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Original article

# The essential oil of *Allium sativum* as an alternative agent against *Candida* isolated from dental prostheses

Alejandro Mendoza-Juache, Saray Aranda-Romo, Josué R. Bermeo-Escalona, Araceli Gómez-Hernández, Amaury Pozos-Guillén, Luis Octavio Sánchez-Vargas*

*Facultad de Estomatología Universidad Autónoma de San Luis Potosí, Mexico*

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ABSTRACT

*Background:* The colonization of the surfaces of dental prostheses by *Candida albicans* is associated with the development of denture stomatitis. In this context, the use of fluconazole has been proposed, but its disadvantage is microbial resistance. Meanwhile, the oil of *Allium sativum* has shown an effect in controlling biofilm formation by *C. albicans*.
*Aims:* The objective of this study was to determine the antifungal activities of the essential oil of *A. sativum* and fluconazole against clinical isolates of *Candida* species obtained from rigid, acrylic-based partial or total dentures and to compare these agents' effects on both biofilm and planktonic cells.
*Methods:* A total of 48 clinical isolates obtained from the acrylic surface of partial or complete dentures were examined, and the following species were identified: *C. albicans*, *Candida glabrata*, *Candida tropicalis*, and *Candida krusei*. For each isolate, the antifungal activities of the essential oil of *A. sativum* and fluconazole against both biofilm and planktonic cells were evaluated using the Clinical & Laboratory Standards Institute (CLSI) M27-A3 method. The isolates were also evaluated by semiquantitative XTT reduction.
*Results:* All planktonic *Candida* isolates were susceptible to the essential oil of *A. sativum*, whereas 4.2% were resistant to fluconazole. Regarding susceptibilities in biofilms, 43.8% of biofilms were resistant to *A. sativum* oil, and 91.7% were resistant to fluconazole.
*Conclusions:* All planktonic cells of the different *Candida* species tested are susceptible to <1 mg/ml *A. sativum* oil, and the majority are susceptible to fluconazole. Susceptibility decreases in biofilm cells, with increased resistance to fluconazole compared with *A. sativum* oil. The essential oil of *A. sativum* is thus active against clinical isolates of *Candida* species obtained from dentures, with effects on both biofilm and planktonic cells *in vitro*.



## Aceite esencial de *Allium sativum* como tratamiento alternativo contra aislamientos de *Candida* procedentes de prótesis dentales

*Palabras clave:*
*Candida albicans*
*Candida glabrata*
Aceite esencial de *Allium sativum*
Ajo
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RESUMEN

*Antecedentes:* La colonización por parte de *Candida albicans* de las superficies de las prótesis dentales se asocia con el desarrollo de estomatitis. Se ha propuesto el uso de fluconazol, pero su desventaja es la resistencia microbiana. El aceite de *Allium sativum* ha mostrado su efectividad al controlar la formación de biopelícula de objetivos.
*Objetivos:* Determinar la sensibilidad de cepas clínicas de especies de *Candida*, obtenidas de prótesis dentales parciales o totales rígidas de base acrílica, al aceite esencial de *A. sativum* y comparar su efecto en células planctónicas y en biopelícula.
*Métodos:* Se incluyeron 48 cepas clínicas de la superficie acrílica de prótesis dentales totales o parciales, identificadas entre las siguientes especies: *C. albicans, Candida glabrata, Candida tropicalis y Candida krusei*. Se evaluó la sensibilidad de cada una al aceite esencial de *A. sativum* y al fluconazol mediante la metodología M27-A3 del CLSI, tanto sobre células planctónicas como en biopelícula, y mediante el método semicuantitativo de la reducción de XTT en el último caso.

* Corresponding author.
*E-mail addresses:* lo.sanchezvargas@gmail.com, cdlosv@gmail.com (L.O. Sánchez-Vargas).

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*Resultados:* Todas las cepas planctónicas de *Candida* fueron sensibles al aceite esencial de *A.sativum*, mientras que el 4,2% fue resistente al fluconazol. En cuanto a su sensibilidad en biopelícula, el 43,8% fue resistente a *A. sativum* y el 91,7% lo fue al fluconazol.
*Conclusiones:* Todas las cepas en forma planctónica de las diferentes especies de *Candida* fueron sensibles a concentraciones inferiores a 1 mg/ml del aceite esencial de *A. sativum* y en menor proporción a fluconazol. La sensibilidad disminuyó en las células en biopelícula, con mayor resistencia al fluconazol en comparación con el aceite esencial de *A. sativum*. Por tanto, el aceite esencial de *A. sativum* es activo frente a cepas clínicas de diferentes especies de *Candida*, obtenidas de dentaduras, con efectos en biopelícula y células planctónicas *in vitro*.


The incidence and prevalence of fungal infections have increased considerably in recent years. The main fungal cause of these infections is the genus *Candida*,[20] comprising 17 species of medical interest that have been associated with infections in humans, and particularly *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida glabrata*.[10,33]

Wearing complete dentures is a risk factor because these prostheses can promote *Candida* biofilm formation and oral candidiasis.[31] *Candida* infections are commonly associated with biofilms on both the mucosa and the plastic surfaces of indwelling devices. These biofilms consist of matrix-enclosed micro-colonies of yeast, hyphae, and pseudohyphae arranged in a complex structure[18] and are inherently resistant to antifungals, so affected devices generally need to be removed.[34,35]

*Candida* pathogenicity has been attributed to several virulence factors, including adhesion to host cells or medical devices; biofilm formation; and the secretion of hydrolytic enzymes such as proteases, phospholipases, and hemolysins.[14,42] Among clinical *Candida* strains, biofilm formation is variable and depends on the species.[25,28]

Colonization of and biofilm formation on the surfaces of dental prostheses comprise important risk factors for the development of denture stomatitis because they support diverse microbial species, promoting the mechanisms that confer resistance and increase pathogenicity in *Candida* in particular. In fact, Bilhan et al. (in 2009) and De la Rosa et al. (in 2012) reported a significant association between the presence of *C. albicans* on prosthetic surfaces and the development of denture stomatitis.[5,17]

Regarding the use and care of dental prostheses, hygienic techniques and the use of medicines, antiseptics, and nanomaterials, among other approaches, have been proposed to control biofilm development and possible secondary conditions associated with the microorganisms present in these prostheses.[21,38] However, among the disadvantages of these proposed approaches are the emergence of strains with antimicrobial resistance and alterations in the prosthetic structure. Therefore, studies of alternative treatments that offer the fewest secondary effects are required. Alternative medicine includes several natural compounds that are effective against certain pathogenic species of interest in dentistry, as in other medical fields.[27,30]

The essential oil of *Allium sativum* comprises one of the natural alternatives most robustly proven to exhibit activity against diverse microorganisms. Previous works report that allicin is one of the active components of *A. sativum* oil and exerts a significant effect in inhibiting the growth of *Pseudomonas aeruginosa* biofilms.[30] In 2011, Khodavandi et al. reported that the oil of *A. sativum* has a similar effect in controlling biofilms of *C. albicans* American Type Culture Collection (ATCC) strains in comparison with fluconazole.[27]

The purpose of the present study was to determine the antifungal activity of the essential oil of *A. sativum* and to compare it with that of fluconazole in the context of clinical isolates of *Candida* species obtained from dental prostheses.

## Materials and methods

### *Study design and clinical isolates*

A cross-sectional study approved by the institutional bioethics committee was conducted. A total of 56 patients aged ≥40 years with total or partial bilateral, rigid, acrylic-based dental prostheses (16 maxillary, 12 mandibular and 28 both maxillary/mandibular) and with a minimum of 6 months of use were included in our study. When the patients had both types of prostheses (maxillary/mandibular), only one sample was taken. All of them had requested diagnostic dental care during the period of July to December 2014.

Patients with flexible dental prostheses were excluded. Those who agreed to participate were given a brief explanation of the protocol and were then asked for authorization by signing an informed consent form.

The patients were asked to remove their dental prosthesis from their oral cavity in order to undergo a clinical examination of the oral mucosa with artificial light and a dental mirror. Any pathological lesions observed were registered. If a patient was diagnosed with sub-prosthetic stomatitis, exfoliative cytology was performed, and the sample was stained using the Gram technique to discern whether yeast, hyphae, and/or pseudohyphae were present. The cytology result was reported to the patient, and the case was then followed until remission.

Samples were taken from the internal surfaces of the acrylic prosthesis basis with a sterile swab (Protec®; México, D.F., México); in particular, after the prosthesis was removed, scraping of all support surfaces that were in contact with the palate and the alveolar ridges was immediately performed. After being obtained, the swabs were suspended in 500 μL of 0.9% saline solution (PiSA, Jalisco, Mexico) and were then immediately taken to the microbiology laboratory for processing.

### *Microbiological assessment and identification of isolates*

Each sample collected was mixed for 20 s with a vortex, after which 100 μl of the suspension were plated on CHROMagar *Candida* (CHROMagar®; Paris, France) and incubated at 36 °C for 2 days. To ensure that seemingly negative samples were in fact negative, these samples were incubated for an additional 7-day period at a temperature of 30 ± 1 °C. Using chromogenic cultures, presumptive identification of *Candida* species was performed according to the colorimetric characteristics described by the manufacturer of the agar for each species (colonies green in color = *C. albicans*, mauve in color = *C. glabrata*, pink in color with a curly texture = *C. krusei*, and blue in color = *C. tropicalis*). The use of this medium allowed the separation of two or more strains from the overgrowth of different species from the same sample. Cultures positive for one or more species and purified cultures were reseeded and purified on Sabouraud glucose

agar plates (Difco®; Detroit, MI, USA) by incubating the cultures at 36 ± 1 °C for 2 days and confirming the species' presence using an optical microscope. Filamentous morphology was observed on cornmeal agar (Difco®). Subsequently, the yeast species were identified using the carbohydrate assimilation ID 32C AUX system and the Apiweb™ database (bioMérieux®; Marcy l'Etoile, France). The phenotypic isolates were preserved in yeast peptone dextrose (YPD) broth (1% w/v yeast extract, 2% w/v peptone, and 2% w/v dextrose; Difco®) with 50% glycerol and frozen for later use.

*Growth conditions*

All organisms were stored in vials of distilled water at room temperature. Later, each strain was grown in YPD medium (yeast extract 1%, w/v, peptone 2%, w/v dextrose 2%, w/v) in threaded centrifuge capped-cone tubes (Falcon # 2095, 17,120 mm, Becton Dickinson, Franklin Lakes, NJ, USA), and incubated overnight on an orbital shaker (Labnet, NJ, USA) at 36 °C. Each culture grew in the budding-yeast phase under these conditions. The cells were harvested on three occasions and washed in sterile phosphate buffer (2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4; Sigma–Aldrich®; St. Louis, MO, USA). Next, the cells were resuspended in RPMI-1640 supplemented with L-glutamine (Sigma–Aldrich®), buffered with morpholino-propane sulfonic acid (MOPS) (Sigma–Aldrich®) and adjusted to the desired cellular density, which was equivalent to 1–1.5 × $10^6$ colony-forming units (CFU) per ml. Cellular density was determined by counting the cells in a hemocytometer (Propper Manufacturing Company, Inc., NY, USA). This cell concentration was selected because previous researchers have demonstrated that optimal biofilm formation occurs at this particular density.[27] The standardized cell suspension was used immediately.

*Biofilm assays*

For biofilm and antifungal activity assays, each experiment was performed in triplicate to confirm the results. The standard deviation (SD) was calculated for three independent experiments. We used three strains (*C. albicans* ATCC 90028, *Candida krusei* ATCC 6258 and *C. parapsilosis* ATCC 96142) as controls in each experiment.

Biofilm assays were performed as previously described,[35] but pre-sterilized, polystyrene, flat-bottomed, 96-well microtiter plates (Costar®, EIA/RIA plate, with a low evaporation lid and high binding; Corning, NY, USA) were used. Biofilms were formed by pipetting standardized cell suspensions (100 μl of a suspension containing $10^6$ cells/ml in RPMI-1640) into selected wells of microtiter plates and incubated for 48 h at 37 °C. After biofilm formation, the medium was aspirated, and non-adherent cells were removed by thoroughly washing the biofilms three times in sterile phosphate-buffered solution (PBS) (137 mM NaCl, 12 mM KCl, 2.7 mM $Na_2HPO_4$, and 2.7 mM $KH_2PO_4$, pH 7.4; Sigma–Aldrich®).

*Measurement of biofilm formation*

A semi-quantitative measurement of biofilm formation was performed using an XTT (2,3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide) (Sigma–Aldrich®; St. Louis, MO, USA) reduction assay, essentially as described previously.[38] Briefly, XTT was prepared as a saturated solution at a concentration of 0.5 g/l in Ringer's lactate (PiSA, Jalisco, Mexico). This solution was filter sterilized through a filter with a 0.22-μm pore size, aliquoted, and then stored at −80 °C. Prior to each assay, an aliquot of XTT stock was thawed, and menadione (Sigma–Aldrich®; 10 nM, prepared in acetone) was dispensed into the aliquot at a final concentration of 1 μM. A 100-μl aliquot of the XTT/menadione was then added to each pre-washed biofilm to measure metabolic activity levels. Specifically, samples were incubated in the dark for 1 h at 37 °C, and the colorimetric change (a reflection of the biofilm's metabolic activity) was measured using a microplate reader system (Thermo Fisher Scientific, Shanghai, China) at 492 nm.

Microscopic examinations of the biofilms that formed on microtiter plates were conducted by light microscopy using an inverted microscope (Zeiss, Oberkochen, Germany).

*Antifungal activity testing*

One clinically utilized antifungal agent, *i.e.*, fluconazole (Pfizer, Toluca, Mexico), and one alternative agent, *i.e.*, the essential oil of *A. sativum*, were used in this study. Fluconazole was prepared at a stock concentration of 1280 μg/ml in RPMI-1640 (Sigma). The essential oil of *A. sativum* was obtained by hydrodistillation at low pressure and low temperature and GC-MS, as described previously[6]; the oil distillation was performed by a distillation company (Javier Morales-López, distiller). Final concentrations of fluconazole and the essential oil of *A. sativum*, ranging from 0.5 to 128 μg/ml and from 7.8 to 1000 μg/ml, respectively, were tested. Prior to susceptibility testing, each isolate was sub-cultured on Sabouraud dextrose agar and CHROMagar *Candida* to ensure purity and viability. Susceptibility to fluconazole and the essential oil of *A. sativum* was tested *via* a broth microdilution assay according to M27-A3 using updates provided by M27-S4 of the Clinical & Laboratory Standards Institute (CLSI).[12,13]

RPMI-1640 medium without bicarbonate was prepared with L-glutamine and buffered at pH 7.0 (with 0.165M MOPS). Each yeast inoculum suspension was prepared using a spectrophotometer to obtain a final concentration of 0.5–2.5 × $10^3$ cells/ml. The trays were incubated in air at 35 °C, and minimal inhibitory concentration (MIC) endpoints were read visually after 24 h. To eliminate the effects of trailing growth, in cases with low growth, spectrophotometric endpoints for fluconazole were also determined at 24 h after stirring with wooden sticks, and readings were performed at 48 h. The visual and spectrophotometric endpoints were defined as the lowest drug concentrations that resulted in a prominent decrease in growth and a 50% reduction in optical density (OD), respectively, when compared with the data for a drug-free growth-control well. Quality control was performed by testing the *Candida* strains recommended by the CLSI, which included *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258.

The interpretive susceptibility criteria employed for fluconazole were the same as those specified by the CLSI in the M27-S4 document[13] and considered the following three categories: S = susceptible, SDD = susceptible dose-dependent, and R = resistant. Isolates of *C. krusei* are considered resistant to fluconazole, irrespective of the MIC.

Biofilms were washed thoroughly three times with sterile PBS prior to the addition of the antifungal agent, and free wells were included to serve as controls. Biofilm MICs were determined at 50% inhibition ($MIC_{50Biofilm}$) compared with drug-free control wells by utilizing the XTT reduction assay described previously. To evaluate the susceptibility of planktonic and biofilm *Candida* species to both the essential oil of *A. sativum* and fluconazole, the following performance criteria were considered: in the case of fluconazole, the breakpoints were those described by the CLSI in the M27-S4 document, and in the case of the oil of *A. sativum*, as there were no previously described cutoffs, the $MIC_{50}$ was considered as being reached when 50% inhibition was determined at the concentrations tested (1–1000 μg/ml).

**Table 1**
Prosthetic stomatitis frequency, biofilm formation (mean optical density $[OD]_{490}$), and filamentation capacity among *Candida* species isolates.

| *Candida* species ($N=48$) | Prosthetic stomatitis $n$ (%) | Biofilm [OD] 490 nm Mean (CI) | Filamentation $n$ (%) |
|---|---|---|---|
| *Candida albicans* ($n=25$) | 14 (56) | 2.0 (1.17–3.25) | 14 (56) |
| *Candida glabrata* ($n=11$) | 3 (27) | 2.23 (1.32–3.17) | NA |
| *Candida tropicalis* ($n=10$) | 2 (20) | 1.81 (1.02–2.95) | 7 (70) |
| *Candida krusei* ($n=2$) | 0 | 2.59 (2.1–3.08) | 1 (50) |

NA = not applicable. CI = confidence interval.

### *Statistical analysis*

A descriptive analysis was performed, categorizing the study population into the following three age groups: (I) 60–69 years, (II) 70–79 years, and (III) ≥80 years. Mann–Whitney *U* tests were performed to assess whether the age of the prosthesis generated differences with respect to the presence of denture stomatitis and filamentation. Kruskal–Wallis tests were also performed to analyze the categorized species of *Candida* and the $MIC_{50}$ of fluconazole.

The Spearman test was used to determine whether there was a correlation between prosthetic age and biofilm formation and between the fluconazole planktonic $MIC_{50}$ and the oil of *A. sativum* planktonic $MIC_{50}$. For all tests, a significance level of $p \leq 0.05$ was considered. Differences between categorical variables were evaluated using the chi-square test. Comparisons of the species distribution and MIC distribution were additionally performed using the chi-square test for categorical variables. For all statistical analyses, we used the statistical software SPSS ver. 20.0 for Windows (IBM; Chicago, IL, USA).

**Table 2**
Chemical composition of the essential oil used.

| Kovats index | Compound | Relative % of abundance |
|---|---|---|
| 904 | Allyl mercaptan | 0.20 |
| 960 | Methyl allyl sulfide | 0.51 |
| 1051 | 2-Butenol | 0.17 |
| 1078 | Dimethyl disulfide | 0.28 |
| 1097 | 2-Methyl-2-butenol | 0.32 |
| 1110 | 2-Methylene-4-pentanol | 0.69 |
| 1145 | Diallyl sulfide | 2.00 |
| 1261 | Methyl allyl disulfide | 9.18 |
| 1269 | 1-Propenyl methyl disulfide | 2.64 |
| 1369 | Dimethyl trisulfide | 2.43 |
| 1496 | Diallyl disulfide | 39.13 |
| 1533 | Methoxymethyl isothiocyanate | 1.75 |
| 1605 | Methyl allyl trisulfide | 15.43 |
| 1740 | 3,5-Dithiol-1,2,4-tritiolano | 0.15 |
| 1750 | 3-Vinyl-1,2-ditiociclohex-4-eno | 1.40 |
| 1763 | Dimethyl tetrasulfide | 0.18 |
| 1819 | Diallyl trisulfide | 21.62 |
| 1874 | 3-Vinyl-1,2-ditiociclohex-5-eno | 1.92 |
| Total | | 99.8 |

## Results

In total, 56 patients were studied, with 66% ($n=37$) female (average age, 61.2 years) and 34% ($n=19$) male (average age, 62.6 years). In all, 39.2% ($n=22$) had denture stomatitis, with 32% ($n=18$) of cases in the palate and 7.2% ($n=4$) in both the palate and the mandibular alveolar ridges. A total of 37 prostheses were colonized by one or more species of *Candida*, including 8 maxillary prostheses, 9 mandibular prostheses, and 20 maxillary/mandibular prostheses, from which 48 isolates were obtained. In all, 41 samples were total dentures, and 15 were partial dentures with acrylic bilateral support. No significant differences were observed in the analysis by type; however, a complete analysis of all prosthetic surfaces is presented. The most common species was *C. albicans*, with 25 isolates, followed by *C. glabrata*, with 11 isolates; *C. tropicalis*, with 10 isolates; and *C. krusei*, with two isolates.

The biofilm-forming capacity was evaluated through semi-quantitative spectrophotometric measurement of the reducing salt XTT at a wavelength of 492 nm, which provides OD values proportional to the number of viable cells forming biofilms. Based on the previously proposed classification,[39] it was determined that all isolates were strong producers of biofilms.

Considering the specific species, *C. krusei* had the highest values for biofilm formation, with an average OD at 490 nm of 2.59. *C. glabrata* was in second place, with the greatest capacity and an average OD at 490 nm of 2.23. Finally, *C. albicans* showed an average OD at 490 nm of 2.0, and *C. tropicalis* had an average OD at 490 nm of 1.81 (Table 1). Regarding the ability of the isolates to exhibit filamentation, there were no significant differences ($p=0.14$, $chi^2$ test) according to their clinical origin.

The characterization of the components of the essential oil of *A. sativum* is presented in Table 2. The $MIC_{50}$ values for the essential oil of *A. sativum* and fluconazole when applied to the clinical isolates of *Candida* in the planktonic cell and biofilm states were determined.

### *Antifungal activity of the essential oil of* A. sativum

#### *Planktonic cells*

The $MIC_{50}$ for all isolates studied in planktonic growth was between 31.2 and 1000 μg/ml. The $MIC_{50}$ values by species were as follows: 188 μg/ml for *C. tropicalis*, 236.2 μg/ml for *C. albicans*, 321 μg/ml for *C. glabrata* and 503 μg/ml for *C. krusei*. There were no essential-oil-resistant isolates at ≥1000 g/ml.

#### *Biofilm cells*

In the biofilm state, by species, the $MIC_{50}$ values were significantly higher: a $MIC_{50Biofilm}$ of 603.1 μg/ml was determined for *C. albicans*, followed by $MIC_{50Biofilm}$ values of 640.6 and 667 μg/ml for *C. glabrata* and *C. tropicalis*, respectively. For *C. krusei*, the $MIC_{50Biofilm}$ was >1000 μg/ml. Among all species, cases of resistance to the essential oil of *A. sativum* occurred, as evidenced by insensitivity to the highest concentration evaluated; accordingly, 24% of all isolates of *C. albicans* were resistant, and 54.5% of *C. glabrata*, 70% of *C. tropicalis*, and 100% of *C. krusei* isolates were resistant.

### *Antifungal activity of fluconazole*

#### *Planktonic cells*

The fluconazole $MIC_{50}$ in the planktonic state (or suspension) was 8.92 μg/ml for *C. albicans*, which was the species most susceptible to fluconazole. The second most sensitive species was *C. tropicalis*, with a $MIC_{50}$ of 11.1 μg/ml, and the least sensitive species were *C. glabrata* and *C. krusei*, with $MIC_{50}$ values of 13.6 and 19 μg/ml, respectively. In total, 60% of all *C. albicans* isolates and 18% of *C. glabrata* isolates were fluconazole resistant, and 82% of *C. glabrata* isolates were SDD. Additionally, *C. tropicalis* was

**Table 3**
Comparison of the antifungal activities of *Allium sativum* essential oil and fluconazole in biofilm and planktonic cells.

| *Candida* species ($N = 48$) | *Allium sativum* oil $MIC_{50}$, μg/ml | | | Fluconazole $MIC_{50}$, μg/ml | | |
|---|---|---|---|---|---|---|
| | Planktonic cells Mean (C.I.) | Biofilm cells Mean (C.I.) | *p* | Planktonic cells Mean (C.I.) | Biofilm cells Mean (C.I.) | *p* |
| *Candida albicans* ($n = 25$) | 236.2 (31.2–500) | 603.1 (62.5–1000) | ≤0.01 | 8.9 (0.5–17) | 64 (64–64) | ≤0.01 |
| *Candida glabrata* ($n = 11$) | 321 (31.2–500) | 640.6 (62.5–1000) | 0.09 | 13.6 (4–32) | 64 (64–64) | ≤0.01 |
| *Candida tropicalis* ($n = 10$) | 188 (125–250) | 667.0 (500–1000) | 0.08 | 11.1 (0.12–64) | >132 | ≤0.01 |
| *Candida krusei* ($n = 2$) | 503 (500–505) | >1000 | – | >132 | >132 | NA |

NA = not applicable. C.I. = confidence interval.

resistant in 30% of cases and SDD in 20% of cases, and overall *C. krusei* was resistant.

*Biofilm cells*

The fluconazole $MIC_{50Biofilm}$ for biofilm isolates was achieved only in two isolates of *C. albicans*, in one *C. glabrata* isolate, and in one *C. krusei* isolate; in all cases, the $MIC_{50Biofilm}$ was 64 μg/ml. According to the fluconazole breakpoints for *Candida* described by the CLSI in the M27-S4 document (the only benchmarks described), 100% resistance was observed for all species.

*The essential oil of* A. sativum *compared with fluconazole*

When comparing the percentages of sensitivity or resistance to the essential oil of *A. sativum* and fluconazole among the planktonic states of the different *Candida* species, a difference was determined without observing statistical significance in the chi-square test ($p = 0.12$), whereas a significant difference was observed in the biofilm state by species ($p < 0.01$). However, the susceptibility of the isolates to both antifungal agents was observed as a decrease in biofilm growth.

By comparing the concentrations required to produce inhibition of at least 50% of the growth of *Candida* in the planktonic and biofilm states, it was determined that the required concentration of the essential oil of *A. sativum* may be up to 300% higher for cells in biofilms. By contrast, the fluconazole concentration required to inhibit biofilms of *Candida* cells may be up to be 700% higher. The analysis of this difference is presented in Table 3.

There was also a significant difference ($p < 0.01$) observed between the $MIC_{50}$ and the $MIC_{50Biofilm}$ of the *A. sativum* oil in the analysis by species, and a significant difference was observed for *C. albicans* compared with the other species. Fluconazole exhibited no significant difference in its inhibition of *C. albicans* in biofilm or planktonic cells, but among the remaining *Candida* species, it was not possible to evaluate whether a difference was present due to the high resistance observed.

Although the range of denture use duration was broad, namely, <1 year to >20 years, when assessing whether the prosthetic age generated differences in the presence of stomatitis ($p = 0.33$) and filamentation ($p = 0.79$), no significant differences were found. With respect to the categorized *Candida* species ($p = 0.91$) and the $MIC_{50}$ of fluconazole ($p = 0.86$), no differences were found either. The Spearman correlation revealed that in our sample, there was no correlation of age with biofilm formation ($p = 0.21$), the $MIC_{50}$ of fluconazole in planktonic cells ($p = 0.75$) or the $MIC_{50}$ of the essential oil of *A. sativum* in planktonic cells ($p = 0.49$).

## Discussion

In this study, the *in vitro* susceptibility of different *Candida* species obtained from isolates of total dentures or partial dentures with acrylic bilateral support to both the oil of *A. sativum* and fluconazole was evaluated. Initially, when the *Candida* species were isolated from the surfaces of the dentures and typed, a prevalence of 52% was found for *C. albicans*, followed by 23% for *C. glabrata*, 21% for *C. tropicalis*, and 4% for *C. krusei*. These prevalence values were similar to those reported in the literature: in 2012, Ribeiro et al. specifically reported a prevalence of 64.4% for *C. albicans*, 14.4% for *C. glabrata*, and 3.3% for *C. tropicalis* when examining colonization of the surfaces of dental prostheses.[37]

Moreover, in 2013, Cavaleiro et al. reported the following frequencies of *Candida* colonizing the oral mucosa of healthy patients: 66% for *C. albicans*, 9% for *C. glabrata*, and 4.5% for *C. tropicalis*.[8] It has been noted that the *Candida* species that colonize prosthetic surfaces usually correspond to those found colonizing the oral mucosa of patients.[8]

Once a dental prosthesis is set in place, the microorganisms present in the mouth colonize its surface and eventually form a biofilm on the acrylic surface. Interestingly, in the current study, the presence of more than one species was observed in 17.8% of the sampled patients, and 30% of these exhibited sub-prosthetic stomatitis. According to de la Rosa-García et al. (2013), the presence of more than one species increases the risk of developing infections.[16]

Meanwhile, the percentage of patients with sub-prosthetic stomatitis was 39.2%; this frequency is low compared with other reports. In particular, De la Rosa-García et al. (2013) reported a frequency of 56.6% in a population of healthy subjects and patients with diabetes ($n = 99$).[16] Moreover, in the present study, it was found that the percentage of prostheses colonized by *Candida* species was 66%, similar to other reports: De la Rosa et al. (2012) found a 62.2% frequency of *Candida* colonization in prostheses.[17] When risk factors associated with the presence of sub-prosthetic stomatitis were analyzed, no statistical associations with a poor fit of the prosthesis ($p = 0.66$), colonization of the prosthesis ($p = 0.24$), or high consumption of carbohydrates ($p = 0.14$) were found. However, poor hygiene and nocturnal use of dental prostheses were factors that were significantly associated with the presence of sub-prosthetic stomatitis ($p < 0.01$ and $p = 0.01$, respectively). The microenvironment associated with poor hygiene at the contact surface of dental prostheses specifically promotes the development of *Candida* in addition to inhibiting the continuous flow of saliva, thereby favoring food accumulation and *Candida* adhesion to the surface. In addition, not removing prostheses at night does not allow the palatal epithelium and alveolar edges to recover from the trauma and maceration that exists under the prostheses. In this respect, our results are consistent with those previously reported in the literature.[43,44]

Regarding the biofilm formation capacity of *Candida* species in dental prostheses, "strong production" of biofilms was observed for all clinical isolates tested. In a previous study,[39] it was determined that for *C. albicans* and *C. tropicalis* isolated from dental prostheses, the average capacity to produce biofilms at a time point of 24 h was characterized by "moderate production", whereas in a similar time frame, *C. glabrata* was classified as a "strong producer" of biofilms.

In that work, a comparison was performed between isolates from the prostheses and the buccal mucosa of patients with diabetes mellitus, and slight differences regarding biofilm production between the isolates from the two groups were mentioned.[39] The

difference in the ability of the biofilm-forming isolates previously reported and the ability observed in the present study might be due to the differing origins of the isolates. It has been noted that isolates recovered from dental prostheses are found in biofilms, which increases their adhesiveness. In addition, the condition and age of prostheses are decisive in activating microorganisms' virulence factors and their capacity for survival in a complex ecosystem. In the present study, biofilms were quantified after 48 h of formation; this production time was higher than the previously reported time of 24 h.

For each of the clinical isolates in the present study, the $MIC_{50}$ of the essential oil of *A. sativum* was determined in both planktonic and biofilm cells. The $MIC_{50}$ of the *A. sativum* oil in planktonic growth of *C. albicans* was 236.2 μg/ml; for *C. glabrata*, it was 321 μg/ml; for *C. tropicalis*, it was 188 μg/ml; and for *C. krusei*, it was 503 μg/ml. Previous reports related to *A. sativum* refer to extracts of fresh garlic in aqueous medium and suspensions of garlic powder.[1,23,26,29,41]

Few reports have examined the concentrations of *A. sativum* extract required for a fungistatic or fungicidal effect on *Candida* species. Moreover, in the studies conducted to date, there is no consensus regarding MIC determination or the method of producing the garlic extract. In 2006, Shams-Ghahfarokhi et al. reported that the $MIC_{50}$ of an aqueous extract of *A. sativum* in planktonic isolates of *C. albicans*, *C. glabrata*, and *C. tropicalis* was 31.2 μg/ml.[41] In 2002, Lemar et al. conducted a study of a *C. albicans* strain and noted that its growth was inhibited by fresh garlic extract at a $MIC_{50}$ of 290 μg/ml.[29] Ghannoum reported a $MIC_{50}$ for an aqueous extract of *A. sativum* within a range of 800–1600 μg/ml for ATCC strains of *C. albicans* and *C. tropicalis*,[23] with these being the effective concentrations of the *A. sativum* essential oil obtained by hydrodistillation.[6] These values were determined in the studies by the method of microdilution and were able to affect clinical isolates obtained from biofilms developed on dental prostheses.

Other studies have evaluated uncharacterized extracts and employed different methodologies. In the present study, the $MIC_{50}$ for *C. albicans* was found to occur at a greater concentration than that reported by Lemar et al. in 2002.[29] However, those authors used YPD culture medium, whereas in the present study, we used RPMI-1640, which is the medium recommended by the standardized CLSI M27-A3 method.[13] In addition, in the present study, a characterized essential oil of *A. sativum* was evaluated, in contrast to the aqueous extract employed in the previous work by Lemar et al. (2002)[29]; differences in the components of the two substances remain to be analyzed. In the current study, an *A. sativum* essential oil obtained by a hydrodistillation process, in which 47 compounds were detected, was evaluated. Of the compounds, sulfur compounds prevailed in the composition, and these have been reported to possess therapeutic effects.[6] With respect to the biologically active components of *A. sativum* and their mechanism, allicin is a substance formed by exposing the organism's tissue to the oxygen in the air. This exposure releases the alliinase enzyme localized in the cell membrane, which acts on the substrate denominated alliin, subsequently releasing allicin. Allicin is an unstable substance that is a precursor of tri- and di-diallyl sulfide, the most abundant compounds in the essential oil.[2,3]

In the current study, the $MIC_{50}$ of the essential oil of *A. sativum* in *C. albicans* biofilm growth was 603.1 μg/ml; for *C. glabrata*, it was 640.6 μg/ml; and for *C. tropicalis*, it was 667 μg/ml. Furthermore, *C. krusei* demonstrated resistance to the concentrations employed. To our knowledge, there are no reports on the susceptibility of *Candida* species in biofilms to the essential oil of *A. sativum*. However, studies have examined the MICs of specific, active elements of garlic oil.[40] In the present study, the specific concentration of each of the active compounds in the essential oil of *A. sativum* was not determined, but the $MIC_{50}$ was clearly increased when cells were in the biofilm state because the structure of the biofilm delayed the flow of the compounds in question, together with virulence factors expressed by *Candida* when within a biofilm.[15,19,24,40]

When the $MIC_{50}$ of fluconazole in isolates in planktonic form was determined, the average $MIC_{50}$ of fluconazole for *C. albicans* was 8.92 μg/ml; for *C. glabrata*, it was 13.6 μg/ml; and for *C. tropicalis*, it was 11.11 μg/ml. The MICs of these *Candida* strains can be interpreted as follows based on the cutoffs proposed by the CLSI[12]: *C. albicans* was fluconazole resistant in 60% of cases and SDD in 16% of cases, the susceptibility of *C. glabrata* was dose dependent (SDD) in 82% of cases and resistant in 18% of cases, and *C. tropicalis* was resistant in 30% of cases and SDD in 20% of cases according to the latest CLSI report (2012). The CLSI does not consider fluconazole cutoffs for *C. krusei* because this species is considered intrinsically resistant to the antifungal agent. Ben-Ami et al. reported a resistance to fluconazole of 1.6% for *C. glabrata* in their total samples[4]; in the current study, fluconazole resistance was found in 46% of cases, with 31% SDD for this species in particular. This difference in the percentage of resistance was probably due to the characteristics of the isolated strains, associated with the site of sample collection: our samples were specifically taken from the surfaces of dental prostheses. It is likely that species isolated from a biofilm express genes that enhance their resistance and virulence due to the different types of stresses that occur in the medium in which they grow.[7]

In 2012, Cleveland et al. conducted a study in which two populations from different regions of the U.S. were sampled. The authors reported that there were differences in the percentages of resistance of *Candida* species isolated from the different regions; however, the percentage of resistance reported for *C. albicans* was 2% in both populations. Meanwhile, the percentages of resistance reported for *C. glabrata, C. tropicalis*, and *C. krusei* were 11 and 13%, 4 and 9%, and 100% and 100%, respectively, for the two populations.[11] As mentioned previously, the differences in the percentages of resistance may be due to differences in the sampling site and changes in the cutoffs recently proposed by the CLSI. In the present study, the samples were taken from patients at risk for candidemia, similarly to what was performed in the report by Cleveland et al., who reported differences in resistance between the populations of different geographical areas, but the percentage of difference is not large.[11]

Regarding the $MIC_{50}$ of fluconazole for *Candida* species in biofilms, the $MIC_{50}$ was 64 μg/ml and was only effective against isolates of *C. albicans* and *C. glabrata.* The percentages of resistance were markedly higher: all species showed fluconazole resistance of 100%. The $MIC_{50}$ necessary to reach and inhibit *Candida* species in sessile growth is up to 7 times higher than the $MIC_{50}$ necessary to inhibit planktonic growth. In 2001, Chandra et al. reported that resistance to fluconazole is related to the phases of biofilm development.[9] In the current study, the phase in which we applied the antifungal agent was during early maturation. In 2003, Mukherjee et al. reported that in certain species of *Candida*, the MIC can decrease during the first phase of biofilm formation (the first 6 h)[32]; this may explain why the $MIC_{50}$ values were so high in our study. In 2001, Ramage et al. reported $MIC_{50}$ values >1024 μg/ml for an ATCC strain of *C. albicans*.[36,39] Fu et al. (2014) reported a gradual increase in the $MIC_{50}$ in relation to the time of biofilm formation: at 2 and 4 h, the $MIC_{50}$ was 1 μg/ml; at 8 h, it was 8 μg/ml; and at 24 h, it was >1024 μg/ml.[22] As previously mentioned, resistance is related to the phase of biofilm formation; according to what has been described, as the biofilm matures, higher concentrations are required to inhibit *Candida*, and this observed mechanism is affected by the arrangement of the drug on the cell in question.

Due to the rigorous methodology employed and the extensive validation by different authors, the present study provides important microbiological data on the *in vitro* sensitivity of *Candida*

species to the essential oil of *A. sativum*, allowing us to determine the specific concentrations at which the species' growth is inhibited not only as cells in suspension but also as cells in biofilms.

## Conclusion

The essential oil of *A. sativum* is more effective than fluconazole in inhibiting both planktonic cells *in vitro* and biofilms of *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. krusei* isolated from dental prostheses.

## Conflict of interest

There is no conflict of interest, either real or potential, for any of the authors; that is, no financial relationships, personal relationships, academic or intellectual competition is present.

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