



Chemical composition of essential oils from the apiaceae family, cytotoxicity, and their antifungal activity *in vitro* against *Candida* species from oral cavity

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Abstract

The aims of this research were: evaluate the chemical composition and the cytotoxicity of the *Cuminum cyminum* (cumin), *Anethum graveolens* (dill), *Pimpinella anisum* (anise) and *Foeniculum vulgare* (fennel) essential oils, as well as their antifungal activity *in vitro* against ten *Candida* spp. isolates. The chemical composition of the oils was analyzed by means of gas chromatography coupled with mass spectrometry (GC/MS). The cytotoxicity assays were performed, using the cell proliferation reagent WST-1 in L929 mouse fibroblasts (20×10^3 well⁻¹). The determinate the Minimum Inhibitory Concentration (MIC), was performed through the Broth Microdilution technique (CLSI). The chemical main components were the cuminaldehyde (32.66%) for cumin, carvone (34.89%) for the dill, *trans*-anethole (94.01%) for the anise and anethole (79.62%) for the fennel. Anise and fennel did not were cytotoxic in all the tested concentrations, however the cumin oil was cytotoxic in the concentration of 20 mg.mL⁻¹ and the dill in the concentrations of 20 and 8 mg.mL⁻¹. All yeasts were susceptible against the evaluated essential oils. Cumin presented the lowest MIC against yeasts. We concluded that all the essential oils presented inhibitory action against *Candida* spp., and *C. cyminum*, *P. anisum* and *F. vulgare* were not cytotoxic in the same minimum inhibitory concentrations for the fungi.

Keywords: *Cuminum cyminum*, Chronic Atrophic Candidiasis, *Candida* spp., antifungal.

Composição química de óleos essenciais da família apiaceae, citotoxicidade e sua atividade antifúngica *in vitro* contra espécies de *Candida* da cavidade oral

Resumo

Os objetivos desta pesquisa foram: avaliar a composição química e a citotoxicidade dos óleos essenciais de *Cuminum cyminum* (cominho), *Anethum graveolens* (endro), *Pimpinella anisum* (erva-doce) e *Foeniculum vulgare* (funcho), bem como sua atividade antifúngica *in vitro* contra dez isolados de *Candida* spp.. A composição química dos óleos foi analisada por meio de cromatografia gasosa acoplada à espectrometria de massa (GC / MS). Os ensaios de citotoxicidade foram realizados, utilizando o reagente de proliferação celular WST-1 em fibroblastos de ratinho L929 (20×10^3 poço-1). A determinação da Concentração Inibitória Mínima (MIC) foi realizada através da técnica de microdiluição em caldo (CLSI). Os principais componentes químicos foram o cuminaldeído (32.66%) para cominho, carvona (34.89%) para o endro, *trans*-anetol (94.01%) para erva-doce e anetol (79.62%) para a funcho. O endro e a erva-doce não foram citotóxicos em todas as concentrações testadas, no entanto, o óleo de cominho foi citotóxico na concentração de 20 mg.mL⁻¹ e o endro nas concentrações de 20 e 8 mg.mL⁻¹. Todas as leveduras foram suscetíveis aos óleos essenciais avaliados. O cominho apresentou a menor CIM contra as leveduras. Concluímos que todos os óleos essenciais apresentaram ação inibidora contra *Candida* spp., e *C. cyminum*, *P. anisum* e *F. vulgare* não foram citotóxicos nas mesmas concentrações inibitórias mínimas para os fungos.

Palavras-chave: *Cuminum cyminum*, Candidíase atrófica crônica, *Candida* spp., antifúngico.

1. Introduction

Candida spp. are the most common cause of fungal infections in humans, are important commensals microorganisms from the oral cavity, turning them pathogenic, if favorable conditions related to the host low immunity exist. Infections commonly known as oral candidiasis or moniliasis, have different clinical forms, such as pseudomembranous candidiasis, commonly known as thrush, erythematous candidiasis, hyperplastic and mucocutaneous candidiasis, being common problems faced by dental professionals (Simões et al., 2013).

Clinical Chronic Atrophic Oral Candidiasis or denture stomatitis is an erythematous oral candidiasis, characterized by different degrees of erythema and hemorrhagic petechiae, localized in the edge of the denture area of a superior removable prosthesis. This reaction can result from; an inappropriate denture design, an allergy at its base or by an acrylic inadequate polymerization (Moreira et al., 2002).

In the last decade, the infections by *Candida* species had been growing in an alarming speed, as well as their resistance development towards antifungal drugs commonly used with unwished reactions in patients. There by, new alternative products are proposed to minimized such of these negative facts (Lima et al., 2006; Pozzatti, 2007).

By the above, fitoherapeutic alternatives, such as essential oils, have entered into the market, with the purpose to reduce the costs of production, this is why rigorous tests are required to control their quality in relation to their effectiveness, antimicrobial activity, security and toxicity when compared to others types of synthetic drugs, as well as the reduce the side effects in patients (Castro and Lima, 2010; Kamble, 2015).

Among the many species of aromatic plants, the ones from the family Apiaceae had been investigated for medical use, due to their antimicrobial properties. These plants produce essential oils, characterized for releasing a relative high amount of volatile compounds (Simões et al., 2010). Among some representatives species of the Apiaceae family, we can mention; the *Cuminum cyminum* (cumin), *Anethum graveolens* (dill), *Pimpinella anisum* (anise) and *Foeniculum vulgare* (fennel) and the fennel (*Foeniculum vulgare*), plants with culinary and medical applications (Downie et al., 2000).

The aim of this research was to evaluate the cytotoxicity and chemical composition of the *C. cyminum*, *A. graveolens*, *P. anisum* and *F. vulgare* essential oils, as well as their *in vitro* antifungal activity against *Candida* spp. isolates from clinical Chronic Atrophic Oral Candidiasis cases.

2. Material and Methods

2.1. Research location and fungal isolates

The microbial assays were performed in the Laboratory of Mycology located in the Biology Institute from the Federal University of Pelotas (UFPel). The isolates tested were obtained from the mycology collection of the Oral Microbiology Laboratory in the Faculty of Odontology,

from clinical Chronic Atrophic Oral Candidiasis cases, being the following *Candida* species: *Candida albicans* (23778, 23651, 23609, 24073 e 23536), *Candida parapsilosis* (2340 e 23459), *Candida glabrata* (23453), *Candida krusei* (23932) and *C. albicans* ATCC (62342).

2.2. Essential oils

The *C. cyminum*, *A. graveolens*, *P. anisum* and *F. vulgare* essential oils were obtained through the Department of Organic Chemistry of the Chemical Science, Pharmaceutics and Food Center (CCQFA) of the UFPel, by means of hydrodistillation (1.5 L of distilled H₂O / 100 g of plant material) in a Clevenger apparatus. The plants were commercially acquired from the Luar Sul L.T.D.A., company and the parts used were: seeds, for the cumin, dill and anise; and leaves for the fennel. The essential oil yield for each plant was of 2.5 mL for the cumin and anise, 1.9mL for the dill and 2.3 mL for the fennel.

2.3. Chromatographic analysis of the essential oils

The essential oils were submitted to chromatography analysis coupled with mass spectrometry (GC/MS) in a Shimadzu QP2010 apparatus, equipped with split/splitless injector with capillary column Rtx-5MS RESTEK (30 m × 0,25 mm × 0,25 µm), in the following chromatographic conditions: Helium gas as carrier, obtained fragments by the impact of the electrons at the energy of 70 eV, flow 1.2 ml/min, split 1:10, 1 µL of sample injected volume. Oven programmed temperature: Initial temperature of 40 °C, with a heating ramp at 5 °C/min to 280 °C, remaining stable in this temperature for 10 minutes, with a total time of 8 minutes, being the injector temperature of 58°C and the interface of 200 °C. The compounds were analyzed according to the chromatographic standards, based on the GC/MS NIST08 library. The oil was diluted in hexane (analytical grade, ultra-pure).

2.4. Cytotoxicity assay

The cytotoxicity assay was realized by the Department of Restorative Dentistry, School of Dentistry, from the Federal University of Pelotas (UFPel). The cell viability was performed according to the ISO 10993-5 (ISO, 2009). Mouse fibroblasts cell line L929 (20 × 10³ well⁻¹) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% of fetal bovine serum (FBS), 2% of L -glutamine, penicillin (100 U.ml⁻¹) and streptomycin (100 mg.ml⁻¹) using a plate of 96-wells, incubated for 24 h at 37 °C in a humidified atmosphere of CO₂ 5% in air until sub confluence was achieved. The essential oils were made by a mold (6 mm diameter and 1 mm depth), and photo-activated by Radium Cal (n = 5). The essential oils were diluted and tested at concentrations of 20, 8, 4 e 2 mg.mL⁻¹. After 24 hours, 200 µl of each dilution oils were transferred to the 96-well plates previously prepared and incubated for 24 h. WST-1 assay was used to assess cell metabolic function by mitochondrial dehydrogenase activity. The absorbance at 450nm was measured via a microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA, USA). Each assay was performed in triplicate.

Statistical analysis was performed using Kruskal-Wallis (One Way Analysis of Variance on Ranks) followed by Turkey complementary test. Cell viability values above 70% indicate that the chemical compound was not cytotoxic, according ISO 10993-5 (2009).

2.5. Minimum Inhibitory Concentration (MIC)

The determination of the Minimum Inhibitory Concentrations of the essential oils was performed using the Broth Microdilution technique, in accordance with the document M27-A4 by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2013), adapted to phyto pharmaceuticals. Initially, 100 μ L of RPMI media - 1640 (DIFCO®, Detroit, Michigan, EUA) were distributed in each well of the microdilution plate which contains 96 wells with a “U” bottom form. For the MIC evaluation of the essential oils, 10 successive dilutions in RPMI medium were performed; obtaining concentrations of 70 to 0.137 mg.mL⁻¹, arranged in a volume of 100 μ L according to the sense of the lines in the microdilution plate. In the each well of each column, aliquots of 100 μ L of each inoculum of each corresponding *Candida* species were added, for further analysis. The yeast's suspension was adjusted in a spectrophotometer (530 nm) to obtain a final concentration of approximately 5×10^6 cells mL⁻¹.

The tests were performed by duplicate, with three repetitions; a column used for the positive control (inoculum/medium)

and another column for the negative control (essential oil/medium).

Jointly the same procedure was performed with the fluconazole, considered a model in clinical use, in successive concentrations, from 16 μ g.mL⁻¹ to 0.0313 μ g.mL⁻¹ (Clinical and Laboratory Standards Institute, 2013), by the microdilution technique.

The microplates were incubated at 35 °C for 48 hours. The lecture for the MIC determination of the essential oils against the yeasts strains was performed through turbidity visualization, related to the microorganism growth and compared to the positive and negative controls. It was considered as MIC, the minor concentration of the drug capable to produce growth inhibition of the yeasts, compared with the positive control well (Clinical and Laboratory Standards Institute, 2013).

3. Results

In Table 1, the majority compound present in the cumin essential oil was the cuminaldehyde (32.66%), for the dill the carvone (34.89%), for the anise the *trans*-anethole (94.01%) and for the fennel the anethole (79.62%).

In the present study, cytotoxicity assays showed that only cumin and dill oils were cytotoxicity, since present below of 70% of cell viability (Figure 1). In relation to the yeasts' MIC, the cumin, anise and fennel did not shown

Table 1. Main chemical compounds identified in the *Cuminum cyminum*, *Anethum graveolens*, *Pimpinella anisum* and *Foeniculum vulgare* essential oils, using the chromatography GC/MS technic.

Chemical Compounds	Essential Oils			
	Cumin	Dill	Anise	Fennel
Cuminaldehyde	32.66%			
γ -terpinene	19.87%			
β -pinene	15.22%			
o-cymene	14.00%			
2-careen-10-al	8.54%			
1-fenil-1-butanol	8.01%			
D-limonene	1.7%	18.92%		
<i>trans</i> -anethole			94.01%	
Estragole <i>cis</i> -anethole			2.46%	3.65%
<i>cis</i> -(-)-2,4a,5,6,9a-Hexahydro-3,5,5,9-tetramethyl(1H)benzocycloheptene <i>cis</i> -anethole			1.7%	
(+)-Carvone			0.83%	
Di-epi-alpha-cedrene			0.29%	
Limonene			0.24%	
p-Cumic aldehyde			0.18%	
beta-Chamigrene			0.16%	
Carvone		34.89%		
Apiol		22.02%		
Dihydrocarvone		16.91%		
Trans-dihydrocarvone Anethole		7.26%		79.62%
Fenchone				12.19%
Methyl nonadecanoate				2.89%
1R- α -pinene				1.65%

cytotoxicity, however the dill oil in the concentrations of 20 and 8 mg.mL⁻¹ were cytotoxic related to the MIC.

The antifungal tests with ten *Candida* species demonstrated that all tested yeasts showed susceptibility against the evaluated essential oils, being that the cumin (*Cuminum cyminum*) essential oil, showed the best antifungal activity, with the lowest MICs, that varied from 2.188 to 4.375 mg.mL⁻¹. The essential oil of fennel (*Foeniculum vulgare*) also showed low minimum inhibitory concentration (MIC: 4.375 mg.mL⁻¹) against *Candida albicans* isolate (24073), however, this was equal to the CMI verified for cumin (Table 2).

4. Discussion

Due to the problems faced by patients carrying oral candidiasis, especially clinical Chronic Atrophic oral Candidiasis, mainly by the *Candida* species resistance against antifungal drugs commonly use, the search of new compounds, such as essential oils, which have antimicrobial actions against resistant microorganisms, are necessary. As result of the present study, it was verified that the essential oils of *C. cyminum*, *A. graveolens*, *P. anisum* and *F. vulgare* showed antifungal activity against ten

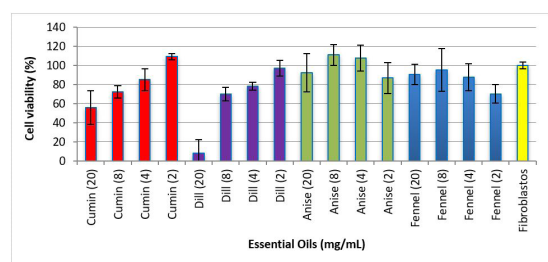


Figure 1. Cytotoxicity activity *in vitro* of the *Cuminum cyminum*, *Anethum graveolens*, *Pimpinella anisum* and *Foeniculum vulgare* essential oils in Mouse fibroblasts L929 (20×10³ well⁻¹) cells.

Table 2. Inhibitory Minimum Concentration (mg.mL⁻¹) of the *Cuminum cyminum*, *Anethum graveolens*, *Pimpinella anisum* and *Foeniculum vulgare* essential oils against the ten *Candida* species isolates.

Essential Oils Yeasts	Cumin	Dill	Anise	Fennel
<i>C. albicans</i> ATCC (62342)	8.75	8.75	8.75	8.75
<i>C. albicans</i> (23778)	4.375	8.75	8.75	8.75
<i>C. albicans</i> (23651)	4.375	8.75	8.75	8.75
<i>C. albicans</i> (23609)	4.375	8.75	8.75	8.75
<i>C. albicans</i> (24073)	4.375	8.75	8.75	4.375
<i>C. albicans</i> (23536)	4.375	8.75	8.75	8.75
<i>C. parapsilosis</i> (2340)	4.375	8.75	8.75	8.75
<i>C. parapsilosis</i> (23459)	2.188	8.75	8.75	8.75
<i>C. glabrata</i> (23453)	4.375	17.5	8.75	8.75
<i>C. krusei</i> (23932)	4.375	8.75	8.75	8.75

tested oral *Candida* species, all isolates were sensitive to the synthetic antifungal fluconazole, used as control.

Several studies have shown the excellent therapeutic properties of essential oils, meanwhile, in the assays of antifungal *in vitro* activity of the oils, it is possible to verified a variety of methodologies proposed, such are the agar diffusion, the disk diffusion and the broth dilution (Kosalec et al., 2005; Gulfranz et al., 2008; Pai et al., 2010; Wanner et al., 2010; Cavalcanti et al., 2011; Cleff et al., 2013; Kamble, 2015), making hard to compared between the present study and these studies. There by, the recognition of the pharmacologic activity of the presented plants in this study represents important information to evaluate the obtained viability of the product in clinical use.

In our results, the *C. cyminum* essential oil had the lowest inhibitory concentration against *Candida* species, compared to the other oils. Results with inhibition values and good antimicrobial activity of *C. cyminum*, against the *Candida* species, were also registered by other authors. Shetty et al. (1994), using the agar diffusion method, founding positive results, however with a minor inhibitory concentration of 0,10 mg.mL⁻¹. Pai et al. (2010) and Wanner et al. (2010) used the disk diffusion method, confirming the cumin as an anti-*Candida* potential agent. Kamble (2015), had good inhibition results against *Candida albicans* and not *albicans* using the disk diffusion and the broth micro and macrodilution techniques (Minimum Inhibitory Concentration).

C. parapsilosis (23459) had the highest susceptibility against cumin essential oil (CIM de 2,188 mg.mL⁻¹), meanwhile the others isolates showed inhibition at higher concentrations, to this and the others essential oils.

In the dill, anise and fennel essential oils, the antifungal activity was practically the same against *Candida* spp. isolates, having few variations. In other works using the dill essential oil, the antifungal activity against *A. graveolens* was analyzed and confirmed against the *Candida* spp. clinical isolates. In the study by Zeng et al. (2011), using the broth microdilution technic (MIC), microbiological and histological technics in mice, demonstrated that dill was active *in vitro* and *in vivo* against all the *Candida* species tested. Yili et al. (2009), analyzed the MIC through the Barry method, with an ethylene oxide addition, and they found inhibition results of 0.00273 mg.mL⁻¹ in *C. albicans*, affirming that the yeast growth, sharply decreased with this component addition in the nutrient medium.

In studies using anise, Kosalec et al. (2005), evaluated the antifungal activity of the extract fluid and essential oil of *P. anisum* in *Candida* species, through the agar diffusion and broth dilution methods and verified that the essential oil exhibit a low minimum inhibitory concentration *in vitro* against yeasts.

As for the fennel essential oil, Gulfranz et al. (2008) and Pai et al. (2010) also confirmed the effective antifungal effect of the oil of *F. vulgare* against *C. albicans*, through the disk diffusion method, however, Skrobonja et al. (2013), in one of the used technics, found results *in vitro* with MICs of 0.06 and 0.02 mg. mL⁻¹ against this yeast.

In this research, the antifungal activity determination of the oils was performed through the broth microdilution technic (Clinical and Laboratory Standards Institute, 2013), with the aim to proportionate a major contact between the tested products and the fungic cells (Cavalcanti et al., 2011), being the MIC obtained one of the better patterns in evaluating the antifungal activity of the essential oils (Pozzatti, 2007).

According to Menezes et al. (2009) and Cavalcanti et al. (2011), vegetal products with good antimicrobial activity must present a MIC equal or inferior to 100 mg.mL⁻¹, and in the present study, 97.5% of essential oils were equal to or below 8.75 mg.mL⁻¹, demonstrating an adequate inhibitory activity in low concentrations and appropriate antifungal activity, with the application capacity of these compounds as antimicrobial substances in dental practice.

The higher and lower biological activity of the essential oils has been dependent to its composition, in other words of his chemicals constituents (Lima et al., 2006).

Elevated concentrations of these constituents and their proportions are of great importance in the product efficacy, confirming their properties and medicals applications. However, studies demonstrated that these isolated compounds don't have the same efficacy of the essential oil (Simões et al., 2010; Yili et al., 2009).

In the chromatographic analysis of the *C. cyminum* essential oil, the majority constituents were Cuminaldehyde, γ -terpinene, β -pinene, *o*-cymene, 2-carene-10-al, 1-phenyl-1-butanol and D-limonene agreeing with the results found by others authors, only with few variations (Shetty et al., 1994; Wanner et al., 2010; Kamble, 2015).

In the chromatography analysis of the essential oil of *A. graveolens*, the principal identified constituents were Carvone, Apiol, D-limonene, Dihydrocarvone and Trans-dihydrocarvone agreeing with the studies by Zeng et al. (2011); Tian et al. (2011), featuring the carvone, limonene and apiol as the majority compounds.

In the essential oil of *P. anisum*, the principal compounds found were *trans*-anethole and Estragole. Other constituents of the oil present in lower quantity were *cis*-(-)-2,4a,5,6,9a-Hexahydro-3,5,5,9-tetramethyl(1H) benzocycloheptene, *cis*-anethole, (+)-Carvone, Di-epi-alpha-cedrene, Limonene, p-Cumic aldehyde and beta-Chamigrene. Similar results were reported in other Works (Shojaii and Fard, 2012; Ozcan and Chalchat, 2006).

In the chromatography result of *F. vulgare*, the following constituents were verified in higher quantity: Anethole, Fenchone, Estragol, methyl nonadecanoate and 1R- α -pinene. Other studies also confirmed such identified components as the main (Anwar et al., 2009; Peixoto et al., 2014).

The antimicrobial activity of the essential oils is attributed to their principal components and to the resulted synergic or antagonist action. However, the minor components also contribute to their biologic activity (Iacobellis et al., 2005).

In the present study, the cell viability test WST-1 in the Mouse fibroblasts L929 (20 × 10³ well⁻¹) was made for cytotoxicity evaluation. We sought, with this analysis; information in relation to the biological behavior of the

oils, so that they can subsequently be used for *in vivo* studies. The cytotoxicity evaluation provides a security parameter in the compounds utilization having potential therapeutic activity (Yamaguchi et al., 2013). As for the cytotoxicity assay, the oils of cumin, anise and fennel were not cytotoxic in the same minimum inhibitory concentrations found for the tested yeast. Such results suggest that these oils showed to be promising models in the selection of natural products with antifungal activity, to prevent and treat fungal infections in oral tissue.

Given the above, it is possible note the need to investigate the potential application of these phytotherapeutic oils in the formulation of dental products such as in the prevention and treatment of candidiasis on dental prostheses, which indicates conducting further research to knowlede the effectiveness of these oil constituents in the formulation of these new dental products.

The essential oils of different species of the Apiaceae family, proved to be a promising source of biomolecules, with antifungal potential activity. Inside the experimental conditions of the present study, it was possible to conclude that the *C. cyminum* essential oil exhibit the lowest MIC against the tested *Candida* species, and cumin, anise and fennel essential oils were not cytotoxic to Mouse fibroblasts in the same minimum inhibitory concentrations found for the tested yeast. However, other specific *in vivo* studies and clinical assays about the security and efficacy of these oils are necessary, to evaluate the relevance practice of the results obtained *in vitro*, for the future development of new products with possible therapeutic applications.

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