

Antimycotic Effect of the Essential Oil of *Aloysia triphylla* against *Candida* Species Obtained from Human Pathologies

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The research of alternative substances to treat infections caused by *Candida* species is a need. Aromatic plants have the ability to produce secondary metabolites, such as essential oils (EO). The antimicrobial properties of *Aloysia triphylla* (L'Her.) Britton (cedrón) EO has been previously described. The aims of this work were to determine the antimicrobial activity and the effect on the cell structure of the EO of *A. triphylla* against *Candida* sp isolated from human illnesses. The EO was obtained by hydrodistillation of *A. triphylla* leaves. The minimum inhibitory concentration (MIC) was performed with microdilution method and the minimum fungicidal concentration (MFC) was determined. *A. triphylla* EO's showed antifungal activity against all yeast: *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. guilliermondii*, *C. parapsilosis* and *C. tropicalis* which were resistant to fluconazol (150 mg/mL). The range of MIC values was from: 35 to 140 µg/mL and the MFC: 1842 to 2300 µg/mL. The time of killing at the MFC against *C. albicans* (3×10^5 UFC/mL) was 140 min. The dates of OD₆₂₀ and OD₂₆₀ suggest lysis and loss of absorbing material, respectively. The HROM shows distortion in morphology and shape of the cell, with large vacuoles in the cytoplasm. These studies clearly show that *A. triphylla* EO is a promising alternative for the treatment of candidiasis.

Keywords: candidiasis, *Aloysia triphylla*, essential oil, antimycotic activity.

Candida species are commonly part of the normal flora in the digestive tract of healthy humans; however they have been described as responsible for opportunistic infections, particularly in neonates and immunocompromised patients [1]. These infections are difficult to treat with traditional drugs because they have multiple side effects, high toxicity and yeasts develop resistance against antifungal chemotherapies. Thus, searching for alternative antifungal compounds has been a major concern in recent years [2,3]. The investigation of alternative substances to treat these infections is necessary to find a solution to these problematic. Several medicinal plants have been extensively studied in order to find more effective and less toxic compounds [4].

Aromatic plants constitute an interesting group of vegetables with ability to produce secondary metabolites, such as essential oils (EO). *Aloysia triphylla* (L'Her.) Britton, (*Aloysia citriodora* Palau.) popularly known as "cedrón", is a member of the Verbenaceae Family. It is perennial and grows widely in North and South America and also in northeast, northwest and central regions of Argentina. It is cultivated from Mexico till the South region of the continent. It is a bush with white flowers and fruits, with an intense scent lemon-like, sweet, lightly floral, and herbaceous [5,6]. This specie is used in folk medicine to treat many digestive disorders, as

anti-inflammatory, analgesic, antipyretic, tonic and stimulating. It shares an important place on the international herbal market due to the sensory and medicinal properties of it EO. These attributes determine its use as a primary ingredient for infusions and nonalcoholic beverages as well as aromatic ingredient for the flavor and fragrance industries. The pharmaceutical industry uses *A. triphylla* for its carminative, antispasmodic and sedative properties [7,8]. EO are constituted by a complex mixture of organic compounds including monoterpenes, diterpenes, carbonylated products and polyenes. There are many studies that suggest the antibacterial and antifungal activity of these compounds [9,10]. *A. triphylla* could be used to treat infections produced by *Candida* species.

The aims of this work were to determine the antimicrobial activity and the effect on the cell structure of the EO of *A. triphylla* against *Candida* species isolated from human illnesses.

The *A. triphylla* EO's were analyzed with GC-MS. The average yield obtained in the hydrodistillation process was 0.4% (w/v) and the main components identified were: limonene (2.9%), neral (20%), geranial (29.2%), spathulenol (8.9%) and caryophyllene oxide (7%), in concordance with other authors that previously described

all of them as the characteristic constituents of the EO of *A. triphylla* [6,10-12].

The antifungal activity of *A. triphylla* EO was tested using a microdilution broth method. The EO presented antifungal activity against all yeast. The range of MIC values was from 35 µg/mL to 143 µg/mL (Table 1). It is interesting to note low values of MIC necessary to inhibit *C. albicans* and *C. dubliniensis* (MIC: 35 µg/mL). In addition to this, the EO was able to cause the death of all *Candida* species. The fungicidal effect of the EO against the yeasts reached values of MFC from 230 µg/mL to 1842 µg/mL. (Table 1)

Table 1: Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of *A. triphylla* essential oil against *Candida* species

Species	MIC (µg/mL)	MFC (µg/mL)
<i>C. albicans</i>	35	460
<i>C. dubliniensis</i>	35	921
<i>C. glabrata</i>	143	230
<i>C. krusei</i>	71	230
<i>C. guilliermondii</i>	71	1842
<i>C. parapsilopsis</i>	71	1842
<i>C. tropicalis</i>	143	230

For more accurate evaluation of the antifungal activity of the EO, time-kill assays were performed using the yeast *C. albicans*. The killing time was tested at different cell concentration and results are shown in Figure 1. For 3×10^5 CFU/mL, killing time was 140 min. For a concentration of 3×10^6 CFU/mL the killing time was 300 min, for 3×10^8 CFU/mL was 480 min. and for 3×10^{12} CFU/mL was 1140 min. (Figure 1). These data shows that it is necessary more time to kill a bigger cell population; this means that killing time of the EO is directly proportional to number of cell. In all cases the viability control of the yeast presented a macroscopically visible growth while treated cells did not show visible growth at each killing time.

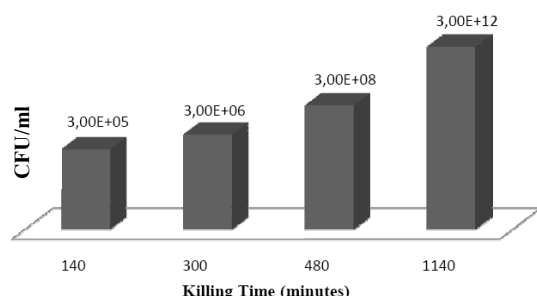


Figure 1: Killing time of *Aloysia triphylla* EO (MFC=460µg/mL) at different cell concentrations of *Candida albicans* (CFU/mL)

Table 2 shows the results obtained in the cellular lyses assay with *C. albicans* cells treated at the MFC. The control showed an increase in OD₆₂₀ what means that the yeast continued with its cellular growth. In contrast, in treated cells the OD₆₂₀ diminished almost at not

Table 2: OD₆₂₀ lectures in *C. albicans* cells treated with *A. triphylla* EO and not treated

Treatment	OD _{620nm}		
	0 h	Not centrifuged (18 h)	Centrifuged (18 h)
Control	0.513	1.785	1.605
With EO	0.513	0.010	0.074
CS+EO	0.081	0.009	0.073

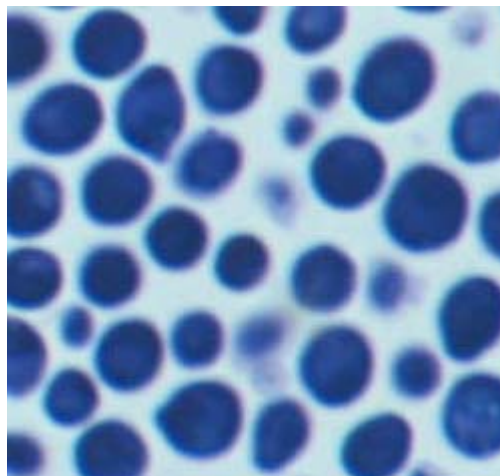


Figure 2: High Resolution Optic Microscopy of *Candida albicans* untreated.

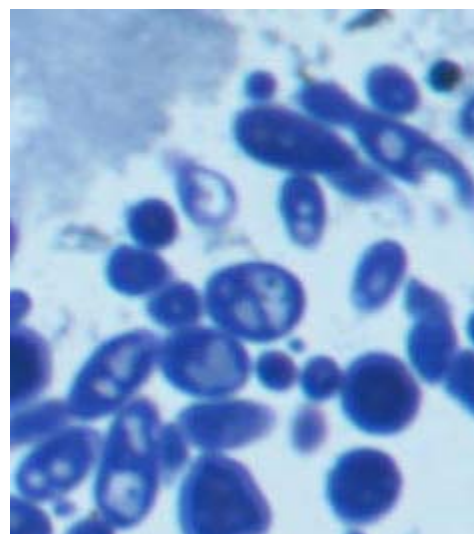


Figure 3: High Resolution Optic Microscopy of *Candida albicans* treated at the MFC (460 µg/mL) of *A. triphylla* EO.

detectable values; this last result could be explained by the possibility that cellular lysis caused by the EO occurred.

In the suspensions obtained from the centrifugation of *C. albicans* inoculums treated with EO and not treated (control), the OD₂₆₀ of the treated samples increased (OD₂₆₀ = 0.270) compared to the control suspensions (OD₂₆₀ = 0.008). This suggests that there is a lost of 260-nm-absorbing material.

The HROM showed in control cells that the morphology and the cytoplasmic density were characteristic of a normal cell (Figure 2). After EO treatment at the MFC

(460 µg/mL), morphology and shape of the cell was distorted and a notable structural disorganization was seen within the cytoplasm with the observation of large vacuoles. In addition, the contents of some treated cells appeared depleted and amorphous. Loss of cellular material was also observed (Figure 3).

A. triphylla EO showed antifungal activity against all *Candida* species, particularly in those that acquire resistance to conventional chemotherapies, like *C. albicans* and *C. dubliniensis*. The last one was recently identified as an opportunistic pathogen associated with oral candidiasis, particularly in individuals who are positive for human immunodeficiency virus (HIV) and immunocompromised patients [1,13]. *C. albicans* is the most common fungal pathogen in humans, responsible for skin, oral, esophageal, intestinal tract, vaginal and circulatory diseases commonly affecting immunologically compromised patients and those undergoing prolonged antibiotic treatment [14].

The results presented in this work demonstrate that *A. triphylla* EO had the potential to kill *Candida* cells causing lysis. What is more, this study clearly demonstrates that there was a direct relationship between number of cells and the time that the EO needed to cause killing effect.

C. albicans suspensions treated with *A. triphylla* EO lost significant 260-nm-absorbing material, suggesting that nucleic acids were released through a damaged cytoplasmic membrane. These data are coincident to experiences made with *C. albicans* treated with tea tree oil [15]. Marked leakage of cytoplasmic material is considered indicative of gross and irreversible damage to the cytoplasmic membrane. Many antimicrobial compounds that act on the bacterial cytoplasmic membrane induce the loss of 260-nm absorbing material. Some antimicrobial agents cause gross membrane damage and provoke whole-cell lysis and this has been reported previously for essential oils from oregano, rosewood, and thyme [16].

EO components have the capability to alter cell permeability by entering between the fatty acyl chains making up membrane lipid bilayers and disrupt the lipid packing. Due to this, the membrane properties like permeability, fluidity and consequently its functions may get changed [17]. This may also affect the regulation and function of the membrane bound enzymes that alter the synthesis of many cell wall polysaccharide components and alter the cell growth morphogenesis [18].

The data obtained by Vataru Nakamura, *et al.* 2004, with electronic microscopy showed that *C. albicans* as well as *C. tropicalis*, *C. parapsilosis*, and *C. krusei* underwent remarkable ultrastructural alterations which were visible by electron microscopy, when treated with the essential oil

of *O. gratissimum* [4]. This study is in concordance with our experience in which changes in the morphology of *C. albicans* cells were observed, as well as the formation of large vacuoles in the cytoplasm when they were exposed to the MFC of *A. triphylla* EO. Tyagy *et al.* 2010, worked with *C. albicans* cells treated with lemongrass EO and they observed similar results in experiences with different techniques of electronic microscopy [18].

A. triphylla EO is a promising natural product for the treatment of candidiasis. Therefore further studies on their pharmacokinetics and toxicological behavior are warranted. The results obtained represent a contribution to the characterization of the anti-*Candida* activity of EO of traditional medicinal plants from the Argentinean flora.

Experimental

Plant material and EO extraction: The EO was obtained by hydrodistillation of samples of *A. triphylla* collected from plants growing in farms (plantations) located in La Paz, Córdoba Province (Argentina).

Gas Chromatography: The EO were analyzed with a Shimadzu GC-R1A gas chromatograph equipped with a fused silica column (30 m x 0.25 mm) coated with CBP-1. The temperature of the column was programmed from 60°C to 240°C at 4°C/min. The injector and detector temperatures were at 270°C. The gas carrier was He, at a flow rate of 1 mL/min. Peak areas were measured by electronic integration. The relative amounts of the individual components are based on the peak areas obtained, without FID response factor correction. Programmed temperature retention index of the compounds were determined relative to *n*-alkanes. GC analysis was still performed using a column Supelcowax-10 with the same conditions as described above [19].

Gas Chromatography-Mass Spectrometry: GC-MS analyses were performed on a Perkin Elmer Q-910 using a 30 m x 0.25 mm capillary column coated with CBP-1. The temperature of the column and the injector were the same than those from GC. The carrier gas was He, at a flow rate of 1 mL/min. Mass spectra were recorded at 70 eV. The oil components were identified by comparison of their retention indices, mass spectra with those of authentic samples, by peak enrichment, with published data, mass spectra library of National Institute of Standards and Technology (NIST 3.0) and our mass spectra library which contains references mass spectra and retention indices of volatile compounds. GC-MS analysis was still performed using a column Supelcowax 10 with the same conditions as describe above [20].

Microorganisms: The activity of the EO was tested against the following yeasts: *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. guilliermondii*, *C. parapsilosis* and *C. tropicalis*. These strains were resistant to fluconazol (150 mg/mL). The strains were isolated in the

Central Hospital of Rio Cuarto and identified in the Mycology Area of Department of Microbiology and Immunology of the National University of Rio Cuarto.

Antimicrobial activity: The minimum inhibitory concentration (MIC) of the *A. triphylla* EO was evaluated against yeast species with the broth microdilution method described by Mann and Markham (1998) [21]. The minimum fungicidal concentration (MFC) was determined [23].

Culture methods: Tubes containing Sabouraud Broth (SB) (Britania) with 0.1% (w/v) agar (SBA) were prepared at pH 7 inoculated with each microorganism and incubated overnight (18 h) at 37°C. Optical densities were measured at 620nm in a spectrometer and number of cells was confirmed by the viable plate count on Sabouraud Agar (SA) (Britania).

Firstly, the cell concentration necessary to cause reduction of resazurin within 3.30 h was determined for each of the test microorganisms. Serial 10 fold dilutions of the overnight culture were prepared in SBA and aliquots (170 µL) from these dilutions were dispensed into microplates containing 20 µL of diluent (Dimethylsulphoxide-distilled water 1:1). The resazurin solution (10 µL) was added; then they were incubated for 3.30 h at 37°C. The appropriate dilution to work was the last one unable to reduce resazurin (blue), which was tested, by the plate count method. Resazurin is a redox indicator that is blue in its oxidized form and pink in its reduced form [21].

Determination of the Minimum Inhibitory Concentration (MIC): Serial two fold dilutions of the EO were prepared by vortexing it in the diluent at room-temperature. The resazurin assay medium, SBA, was inoculated with the test organism to yield a final cell density ≈ 1 log cycle lower than the cell density required to reduce resazurin (usually 10^6 cfu/mL). The inoculum density was confirmed by plate count. A sterile 96-well microtitre tray was set up with each of the tested *Candida* sp as follows: column 1-10, 170 µL inoculum plus 20 µL of the EO dilution; column 11, 170 µL inoculum plus 20 µL EO diluent (positive control); column 12, sterile resazurin assay medium plus 20 µL of EO diluent (negative control, respectively). Well contents were thoroughly mixed and were incubated at 37°C for 18h. After incubation 10 µL of resazurin solution was added to all except column 12, to which 10 µL of distilled water was added. After a second incubation of 3 h at 37°C, wells were assessed visually for color change, with the highest dilution remaining blue indicating the MIC. Each experience was made by triplicate [21].

Determination of the Minimum Fungicidal Concentration (MFC): 100 µL of the dilution belonging to the MIC and the previous dilutions were inoculated in SA and incubated at 37°C for 24 h. The MFC was considered as the last dilution that did not show cell growth [22].

Yeast killing assays: The time of killing of *A. triphylla* EO against the yeast *C. albicans* were evaluated by measuring the cellular viability at the MFC. The treatment consisted on a suspension of cells (UFC/mL) in SBA plus EO dilution (MFC) was incubated at 37°C, 120 rpm. A sample (0.1 mL) was removed at 30 min intervals and plated on SA (viability control) and incubated overnight. A suspension of cells (UFC/mL) in SBA without EO was incubated at the same conditions (suspension control).

Cellular lysis: Suspensions of *C. albicans* (10^4 UFC/mL) were prepared in SB (control) and SB supplemented with EO at the MFC and incubated at 37°C for 18 h. The OD_{620nm} was measured at 0 min and at 18 h. Then both of them were centrifuged at 10000 rpm for 5 min. The pellet was resuspended in PBS (Phosphate buffer saline) and OD_{620nm} was measured. Each experience was made by triplicate.

Loss of 260-nm-absorbing material: The supernatant from the suspensions of *C. albicans* prepared for cellular lysis assay was used to measure the loss of OD 260-nm-absorbing material.

High Resolution Optic Microscopy (HROM): Thin cuts (± 0.25 µm) obtained using a manual ultramicrotome (Sorvall MT-1A, DuPont) of *C. albicans* were processed for high resolution optic microscopy. They were placed on a slide and stained with toluidine blue on a thermic platine, allowing the income of the dye in the fungal cell. Thin stained cuts were mounted in DPX (Merk®) and observed with an optic microscope Axiophot (Carl Zeiss, Alemania). Images were obtained with a digital camera Powershot G6, 7.1 megapixels (Canon INC, Japón) joined to the optic microscope. Software AxioVision Release 4.6.3 (Carl Zeiss, Alemania) was used to process the images [23].

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