Ethnopharmacology. v.142, n.3, p.714-722.
15. Gomes, Jr. 2014. Autophages a new perspective on antifungal resistance. Thesis (Doctorate in Biotechnology and Investigative Medicine) - Oswaldo Cruz Foundation.
16. Gupta A K, Del Rosso J Q, Lynde C W, Brow G H, Shear N H 1998. Hepatitis associated with terbinafine therapy: Three case reported and a review of the literature. Clin. Exp. Dermatol., v. 23, p. 64-67.
17. Heisig P. 2001. Inhibitors of bacterial topoisomerases: mechanisms of action and resistence and clinical aspects. Planta. Med. v. 67, p. 3-12.
18. Howard D H, Scott R D, Packard R, Jones D 2003. The global impact of drug resistance. Clin. Infect.Dis. 36 (1), S4–S10.
19. Jeong E M 2009. In vitro activity of the essential oil of Eugenia Uniflora and its major

component (citral) on Candida tropicalis. Microb. Res. 105 (6), 1489–1496
20. Jesus J B,Vannier-Santos M A, Britto C, Godefroy P, Silva-Filho F C, Pinheiro A.A, Rocha-Azevedo B, Lopes A H, Meyer-Fernandes J R 2004. Trichomonas vaginalis virulence against epithelial cells and morphological variability: the comparison between a well-established strain and a fresh isolate. Parasitol Res.; 93(5): 369-77.
21. Khan A, Ahmad A, Khan L A, Manzoor N 2014a. Ocimum sanctum (L.) essential oil and its lead molecules induce apoptosis in Candida albicans. Res.Microbiol 165 (6), 474 411–419.
22. Khan W, Rayirath U P, Subramanian S, Jithesh M N, Rayorsth P R, Hodges M A, Critchley A T, Craigie J S, Norrie J 2014b. Seaweed extracts as biostimulants of plant growth and development. Journal of Plant Growth Regulation. 28:386–399.
23. Lee Y J 2011. Molecular mechanism of SAHA on regulation of autophagic cell death in tamoxifen-resistant MCF-7 breast cancer cells. International journal of medical sciences, v. 9, n. 10, p. 881–893.
24. Low W L, Martin C, Hill D J, Kenward M . 2011. Antimicrobial efficacy of silver ions in combination with tea tree oil against Pseudomonas aeruginosa, Staphylococcus aureus and Candida albicans. Int. J. Antimicrob, 162–165.
25. Lu J J, Pan W, Hu Y J, Wang Y T 2012. Multi-target drugs: the trend of drug research and development. PLOS ONE 7 (6), e 40262.
26. Masquelier J 1959. The bactericidal action of certain phenolics of grapes and wine. In: International journal of microbioly sciences, v. 7, n. 4, p. 88–93.
27. Malele R S 2007. Essential oil of Cymbopogon winterianus Jowitt from Tanzania: composition and antimicrobial activity. Journal of Essential Oil-Bearing Plants, v. 10, n. 04, p. 83-87.
28. Mcchesney J D, Kinghorn A D, Balandrin M.F 1993. In Human medicinal agents from plants Eds.; Amer. Chem. Soc. Symposium Series. v. 534, p. 38-47.
29. Melo A P C 2015. Action of β-Lapachone combined with diethyldithiocarbamate (DETC) in Candida albicans. Dissertation (Master in Biotechnology) - State University of Feira de Santana, National Council of Scientific and Technological Development.
30. Moloney M G 2016 Natural Products as a Source for Novel Antibiotics. Review Trends in Pharmacological Sciences. Rev. 13 (1), 123–134. Available online 4 June.
31. Mukherjee P K, Sheehan D J, Hitchcock C A, Ghannoum M A 2005. Combination treatment of invasive fungal infections. Clin. Microbiol. Rev. 18 (1), 163–194.
32. Noysang C, Mahringer A, Zeino M, Saeed M, Luanratana O, Fricker G, Bauer R, Efferth T 2014 Cytotoxicity and inhibition of P-glycoprotein by selected medicinal plants from Thailand. J Ethnopharmacol. 155(1): 633-41.
33. Pinto L A, Meira,C S, Villarreal C F, Vannier-Santos M A, De Souza C V, Ribeiro I M, Tomassini T C, Galvão-Castro B; Soares M B, Grassi M F 2016. Physalin F, a seco-steroid from Physalis angulata L., has immunosuppressive activity in peripheral blood mononuclear cells from patients with HTLV1-associated myelopathy. Biomed Pharmacother.; 79: 129-34.
34. Olorunnisola S K, Asiyanbi H T, Hammed A M, Simsek S 2014. Biological properties of lemongrass: an overview. Int. Food Res. J. 21 (2), 455–462.
35. Pina-Vaz C, Rodrigues A, Sansonetty F 2014. Antifungal activity of local anesthetics against Candida species. Infect Dis Obstet Gynecology.
36. Santin M R, Dos Santos A O, Nakamura C V, Filho B P D, Ferreira, I C P, Ueda T 2009. In vitro activity of the essential oil of Cymbopogon citratus and its major component (citral) on Leishmania amazonensis. Parasitol. Res. 105 (6), 1489–1496.
37. Santos K K A 2012. Anti-Trypanosoma cruzi and cytotoxic activities of Eugenia uniflora L. Exp. Parasitol. 131:130-132.
38. Serafini L A, Santos A C A, Touguinha L A, Agostini G, Dalfovo V 2002. Extrações e aplicações de óleos essenciais de plantas aromáticas e medicinais. Pharmacol and Pharmacother.; 19: 12-34
39. Vannier-Santos M. A, Menezes D, Oliveira M F, De Mello F G. 2008. The putrescine analogue 1,4-diamino-2-butanone affects polyamine synthesis, transport, ultrastructure and intracellular survival in Leishmania amazonensis. Journal of Microbiology.; 154(Pt 10): 3104-11.
40. Wang P, Yang H L, Yang Y , Wang L, Lee S C 2015. Overcome Cancer Cell Drug Resistance Using Natural Products. Evid Based Complement Alternat Med. 2015:767136.
41. Xiang M J 2013. Erg11 mutations associated with azole resistance in clinical isolates of Candida albicans. FEMS yeast research, v. 13, n. 4, p. 386-393.
42. Zarrin M M, Ahmoudabadi Z A 2009. Invasive candidiasis: a review article. Journal of Microbiology. v. 2 n.1, p.1-6.
43. Zhou H, Xie X,Tang Y 2008. Engineering natural products using combinatorial biosynthesis and biocatalysis. Curr. Opin. Biotechnol. v.19, p. 590–596.