IRLAN DE ALMEIDA FREIRES

CORIANDRUM SATIVUM L. (CORIANDER) ESSENTIAL OIL: ANTIFUNGAL ACTIVITY AND MODE OF ACTION ON *CANDIDA* SPP., AND MOLECULAR TARGETS AFFECTED IN THE HUMAN WHOLE-GENOME EXPRESSION

ATIVIDADE ANTIFÚNGICA E MODO DE AÇÃO DO ÓLEO ESSENCIAL DE *Coriandrum sativum* L. (COENTRO) SOBRE *Candida* spp. E ALVOS MOLECULARES AFETADOS NA EXPRESSÃO DO GENOMA HUMANO

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Faculdade de Odontologia de Piracicaba

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Dissertation presented to Piracicaba School of Dentistry, State University of Campinas, as part of the fulfilments required for the degree of Master in Dentistry, in the area of Pharmacology, Anesthesiology and Therapeutics.

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Orientador: Prof. Dr. Pedro Luiz Rosalen
Co-orientadora: Profa. Dra. Marta Cristina Teixeira Duarte

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RESUMO

Introdução: A candidíase oral é uma infecção fúngica oportunista da cavidade oral, cujas taxas de prevalência e incidência vêm aumentando significativamente em todo o mundo. Assim, novas estratégias orientadas para gerir esta doença têm sido propostas, dentre as quais está o uso de óleos essenciais (OE) com propriedades antifúngicas. Evidências indicam que o OE de *Coriandrum sativum* L. (coentro) é um forte agente antifúngico contra *Candida* e, portanto, investigações devem dar continuidade ao conhecimento gerado. **Objetivo:** Este estudo buscou avaliar a atividade antifúngica e modo de ação do OE de *C. sativum* sobre *Candida* spp., e determinar os alvos moleculares afetados na expressão global do genoma humano. **Material e Métodos:** *C. sativum* foi obtido a partir do Banco de Germoplasmas do Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas (Universidade Estadual de Campinas, SP, Brasil) cujo OE e fração ativa tiveram o perfil fitoquímico determinado por cromatografia gasosa acoplada a espectrometria de massa. Posteriormente, foram realizados testes com cinco cepas de referência de *Candida*: Determinação da Concentração Inibitória e Fungicida Mínima (CIM/CFM); modo de ação antifúngica (ensaio do sorbitol e ergosterol); Microscopia Eletrônica de Varredura (MEV) de biofilmes de *Candida* e testes de inibição de aderência em biofilme. Utilizou-se nistatina, anfotericina B ou caspofungina como controles positivos, além de controles negativos. Também, foi testado o efeito do OE sobre a atividade proteolítica de *C. albicans*. Por fim, um ensaio de farmacogenômica identificou quais alvos moleculares no genoma humano foram afetados pelo OE e fração ativa de *C. sativum*. Os testes foram realizados em triplicata de experimentos independentes e os dados foram tratados estatisticamente (ANOVA, pós-teste de Tukey, α=0,05). Os dados da análise farmacogenômica foram processados nas plataformas GeneGo MetaCore® e David Bioinformatics Resources. **Resultados:** O perfil fitoquímico EO indicou monoterpenos (37,9%) e sesquiterpenos (62,1%) como compostos principais. Os valores de CIM/CFM para o OE variaram de 15,6 a 62,5 µg/mL. Quanto ao modo de ação, o OE de *C. sativum* parece se ligar ao ergosterol da membrana celular fúngica, aumentando a permeabilidade iônica e causando morte celular; entretanto, o OE não atua sobre vias de biossíntese da parede celular. Estes achados confirmam as alterações na integridade da morfologia do biofilme verificadas nas análises por MEV. Além disso, o OE apresentou atividade antiaderente em biofilme em baixas concentrações (15,6-62,5 µg/mL) contra as cepas testadas, bem como atividade contra proteases produzidas por *C. albicans*, sendo estatisticamente significante na CIM (p<0,05). Finalmente, o OE e sua fração ativa apresentaram baixa citotoxicidade em células humanas com CI_{30} de 359,8 e 366,7 µg/mL, respectivamente. As principais vias afetadas estão relacionadas com quimiocinas e MAP-quinase (apoptose, proliferação) bem como proteínas de adesão. **Conclusões:** O OE das folhas de *C. sativum* tem forte atividade antifúngica e antiaderente sobre *Candida* spp. e atividade anti-proteolítica sobre *C. albicans*, e atua aumentando a permeabilidade iônica da membrana celular, provavelmente devido ao efeito sinérgico de mono e sesquiterpenos. Análise farmacogenômica indicou baixa citotoxicidade do OE e sua fração ativa com alvos moleculares específicos afetados no genoma humano, o que incentiva o desenvolvimento de novas pesquisas pré-clínicas toxicológicas e clínicas nesta área.

Palavras-chave: *Candida* spp., Óleos Essenciais, Coentro, Antifúngicos, Farmacogenômica.

ABSTRACT

Introduction: Oral candidiasis is a common opportunistic fungal infection of the oral cavity with increasingly significant worldwide prevalence and incidence rates. Accordingly, novel specific-targeted strategies to manage this ailment have been proposed, among which is the use of essential oils (EO) with antifungal properties. *Coriandrum sativum* L. (coriander) EO has proven antifungal activity against *Candida* species and thus deserves further investigation. **Objective:** This study aimed to evaluate the antifungal activity and mode of action of the EO from *Coriandrum sativum* L. leaves on *Candida* spp., and to determine the molecular targets affected in the whole-genome expression in human cells. **Material and Methods:** *C. sativum* was obtained from the Germoplasm Bank of the Research Center for Chemistry, Biology and Agriculture (University of Campinas, SP, Brazil) whose EO and active fraction had their phytochemical profile determined by Gas Chromatography coupled to Mass Spectrometry. Then, we carried out the following tests with five reference strains of *Candida* spp. (CBS): Minimum Inhibitory and Fungicidal Concentration (MIC/MFC); antifungal mode of action (sorbitol and ergosterol assays); Scanning Electron Microscopy (SEM) analysis of *Candida* biofilm and tests of inhibition of biofilm adherence. We used nystatin, amphotericin B or caspofungin as positive controls, in addition to negative controls. Also, we tested the effect of *C. sativum* EO on the proteolytic activity of *C. albicans.* Next, a pharmacogenomic assay identified which molecular targets in the human genome were affected by *C. sativum* EO and its active fraction. Tests were performed in triplicate of independent experiments and data were statistically treated (ANOVA, Tukey's post-test, α=0.05). Pharmacogenomic data were processed on GeneGo MetaCore® and DAVID Bioinformatics Resources. **Results:** The EO phytochemical profile indicated monoterpenes (37.9 %) and sesquiterpenes (62.1 %) as major compounds. The MIC/MFC values for the EO ranged from 15.6 to 62.5 µg/ml. With regard to the mode of action, *C. sativum* EO may bind to membrane ergosterol, increasing ionic permeability and causing membrane damage to cell death, but it does not act on cell wall biosynthesis-related pathways. This mode of action confirms the changes in the integrity of the biofilm morphology as verified in the analyses by SEM. The EO showed anti-adherent activity at low concentrations (31.2 – 62.5 µg/ml) against all strains tested, as well as activity against proteases produced by *C. albicans*, with statistical significance at MIC ($P < 0.05$). Finally, the EO and its active fraction had low cytotoxicity on human cells with IC_{30} of 359.8 and 366.7 µg/ml, respectively, affecting the pathways of chemokines and MAP-kinase (apoptosis, proliferation), as well as adhesion proteins. **Conclusions:** The EO from *C. sativum* leaves has strong antifungal and anti-adherent activity against *Candida* spp., as well as anti-proteolytic activity against *C. albicans*, and acts by increasing cell membrane ionic permeability, probably due to the synergistic effect of mono- and sesquiterpenes. Pharmacogenomic analyses revealed low cytotoxicity with specific targets affected in the human genome, which encourages further pre-clinical toxicological screening and clinical research in this field.

Key-words: *Candida* spp., Essential Oils, Coriander, Antifungals, Pharmacogenomics.

SUMÁRIO

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[...Gosto de ser gente porque, inacabado, sei que sou um ser condicionado mas, consciente do inacabamento, sei que posso ir mais além dele. Esta é a diferença profunda entre o ser condicionado e o ser determinado].

(Paulo Freire)

LISTA DE ABREVIATURAS E SIGLAS[1]

CBS – *Central Bureau voor Schimmelcultures,* coleção holandesa de cepas fúngicas.

CIM (MIC) – Concentração Inibitória Mínima (Minimum Inhibitory Concentration).

CFM (MFC) – Concentração Fungicida Mínima (Minimum Fungicidal Concentration).

CG-EM (GC-MS) - Cromatografia Gasosa acoplada a Espectrometria de Massa (Gas Chromatography coupled to Mass Spectrometry).

CPMA – Coleção de Plantas Medicinais e Aromáticas.

CPQBA – Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas.

CI_{30} (IC_{30}) – Concentração de Inibição celular a 30 % (30% Inhibition Concentration).

MEV (SEM) - Microscopia Eletrônica de Varredura (Scanning Electron Microscopy).

OE (EO) – Óleos Essenciais (Essential Oils)

SAPs – Aspartil-proteases Secretadas (Secreted Aspartyl-Proteases)

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[1] Abreviaturas entre parêntesis correspondem à versão na língua inglesa.

INTRODUÇÃO

Candidíases são infecções fúngicas que acometem tecidos superficiais ou internos do organismo humano, manifestando-se em diversas formas clínicas de acordo com os sítios afetados (orofaringe, vagina, pele, olhos, dentre outros). A candidíase oral é uma infecção superficial e oportunista que comumente afeta as membranas mucosas da cavidade oral, tendo *Candida albicans* como principal agente etiológico. Diversos fatores locais e sistêmicos podem predispor ou desencadear quadros clínicos de candidíase oral, como: acidez da saliva, hipossalivação, uso noturno de próteses dentárias, endocrinopatias, deficiências nutricionais, uso de tabaco, má higiene oral, drogas imunossupressoras e tratamento radioterápico e quimioterápico em estruturas maxilo-faciais (Dangi *et al*., 2010).

As taxas mundiais de prevalência e incidência de candidíase oral vêm aumentando nas últimas décadas, especialmente na população de indivíduos imunocomprometidos por HIV e hospitalizados (Samaranayake, Fidel & Naglik, 2002). Estima-se que *Candida albicans* seja responsável por mais de 42 % das infecções causadas por fungos em todo o mundo, seguida em menores proporções de espécies não-*albicans* como *C. glabrata, C. parapsilosis, C. tropicalis, C. krusei, C. guilliermondii, C. rugosa* e *C. dubliniensis* (Colombo *et al*., 2006; Pfaller *et al*., 2012). Considerando que leveduras do gênero *Candida* são colonizadoras comensais da cavidade oral de até 75% dos indivíduos saudáveis (Samaranayake, Fidel & Naglik, 2002), sua relevância clínica como potenciais patógenos fúngicos causadores de doença tem sido sobremaneira discutida (Nucci *et al*., 2010; Pfaller *et al.*, 2012; Sardi *et al.*, 2013).

Desde o século passado, tem-se verificado um aumento no número de compostos antifúngicos sintéticos e classes terapêuticas disponíveis para o tratamento de candidíase. Os principais grupos de antifúngicos prescritos atualmente são polienos, azóis, análogos de purina e equinocandinas (Pierce *et al*., 2013). Particularmente, em se tratando de candidíase oral, vários desses fármacos são utilizados por via tópica, por exemplo, suspensão de nistatina e miconazol em gel. Os antifúngicos poliênicos como anfotericina B e nistatina, se ligam ao ergosterol, principal esterol da membrana fúngica, e criam poros que comprometem a integridade da membrana, causando extravasamento de conteúdos intraplasmáticos e morte celular. Por sua vez, os derivados azólicos são drogas fungistáticas que inibem a biossíntese do ergosterol, tendo como alvo a enzima 14-α-lanosterol demetilase (Erg11 ou Cyp51), uma enzima do citocromo P-450 que catalisa um passo chave na via biossintética do ergosterol (Ostrosky-Zeichner *et al*., 2010; Pierce *et al*., 2013). Exemplos desse grupo incluem derivados imidazólicos (cetoconazol, miconazol) e triazólicos (fluconazol, itraconazol, voriconazol,

posaconazol e ravoconazol). Outra classe, mais recente, de antifúngicos são as equinocandinas, representadas pela caspofungina e micafungina. Esses fármacos promovem a inibição específica e não-competitiva do complexo 1,3-β-D-glucano sintetase. Esta enzima é essencial para a síntese dos polímeros de glucano estruturais da parede celular dos fungos, e a sua inibição conduz à depleção dos glucanos da parede celular e à lise da célula fúngica (Perlin, 2011). Os mecanismos de ação dos antifúngicos estão ilustrados na Figura 1.

Figura 1. Principais classes de agentes antifúngicos e seus alvos (Ilustração: Freires IA, 2013).

Apesar dos avanços científicos até o presente momento, é importante notar que, como fungos são eucariontes, eles compartilham alguns de seus processos biológicos com células humanas, o que leva a maioria das drogas antifúngicas a causar efeitos colaterais prejudiciais (Berman & Sudbery, 2002), como erupções cutâneas, alterações na visão (Johnson & Kauffman, 2003), náusea e vômito (Laniado-Laborín & Cabrales-Vargas, 2009). Este fato aliado ao aumento da resistência de patógenos aos sintéticos (Kuhn & Ghannoum, 2004; Ramage *et al.*, 2012) e à necessidade de se agregar estratégias específicas no tratamento de candidíase oral têm impulsionado novas alternativas terapêuticas na clínica médica e odontológica. Os recentes avanços em relação a técnicas de genética molecular e o sequenciamento genômico de *Candida albicans* (Jones *et al.*, 2004) têm acelerado o desenvolvimento de modalidades preventivas e terapêuticas em pacientes suscetíveis (Berman & Sudbery, 2002; Messer *et al*., 2009; Castro, Álvarez & Martínez, 2013).

A patogenicidade das diferentes espécies *Candida* desempenha papel importante no estabelecimento da infecção fúngica em humanos. Assim sendo, uma alternativa razoável para

desenvolver agentes mais eficazes contra infecções por *Candida* consiste em se entender os mecanismos de virulência implicados na capacidade de as leveduras orais evadirem às defesas do hospedeiro e se desenvolverem em condições favoráveis, a exemplo da organização em biofilme (Sardi *et al.*, 2013).

Os biofilmes constituem comunidades específicas e organizadas de células embebidas em uma matriz extracelular autoproduzida sob o controle de moléculas de sinalização, ao invés do simples acúmulo aleatório de células resultantes da divisão celular (Sardi *et al.*, 2013). É sabido que, na maioria dos casos, as células sésseis de biofilmes de *Candida* são menos suscetíveis a agentes antimicrobianos que as células planctônicas (Kuhn & Ghannoum, 2004), indicando um aumento dos níveis de resistência, particularmente a antifúngicos convencionais (Pierce *et al.*, 2013).

A formação do biofilme começa com a aderência de células de levedura a uma superfície biótica ou abiótica, cuja colonização e crescimento levam à produção de uma camada basal. As células da camada basal incluem algumas hifas, ou cadeias longas tubulares de células que se estendem por outras camadas superiores formadas quase que exclusivamente por hifas. À medida que o biofilme cresce, ele produz uma matriz extracelular contendo hidratos de carbono, proteínas, fósforo e hexosaminas (Douglas, 2003). Salienta-se que os estágios iniciais da formação de biofilme e adesão a um material de substrato são mediadas tanto por fatores abióticos, como a hidrofobicidade, e fatores bióticos, por exemplo, aumento da expressão de adesinas e outras proteínas da superfície celular (Ramage *et al.*, 2005).

Assim, os principais fatores de virulência relacionados à *Candida* spp. são: adesão, formação de biofilme em tecidos do hospedeiro ou dispositivos médicos, alteração fenotípica e produção de enzimas hidrolíticas que danificam o tecido, tais como aspartil proteases secretadas (SAPs), fosfolipases e hemolisinas (Sardi *et al.*, 2013). Destas, as proteínas SAPS, codificadas por uma família de 10 genes (SAPS 1 a 10), têm sido extensivamente estudadas como um dos principais determinantes de virulência em *C. albicans* e em algumas espécies não-*albicans*. Os papéis desempenhados pelas proteinases, por meio da quebra de ligações peptídicas, são: prover nutrição para as células fúngicas, facilitar penetração e invasão, bem como evadir às respostas imunológicas (Naglik, Challacombe & Bernhard, 2003). Além deste fator de virulência, algumas espécies de *Candida*, especialmente *C. albicans*, podem converter-se de células de leveduras unicelulares para pseudo-hifas ou hifas verdadeiras, as quais também desempenham um papel fundamental na formação e persistência do biofilme, invasão dos tecidos (Banerjee *et al.*, 2013) e resistência à fagocitose (Jayatilake *et al.*, 2006).

Vale salientar que os mecanismos patogênicos relacionados com infecções por *Candida* spp. são diversos e muitos dos quais ainda não estão bem estabelecidos (Palmeira-de-Oliveira *et al.*, 2009). Portanto, é necessária a busca não apenas de alternativas que afetem a viabilidade fúngica (ex.: fungistáticos e fungicidas), mas principalmente de agentes que possam atenuar a virulência microbiana (ex.: formação de biofilme e produção de enzimas hidrolíticas) sem interferir com o equilíbrio ecológico da microflora bucal.

Com essa perspectiva, os produtos naturais se destacam como uma promissora fonte de moléculas bioativas com potencial aplicação terapêutica nas áreas médica e odontológica (Newman & Cragg, 2012); dentre eles estão os óleos essenciais (OE), que representam compostos naturais complexos e voláteis produzidos pelas plantas aromáticas como metabólitos secundários (Edris, 2007; Bakkali *et al.*, 2008). De modo geral, as estruturas químicas dos diferentes OE compreendem dois grupos com distintas origens biossintéticas: (i) terpenos (monoterpenos e sesquiterpenos) e terpenoides (isoprenoides), e um outro grupo de (ii) componentes aromáticos e alifáticos (por exemplo, aldeídos, fenóis, entre outros), todos caracterizados por baixo peso molecular (Bakkali *et al.*, 2008).

Os OE têm sido utilizados pela humanidade há séculos, devido ao seu poder antisséptico (bactericida, virucida e fungicida), fragrância, capacidade de conservação dos alimentos, e propriedades medicinais, como sedativo, analgésico, anti-inflamatório e antiespasmódico (Bakkali *et al.*, 2008). A atividade antimicrobiana dos OE pode não estar relacionada com um único mecanismo de ação, mas ser o resultado do efeito de compostos diferentes (composição química altamente complexa) em vários alvos celulares no microrganismo. No que diz respeito ao potencial antifúngico, os avanços nas técnicas de isolamento e de perfil fitoquímico de OE têm estimulado mais pesquisas correlacionando sua estrutura química com a intensidade da atividade anti-*Candida* (Palmeira-de-Oliveira et al., 2009). Portanto, a composição química dos OE pode explicar a potente atividade antifúngica observada para o OE bruto (Cleff *et al.*, 2010; Furletti *et al.*, 2011; Victoria *et al.*, 2012; Zuzarte *et al.*, 2012; Palmeira-de-Oliveira *et al.*, 2012; Sookto *et al.*, 2013) ou compostos isolados (Carrasco *et al.*, 2012; Jayant *et al.*, 2013) contra espécies de *Candida* clinicamente relevantes.

Dentre as espécies com promissor potencial antifúngico, destaca-se *Coriandrum sativum* L., popularmente conhecida como coentro. O *C. sativum* (do latim *coriandrum*, pelo grego *koríandron*) é uma planta glabra, da família das umbelíferas, de flores róseas ou alvas, pequenas e aromáticas, cujo fruto é diaquênio, e cuja folha, usada como tempero ou condimento, exala odor característico. O coentro é muito utilizado na culinária brasileira nordestina e em Portugal, na cozinha alentejana e em outras regiões do sul daquele país (Proença, 2007). As folhas e sementes do coentro também têm sido amplamente utilizadas na

medicina popular como hipolipemiantes, estimulante digestivo e anti-hipertensivas (Snigdha; Monika, 2013).

Além da atividade antifúngica contra patógenos orais (Furletti *et al.*, 2011; Silva *et al.*, 2011), o OE de *C. sativum* também tem apresentado considerável atividade antibacteriana e antiproliferativa (Galvão *et al.*, 2012), antioxidante (Harsha & Anilakumar, 2012), hepatoprotetora (Sreelatha, Padma & Umadevi de 2009) e anticonvulsivante (Emamghoreishi & Heidari-Hamedani, 2008), dentre outras de interesse clínico.

Figura 2. Folhas *in natura* de *Coriandrum sativum* L. (coentro).

O OE de *C. sativum* mostrou-se um promissor agente antifúngico contra espécies de *Candida* (Silva *et al.*, 2011; Furletti *et al.*, 2011; Soares *et al.*, 2012). Contudo, a maioria dos estudos têm analisado o OE dos frutos (Soares *et al.*, 2012) e sementes (Silva *et al.*, 2011.), os quais apresentam composição química bastante diferente daquela das folhas (Matasyoh *et al.*, 2009, Furletti *et al.*, 2011). Considerando-se que *C. sativum* é um potencial candidato a ser testado em ensaios clínicos, por exemplo, para o tratamento de candidíase oral relacionada ao uso de prótese, há uma necessidade de se investigar também a atividade antifúngica do OE de suas folhas e melhor caracterizá-lo quanto ao modo de ação, atividade contra biofilmes de *Candida* e análises farmacogenômicas com fins toxicológicos.

Embora a maior parte dos OE seja desprovida de carcinogenicidade, alguns deles ou alguns dos seus compostos isolados podem ser considerados agentes citotóxicos (Guba, 2001; Bakkali *et al.*, 2008), o que torna indispensável a realização de ensaios toxicológicos de qualquer material derivado de plantas previamente ao uso em seres humanos.

Neste contexto, o presente trabalho determinou o perfil fitoquímico do OE das folhas de *C. sativum* e investigou seu efeito antifúngico sobre cepas de *Candida albicans* e não-*albicans* em relação à inibição de crescimento e morte microbiana, modo de ação, e alterações morfológicas e atividade antiaderente em biofilme. Por fim, buscamos determinar os alvos moleculares afetados pelo OE e por sua fração ativa na expressão global do genoma humano.

CAPÍTULO I [1,2]

Coriandrum sativum L. (coriander) Essential oil: antifungal activity and mode of action on Candida spp., and molecular targets affected in the human whole-genome expression

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ABSTRACT

Oral candidiasis is an opportunistic fungal infection of the oral cavity with increasingly worldwide prevalence and incidence rates. Novel specific-targeted strategies to manage this ailment have been proposed by using essential oils (EO) with antifungal properties. This work reports on the antifungal activity and mode of action of the EO from *Coriandrum sativum* L. (coriander) leaves on *Candida* spp. Also, the molecular targets affected in the whole-genome expression in human cells were determined. The EO phytochemical profile indicated monoterpenes and sesquiterpenes as major compounds, which are likely to account for the strong activity observed against yeasts viability. There seems to be a synergistic activity of the EO chemical compounds as their isolation into fractions led to a decreased antimicrobial power, confirmed by the higher inhibitory and fungicidal values found for the selected active fraction in relation to the entire oil. *C. sativum* EO may bind to membrane ergosterol, increasing ionic permeability and causing membrane damage to cell death, but it does not act on cell wall biosynthesis-related pathways. This mode of action is illustrated by photomicrographs showing disruption in biofilm integrity caused by the EO at varied concentrations. It was also found to inhibit *Candida* biofilm adherence onto a polystyrene substrate at low concentrations and to decrease the proteolytic activity of *Candida albicans* at minimum inhibitory concentration. Finally, the EO and its selected active fraction had low cytotoxicity on human cells, whose putative mechanisms affecting the whole-genome expression involve the pathways of chemokines and MAP-kinase (proliferation/apoptosis) as well as adhesion proteins. These findings highlight the auspicious antifungal activity of the EO from *C. sativum* leaves and encourage further toxicological and clinical research in this field.

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[1] Esta dissertação está apresentada no formato alternativo, conforme informação da CPG/01/2008 (Artigo 3º) (Anexo 1).
[2] Este manuscrito está submetido à revista Plos ONE (IF 3,73), como consta no anexo 2.

1. INTRODUCTION

Oral candidiasis is a common opportunistic fungal infection of the oral cavity whose worldwide prevalence and incidence rates have been increasing in the last decades, particularly among the large population of HIV-immunocompromised and hospitalized individuals (Samaranayake, Fidel & Naglik, 2002). It is estimated that *Candida albicans* accounts for over 42 % of fungal infections worldwide, followed in lower proportions by non-*albicans* species such as *C. glabrata, C. parapsilosis, C. tropicalis, C. krusei, C. guilliermondii, C. rugosa* and *C. dubliniensis* (Colombo *et al.*, 2006; Pfaller *et al.*, 2012).

Overall, the number of synthetic antifungal compounds and therapeutic classes available to treat candidiasis has been increasing since the last decades, including polyenes, azoles, purine analogues and echinocandins (Messer *et al.*, 2009). Nevertheless, as fungal pathogens are eukaryotes, they share some of their biological processes with human cells, which leads the majority of antifungal drugs to cause harmful side effects (Berman & Sudbery, 2002). This fact allied to the increased resistance of pathogens to the synthetics (Kuhn & Ghannoum, 2004; Ramage *et al.*, 2012), cost-effectiveness and need for specific targeted strategies to manage oral candidiasis have driven novel alternatives in this field.

With this perspective, naturally-occurring agents stand out as a source of bioactive molecules with potential therapeutic application in the medical and dental fields (Newman & Cragg, 2012). Among them, essential oils (EO) are one of the most auspicious groups of natural compounds for usage in the prevention and treatment of fungal infections (Silva *et al.*, 2011).

One of the species with promising antifungal activity is *Coriandrum sativum* L., popularly known as coriander. It is an annual small plant dating back to around 1,550 BC that belongs to Apiaceae family in the order of Apiales (Asgarpanah *et al.*, 2012). Coriander leaves and seeds are widely used in folk medicine as cholesterol-lowering, digestive stimulant and anti-hypertensive (Snigdha & Monika, 2013), and also as condiment in food preparation. Pharmaceutical applications of *C. sativum* have also revealed antibacterial (Galvão *et al.,* 2012), antioxidant (Harsha & Anilakumar, 2012), hepatoprotective (Sreelatha, Padma & Umadevi, 2009) and anticonvulsant (Emamghoreishi & Heidari-Hamedani, 2008) activities, among others of clinical interest.

The EO from *C. sativum* proved to be a strong antifungal agent against *Candida* species (Silva *et al.*, 2011; Furletti *et al.*, 2011; Soares *et al.*, 2012). However, the majority of studies have analyzed the EO from fruits (Soares *et al.*, 2012) and seeds (Silva *et al.*, 2011), which present chemical composition different from that of the leaves (Matasyoh *et al.*, 2009; Furletti *et al.*, 2011). Considering that *C. sativum* is a potential candidate to be tested in clinical trials,

e.g., for the treatment of denture-related oral candidiasis, there is a need to further investigate the antifungal activity of its leaves EO with regard to mode of action, activity against *Candida* biofilms and pharmacogenomic analyses with toxicological purposes.

Thus, the present study aimed to investigate the antifungal activity and mode of action of the EO from *Coriandrum sativum* L. (coriander) leaves on clinically relevant *Candida* species, and to determine the molecular targets affected in the whole-genome expression in human cells.

2. MATERIAL AND METHODS

2.1 Plant Material. The species of *Coriandrum sativum* L. was obtained from the germoplasm bank of the Collection of Medicinal and Aromatic Plants (CPMA) at the Research Center for Chemistry, Biology and Agriculture (CPQBA), University of Campinas (UNICAMP), SP, Brazil (http://webdrm.cpqba.unicamp.br/cpma/), and identified by G. M. Figueira, CPMA curator.

The plant material was collected between November 2012 and January 2013, during the morning after the dew-point. Voucher specimen was deposited in the herbarium of the Institute of Biology at UNICAMP (Campinas, SP, Brazil) and also registered in the herbarium of CPQBA, receiving an identification number (CPMA voucher # 644).

2.2 Essential Oil Extraction. The EO was obtained using 100 g of the leaves several times by hydrodistillation in the Clevenger-type system for 3 hours. The aqueous phase was extracted three times with 50 ml of dichloromethane. Then, the organic layer was separated, dried with anhydrous sodium sulphate (Na_2SO_4) and filtered; the solvent was evaporated until dryness, resulting in the EO. The oil content was stored at -20 ^{o}C in amber sealed glass vials. Stock dilutions of the EO were prepared using 6.25% propylene glycol (v/v) as vehicle.

A previous study (Furletti *et al.*, 2011) fractionated the EO from *C. sativum* leaves using the dry column fractionation method, and identified 10 fractions by thin layer chromatography, which were grouped to 5 fractions due to chemical similarity (F_3-F_4, F_5, F_6, F_7, F_8-F_{10}). These fractions were tested against *Candida* clinical isolates and culture collection strains, and that fraction showing the lowest MIC/MFC values was considered as the "active" one *(F_8-F_{10})*. In our study, we extracted the EO and made the fractionation process (Furletti *et al.*, 2011) to obtain this "active fraction" *(F_8-F_{10})* to further study it based on microbiological and pharmacogenomic testing.

2.3 Phytochemical Analysis by Gas Chromatography Coupled to Mass Spectrometry (GC-MS).

The profile of volatile constituents was determined using a Hewlett-Packard 6890 gas chromatograph coupled with an HP-5975 mass selective detector and HP-5 capillary column (30 m × 0.25 mm × 0.25 µm diameter). GC-MS analysis was performed using split injection (40:1), with the injector set at 220 ^{o}C, column set at 60 ^{o}C, with a heating ramp of 3 ^{o}C/min and a final temperature of 240 ^{o}C, and the MS detector set at 250 ^{o}C. Helium was used as a carrier gas at 1 ml.min^{-1}. The mass ranging was between 40 and 60 a.m.u. (atomic mass unit). The GC-MS electron ionization system was set at 70 eV. A sample of the EO and active fraction was solubilized in ethyl acetate for the analyses. Retention indices (RIs) were determined by co-injection of hydrocarbon standards (alkanes C_8-C_{30}) and the EO and active fraction samples under the same aforementioned conditions. The oil components were identified by comparison with data from the literature (The Pherobase, available from http://www.pherobase.com/database/kovats/kovats-index.php), and NIST 05 library profiles, and by co-injection of authentic standards (Adams, 2007; Galvão *et al.*, 2012).

2.4 Microorganisms. Reference strains of *Candida* spp. used in this study were obtained from the Netherlands Collection – *Central Bureau voor Schimmelcultures* (CBS): *C. albicans CBS 562, C. tropicalis CBS 94, C. krusei CBS 573, dubliniensis CBS 7987* and *Candida rugosa CBS 12.*

2.5 Determination of Minimum Inhibitory and Fungicidal Concentration (MIC/MFC). This was a bioguided study investigating the antifungal susceptibility of *Candida* spp. to the action of *C. sativum* EO and its active fraction. MIC test was carried out using 96-well U-bottom tissue culture microplates containing 100 µl/well of RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA). A stock solution of EO emulsion and active fraction was transferred to the first well and serially diluted to obtain concentrations ranging from 1,000 to 0.48 µg/ml. Nystatin (Sigma-Aldrich, St. Louis, MO, USA) and 6.25% propylene glycol (vehicle, v/v) were used as positive and negative controls, respectively. Sterility of culture medium and EO emulsion, and yeast viability were also checked. Fungal inocula were prepared (530 nm, abs 0.08-0.1) and diluted to reach a final concentration of 2.5 x 10^3 CFU/ml in the wells. Plates were incubated at 35 ^{o}C for 24-48 h. MIC was defined as the lowest concentration of the oil that inhibited visible fungal growth (CLSI, 2002).

MFC was determined by subculturing an aliquot from each incubated well with concentration higher than the MIC on Sabouraud Dextrose Agar plates (HIMEDIA® Laboratories Pvt. Ltd., Mumbai, India), which were incubated at 35 ^{o}C for 48-72 h. MFC was defined as the lowest concentration of the EO or active fraction that allowed no visible growth on the solid medium (CLSI, 2002).

The ratio MFC/MIC was calculated in order to determine if the EO and its active fraction had a fungistatic (MFC/MIC ≥ 4) or fungicidal (MFC/MIC < 4) activity (Siddiqui *et al.*, 2013).

2.6 Mode of Action of the Essential Oil. These tests indicated if the antifungal effect observed for *C. sativum* EO somehow involved a direct interaction with *Candida* spp. cell wall structure (sorbitol assay) or membrane ionic permeability (ergosterol assay).

2.6.1 Sorbitol assay. The MIC of the EO was determined in presence of sorbitol (osmotic protector) by the microdilution technique (CLSI, 2002), in triplicate of independent experiments. Initially, 100 μL of RPMI-1640 were added to each well of the plate. Subsequently, 100 μl of the EO were transferred to the first well and serially diluted at a ratio of two, in order to obtain concentrations between 1,000 and 0.48 μg/ml. Finally, 100 μl of inoculum (2.5 x 10^5 CFU/ml) prepared with RPMI-1640 previously supplemented with sorbitol (0.8M final concentration) (Sigma-Aldrich, St. Louis, MO, USA) were transferred to each well. Caspofungin (Sigma-Aldrich, St. Louis, MO, USA) was used as standard antifungal (positive control) and yeast growth, media sterility and vehicle were also controlled. Plates were incubated at 35 ^{o}C and read after 48 h and 7 days (Frost *et al.*, 1995; Escalante *et al.*, 2008; Lima *et al.,* 2013).

2.6.2 Ergosterol assay. In order to determine whether the EO interacts with ergosterol, MIC was determined against *Candida* spp. by the microdilution method (CLSI, 2002), in absence and presence of exogenous ergosterol at concentrations of 100, 200 and 400 μg/ml. Amphotericin B (União Química Farmacêutica Nacional, SP, Brazil) was used as positive control, and the vehicle and yeast viability (in presence of ergosterol) were also checked. Plates were incubated at 35 ^{o}C for 24 h and read afterwards. This assay was carried out in triplicate of independent experiments (Frost *et al.*, 1995; Escalante *et al.*, 2008; Lima *et al.,* 2013).

2.7 Scanning Electron Microscopy (SEM) Analysis. In order to evaluate the integrity of *Candida* spp. cells using SEM, the biofilms were first developed (2.5 x 10^5 CFU/ml) in tissue culture treated chambered glass slides (BD Falcon™, Bedford, MA, USA) and treated with the EO (MIC, MFC, MIC times 10, MIC times 20) or nystatin (MIC). The chambered slides were taken to a shaking incubator (100 rpm) at 35 ^{o}C for 72 h. Then the samples were washed twice and kept in glutaraldehyde/PBS 3% (v/v, pH 7.4) for 12 hours at room temperature. After this time, the chambered slides were serially dehydrated with ethanol (50%, 70% and 90%) for 10 minutes

and dried for 30 minutes to a critical point. Finally, the chambers were removed and the remaining slides were coated with gold in a metallizer machine and then observed using a scanning electron microscope (Jeon® JSM 5600LV, Tokyo, Japan) (Furletti *et al.,* 2011).

2.8 Inhibition of Adherence of Candida spp. Biofilms. Adherence tests were carried out using 96-well U-bottom non-treated plates. In order to evaluate the adherence of *Candida* mono-species biofilm, 100 μL of Sabouraud Broth were added to each well; then, 100 μL of EO emulsion were transferred to the first well and serially diluted at a ratio of two to obtain MIC and sub-MIC concentrations. Finally, 100 μL of yeast inoculum (2.5×10^5 CFU/ml) prepared with Sabouraud Broth plus sucrose (2 %) were added to all wells. The control groups used in this test were: sterility of EO emulsion and culture medium, yeasts growth (also used as standard of adherence for comparisons), nystatin (positive control) and vehicle (negative control). Plates were incubated in a shaker (125 rpm) at 35 ^{o}C for 72 h.

2.8.1 Quantification of Adhered Yeasts. After the incubation period, the content of all wells was discarded and plates were washed twice and dried for 45 minutes at room temperature. Then 200 μL of 0.4 % crystal violet were added to the wells and remained therein for 45 minutes. After this procedure, the wells were washed again and immediately discolored with 200 μL of 95% ethanol. Elapsed 45 minutes, 100 μL of such discolored solution were transferred to the wells of new flat-bottom plates, and crystal violet was measured at 595nm using the microplate reader SpectraMax 340 tunable (Molecular Devices Ltd, Sunnyvale, CA, USA). The inhibition of adherence was measured indirectly considering the yeast growth group as with 100 % of fungal adherence (adapted from Djordjevic; Wiedmann; McLandsborough, 2002; Furletti *et al.,* 2011; Galvão *et al.*, 2012).

2.9 Effect of C. sativum EO on the whole proteolytic activity of C. albicans. The strain of *C. albicans* CBS 562 was previously assessed in solid culture medium for its ability to produce proteases. By using 8-mm-sized circular bottom of glass rods properly sterilized, the yeasts colonies were withdrawn from Agar Sabouraud Dextrose plates and then *printed* on Petri plates containing BSA/YNB agar (0.2 % bovine-serum albumin, 1.45 g Yeast Nitrogen Base, without ammonium sulfate and amino acids, 20 g glucose, 20 g agar *per* liter) with pH 4.0. Plates were incubated at 37 ^{o}C for 72 h (Rodrigues *et al.*, 2007). The presence of proteases was detected with the production of a translucent zone around the yeast colony. The enzymatic activity, named Pz interval, was established by the ratio between the colony diameter (cd) and the colony diameter + translucent zone (cdz), and thereby classified as: absence of proteolytic

activity (Pz = 1.0); positive activity (1.0>Pz≥0.64); or strongly positive activity (Pz<0.64) (Price *et al.*, 1982). Following these same procedures, we tested the anti-proteolytic effect of *C. sativum* EO added to the culture medium to be at MFC, MIC and sub-MIC (MIC/2, MIC/4, MIC/8) concentrations. Pepstatin A (5.0 µM) (protease inhibitor) was used as positive control and the vehicle was also tested. A control with untreated microorganisms was made as standard of 100% proteolytic activity for that strain. All tests were performed in triplicate.

2.10 Effects of the Essential Oil on the Human Whole-Genome Expression. We carried out a translational pharmacogenomic study investigating the effect of *C. sativum* EO and its active fraction on the modulation of human whole-genome expression as a way to assist in the elucidation of potential mechanisms of toxic effects in human cells. These assays were undertaken at the University of Southern California (USC), Ostrow School of Dentistry, Division of Periodontology, Diagnostic Sciences, and Dental Hygiene and Division of Biomedical Sciences (Los Angeles, CA, USA).

Total RNA was isolated from human cells (HeLa CCL-2) treated with the EO and active fraction at IC_{30} (30% Inhibitory Concentration) and subjected to the *Whole-Genome Gene Expression* system using the HumanHT-12 BeadChip V4 (Illumina Inc., San Diego, CA, USA). In each chip, it was possible to analyze 34,602 genes and 47,231 probes (human transcriptome) so determining the effect of the naturally-occurring agents on the modulation of gene expression. Bioinformatics analyses were made using the software GeneGo MetaCore® (Thomson Reuters, New York, NY, USA), generating canonical maps and associative networks. These allowed us for determining the specific molecular targets in the human genome affected by the EO and its active fraction.

2.11 *Statistical Analysis.* An exploratory data analysis was initially performed to determine the most appropriate statistical approach for each assay. Data were statistically treated using Graphpad Prism version 5.0 (San Diego, CA, USA). In the test of inhibition of adherence and anti-proteolytic activity, one-way Analysis of Variance and Tukey's post-test were used, with type I error (α) set as 0.05. Pharmacogenomic data were processed on GeneGo MetaCore software (Thomson Reuters, New York, NY) and DAVID Bioinformatics Resources 6.7 (NIAID/NIH, ML, USA, available from http://david.abcc.ncifcrf.gov/).

3. RESULTS

3.1 Essential Oil and Fraction Yields. *C. sativum* EO yield, expressed in relation to dry weight of plant material (%, w/w), was 0.29 %, and the active fraction yield, expressed as a function of the EO yield (%, w/w), was 7.5 %.

3.2 Phytochemical Analysis by GC-MS. The chemical composition of the EO and its active fraction is shown in Tables 1 and 2, respectively. The analyses of their constituents indicated the presence of volatile compounds, mainly mono- and sesquiterpenes hydrocarbons. We identified 12 compounds in the EO from *C. sativum*, representing 82.52 % of its total composition. Also, six compounds were identified in the active fraction of the EO, which accounted for 79.52 % of the fraction content. The major compounds identified in the EO were decanal (19.09 %), trans-2-decenal (17.54 %), 2-decen-1-ol (12.33 %) and cyclodecane (12.15 %), with similar chemical profile for the active fraction (Table 1).

Table 1. Analytes identified by GC-MS in the essential oil and its active fraction from *Coriandrum sativum* leaves*.*

R_t (min)	R_I [a]	Analytes identified*	Relative Percentage [b]	
			EO	Active Fraction
7.62	1004	octanal	0.34	—
15.00	1198	trans-4-decenal	0.47	—
15.38	1207	decanal	19.09	4.91
17.74	1263	trans-2-decenal	17.54	24.11
18.15	1272	2-decen-1-ol	12.33	16.57
18.26	1275	cyclodecane	12.15	16.62
19.61	1307	undecanal	0.99	—
21.97	1364	2-undecenal	1.17	1.50
22.31	1372	trans-2-undecen-1-ol	0.49	—
23.84	1409	dodecanal	4.10	—
26.19	1467	cis-2-dodecenal	10.72	15.81
26.44	1473	dodecan-1-ol	3.13	—
26.46	1474	M[c] = 184	—	6.40
31.81	1612	M = 194	0.94	—
34.02	1671	M = 210	11.51	14.09
34.18	1675	M = 194	1.92	—

*Notes: (a) Retention Index; (b) Percentage fraction of the total area integrated for the chromatogram; (c) M: molecular weight of a non-identified compound. *Only compounds identified or those indicated by their molecular weight are listed.*

3.3 Antifungal activity and mode of action of the EO. MIC and MFC values for the EO and active fraction of *C. sativum* and standard antifungals on *Candida* spp. are expressed in Table 3. For the EO, MIC values ranged from 15.6 to 31.2 μg/ml, and MFC values ranged from 31.2 to 62.5 μg/ml. The active fraction showed higher MIC and MFC values, which ranged from 31.2 to

250 µg/ml and 125 to 1,000 µg/ml, respectively. The vehicle used in the EO emulsion did not affect yeast growth.

The ratio MFC/MIC found for the EO and active fraction demonstrated a fungicidal effect of these samples against the majority of the yeast species tested. As the crude EO was proven to show a better antifungal effect than its active fraction, we decided to perform further antimicrobial assays only with the EO, taking into account the enhanced anti-*Candida* activity of the EO, costs of the fractionation process, and potentially higher toxic effects of fractions in relation to their crude oils (Galvão *et al.*, 2012).

Given the promising antifungal effects of the EO, we next studied the mode of action by which yeast cells had their viability affected. Accordingly, our results showed that the antifungal properties of *C. sativum* EO are not related to cell wall biosynthesis pathways, as the findings of the antifungal test were unaltered either in presence or absence of an osmotic protector (sorbitol) (Table 4). Instead, EO MIC values folded between 8 and 16 times with the increase of exogenous ergosterol concentration, what indicates that the EO seems to bind to membrane ergosterol, increasing ionic permeability, ultimately resulting in cell death. This same mode of action was observed for amphotericin B used as positive control (Table 5).

3.4 Scanning Electron Microscopy (SEM) Analysis. The effect of *C. sativum* EO on oral *Candida* biofilm integrity was assessed by SEM. Summarily, yeast morphology was disrupted at different concentrations with cell damage intensity depending on the species (Figure 1A-E). The most representative injuries were caused by the following concentrations of the EO: *C. albicans* – MIC times 20 (312.5 µg/ml); *C. tropicalis* – MIC times 10 (312.5 µg/ml); *C. krusei* – MIC times 10 (156.0 µg/ml); *C. dubliniensis* – MIC times 20 (625 µg/ml); and *C. rugosa* – MIC times 20 (312.5 µg/ml). At these concentrations, the EO was able to substantially affect biofilm cells structure, most of which turned from turgid to withered appearance, similarly to what was observed for nystatin (positive control) at MIC. The vehicle did not affect the biofilm integrity.

Table 3. Antifungal activity of the essential oil and active fraction of *C. sativum* on *Candida* spp. (values are expressed as µg/ml).

Strain	*C. sativum* EO			Active Fraction from *C. sativum* EO			Nystatin			Amphotericin B		
	MIC	MFC	MFC/MIC ratio	MIC	MFC	MFC/MIC ratio	MIC	MFC	MFC/MIC ratio	MIC	MFC	MFC/MIC ratio
C. albicans CBS 562	15.6	31.2	2	250	1,000	4	7.8	15.6	2	0.038	0.07	2
C. tropicalis CBS 94	31.2	62.5	2	250	500	2	3.9	7.8	2	0.01	0.02	2
C. krusei CBS 573	15.6	31.2	2	125	250	2	3.9	15.6	4	0.15	0.15	1
C. dubliniensis CBS 7987	31.2	62.5	2	31.2	125	4	3.9	3.9	1	0.002	0.004	2
C. rugosa CBS 12	15.6	31.2	2	62.5	125	2	1.9	15.6	8	0.07	0.61	8

Table 4. Effect of *C. sativum* EO on *Candida* spp. cell wall biosynthesis (*sorbitol assay*).

Concentration (µg/ml)	*Candida albicans* (CBS 562)		*Candida tropicalis* (CBS 94)		*Candida krusei* (CBS 573)		*Candida dubliniensis* (CBS 7987)		*Candida rugosa* (CBS 12)	
	With sorbitol	Without sorbitol	With sorbitol	Without sorbitol	With sorbitol	Without sorbitol	With sorbitol	Without sorbitol	With sorbitol	Without sorbitol
1000	-	-	-	-	-	-	-	-	-	-
500	-	-	-	-	-	-	-	-	-	-
250	-	-	-	-	-	-	-	-	-	-
125	-	-	-	-	-	-	-	-	-	-
62.5	-	-	-	-	-	-	-	-	-	-
31.2	-	-	+	+	-	-	+	+	-	-
15.6	+	+	+	+	+	+	+	+	+	+
7.8	+	+	+	+	+	+	+	+	+	+
3.9	+	+	+	+	+	+	+	+	+	+
1.95	+	+	+	+	+	+	+	+	+	+
0.97	+	+	+	+	+	+	+	+	+	+
0.48	+	+	+	+	+	+	+	+	+	+

Note: +, fungal growth; -, no fungal growth.

Table 5. Effect of different concentrations of exogenous ergosterol (100–400 µg/ml) on the MIC of *C. sativum* EO and Amphotericin B against *Candida* spp.

Strain	MIC of the EO in absence of ergosterol	MIC of the EO in presence of ergosterol			MIC of Amphotericin B in absence of ergosterol	MIC of Amphotericin B in presence of ergosterol		
		100 µg/ml	200 µg/ml	400 µg/ml		100 µg/ml	200 µg/ml	400 µg/ml
C. albicans **CBS 562**	15.6	500	500	500	0.038	2.4	2.4	4.8
C. tropicalis **CBS 94**	31.2	250	250	250	0.01	2.4	2.4	9.7
C. krusei **CBS 573**	15.6	62.5	125	125	0.15	4.8	9.7	9.7
C. rugosa **CBS 12**	15.6	125	125	125	0.002	4.8	19.5	19.5
C. dubliniensis **CBS 7987**	31.2	-	-	-	0.07	-	-	-
Yeast growth in presence of ergosterol	+	+	+	+	+	+	+	+
Vehicle	+	+	+	+	+	+	+	+

Note: +, fungal growth; -, no fungal growth or irregular growth due to unclear reasons.
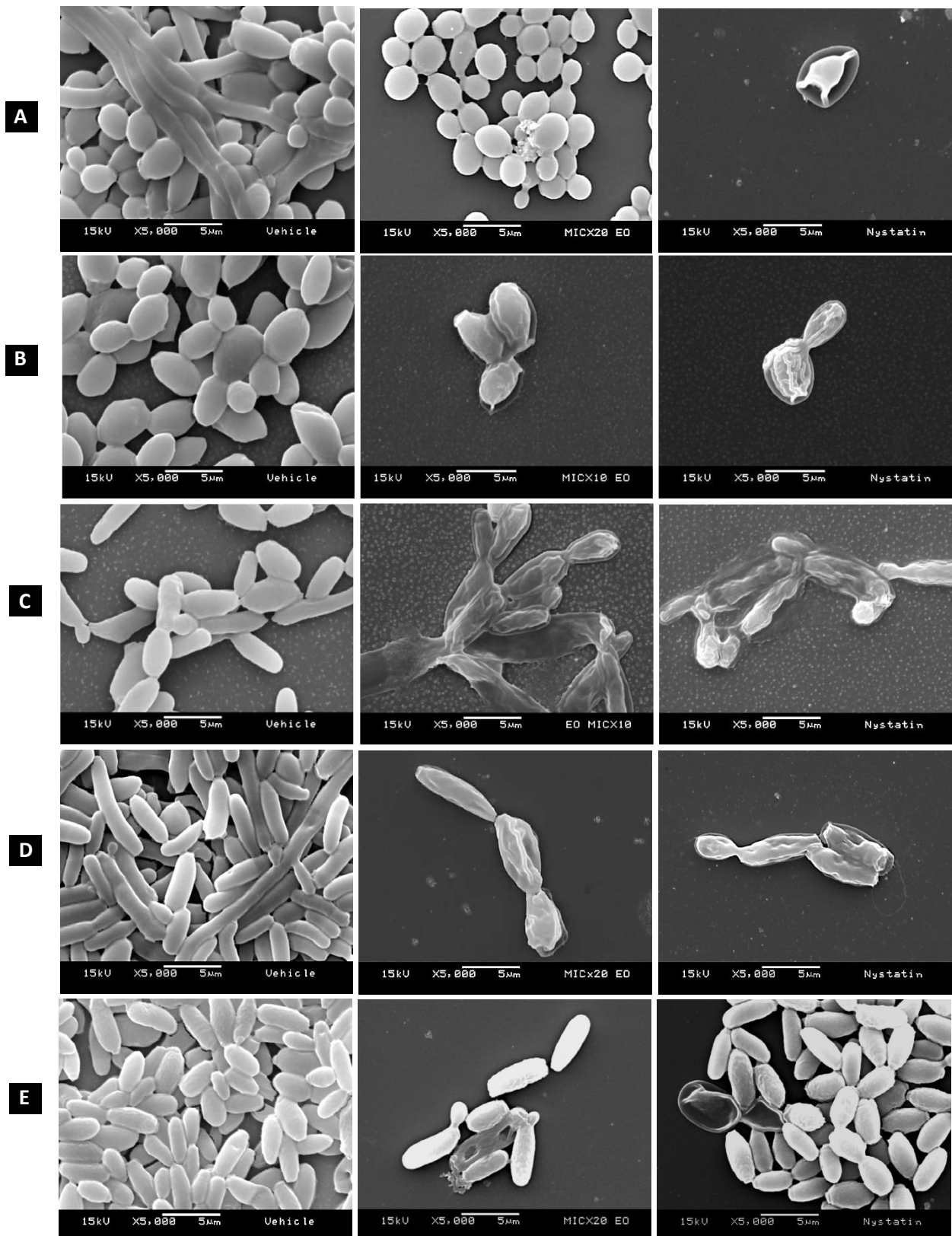
Figure 1. SEM Photomicrographs (5,000x) showing *Candida* biofilm cells after treatment with propylene glycol (vehicle) (left-sided), essential oil from *C. sativum* leaves (middle-sided) and nystatin (right-sided). The most representative cell injuries were caused by the following concentrations of the EO: (A) *C. albicans* – MIC times 20 (312.5 µg/ml); (B) *C. tropicalis* – MIC times 10 (312.5 µg/ml); (C) *C. krusei* – MIC times 10 (156.0 µg/ml); (D) *C. dubliniensis* – MIC times 20 (625 µg/ml); and (E) *C. rugosa* – MIC times 20 (312.5 µg/ml). Nystatin was tested at MIC against all strains (see MIC values in Table 2) and the vehicle did not affect the biofilm integrity.

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3.6 Inhibition of Adherence of Candida spp. Biofilms. The EO had anti-adherent activity (42-85 %) at low concentrations (62.5 µg/ml) against all strains tested (Figures 2-6). The best results were found for *C. tropicalis*, on which *C. sativum* EO and nystatin significantly inhibit biofilm adherence at concentrations lower than their MICs: 15.6 and 0.9 µg/ml, respectively ($P < 0.05$) (Figure 3). The vehicle did not affect biofilm adherence.
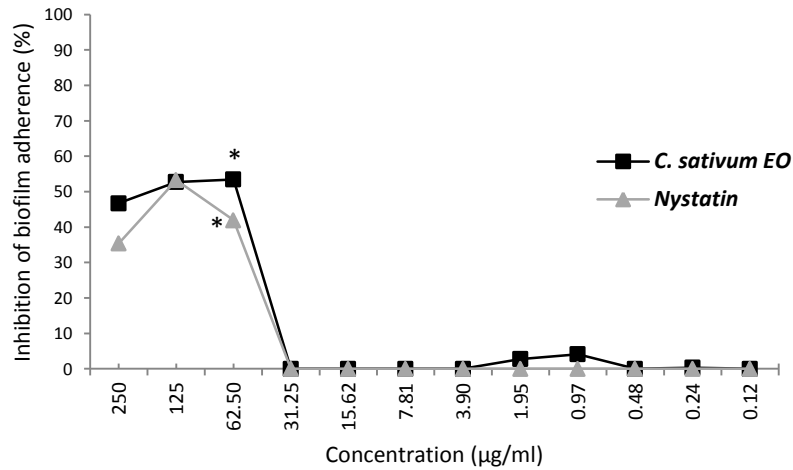

Figure 2. Inhibitory effects of *C. sativum* EO and nystatin on biofilm adherence of *Candida albicans* (CBS 562) after 72h. Both groups were significantly different from the vehicle (One-way ANOVA with Tukey's post-test, $P < 0.05$). *Statistically significant inhibition of biofilm adherence.

Figure 3. Inhibitory effects of *C. sativum* EO and nystatin on biofilm adherence of *Candida tropicalis* (CBS 94) after 72h. Both groups were significantly different from the vehicle (One-way ANOVA with Tukey's post-test, $P < 0.05$). *Statistically significant inhibition of biofilm adherence.

Figure 4. Inhibitory effects of *C. sativum* EO and nystatin on biofilm adherence of *Candida krusei* (CBS 573) after 72h. Both groups were significantly different from the vehicle (One-way ANOVA with Tukey's post-test, $P < 0.05$). *Statistically significant inhibition of biofilm adherence.
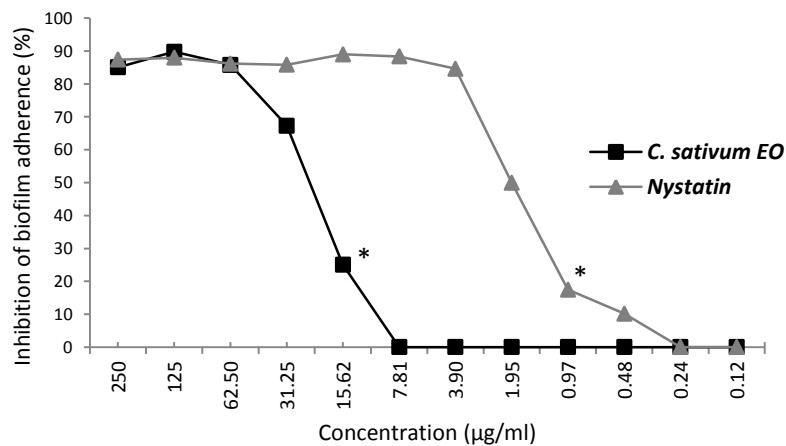

Figure 5. Inhibitory effects of *C. sativum* EO and nystatin on biofilm adherence of *Candida dubliniensis* (CBS 7987) after 72h. Both groups were significantly different from the vehicle (One-way ANOVA with Tukey's post-test, $P < 0.05$). *Statistically significant inhibition of biofilm adherence.

Figure 6. Inhibitory effects of *C. sativum* EO and nystatin on biofilm adherence of *Candida rugosa* (CBS 12) after 72h. Both groups were significantly different from the vehicle (One-way ANOVA with Tukey's post-test, $P < 0.05$). *Statistically significant inhibition of biofilm adherence.
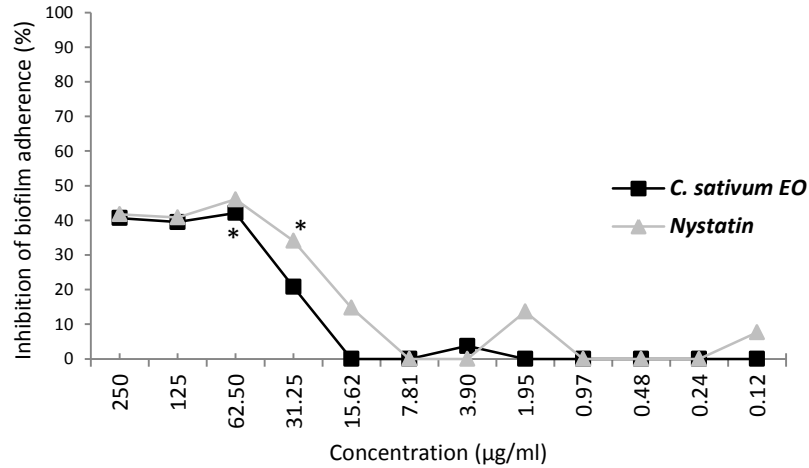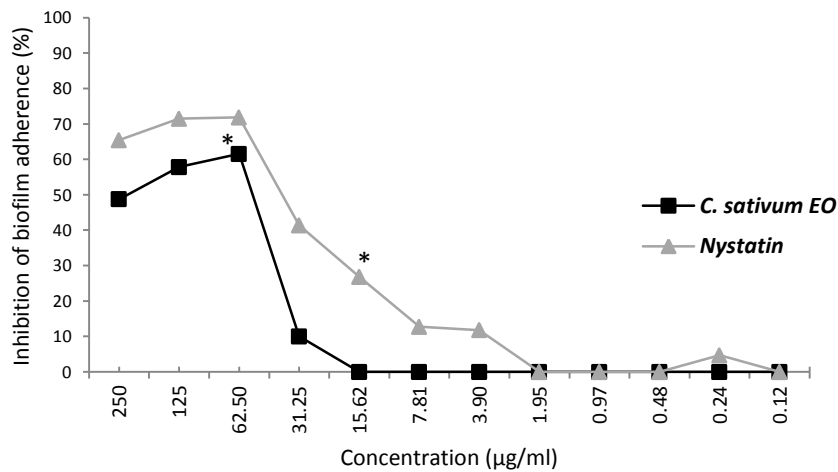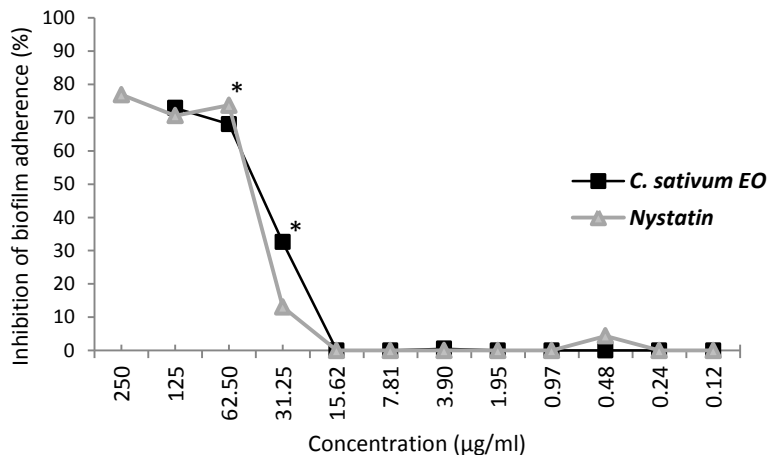
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3.7 Effect of C. sativum EO on the proteolytic activity of C. albicans. The strain of *C. albicans* CBS 562 had a Pz interval of 0.84, indicating positive activity for proteases. Then, we found that the EO from *C. sativum* leaves decreased the proteolytic activity of *C. albicans*, with statistically significant difference at MIC compared to the group of untreated microorganisms (100 % proteolytic activity) ($P < 0.05$).
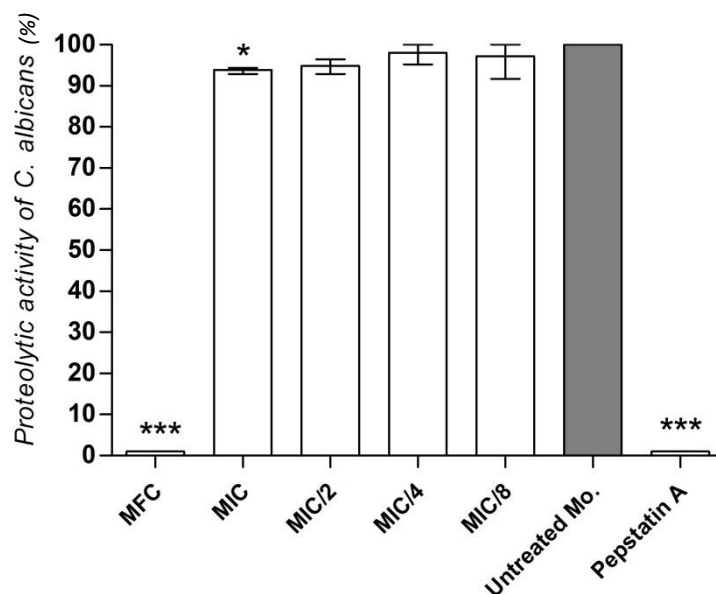

Figure 7. Effect of different concentrations of the EO from *C. sativum* leaves and Pepstatin A (protease inhibitor) on the whole proteolytic activity of *Candida albicans* CBS 562 (One-way ANOVA with Tukey's post-test, *$P < 0.05$; ***$P < 0.0001$). The group of untreated microorganism was considered as standard of 100% proteolytic activity.

3.8 Effects of the Essential Oil on the Whole Human Genome Expression. The OE and active fraction from *C. sativum* L. had low cytotoxicity upon human cells with IC_{30} of 359.76 and 366.69 µg/ml, respectively. Gene expression was up-regulated by the EO and active fraction in 109 and 134 genes, respectively, mostly related to chemotaxis (IL-6, IL-8) and proliferation / apoptosis (c-Jun , c-Fos, c-Jun/C-Fos and AP-1), and down-regulated in 28 and 30 genes, respectively, related to 11 adhesion proteins (alpha-4/beta-1-integrin; ITGB1; phosphoinositide-3-kinase; regulatory subunit 2 (beta); alpha-5/beta-1-integrin; alpha-6/beta-1-integrin; alpha-10/beta-1-integrin; alpha-V/beta 3-integrin; alpha-8/beta-1-integrin; integrin-alpha-V (ITGAV); alpha-11/beta-1-integrin; alpha-2/beta 1-integrin), as seen in Figures 8 and 9. Similarly to the findings processed on Metacore®, the same modulation patterns were found in the DAVID Bioinformatics database (graphs not shown).

Figure 8. Representative map of the pathways whose genes involved were up-regulated by the OE (❶) and active fraction (❷) from *C. sativum*. The thermometer next to each component indicates the type (red color means up-regulation) and the magnitude of the modulation of gene expression (MetaCore®, Thomson Reuters).
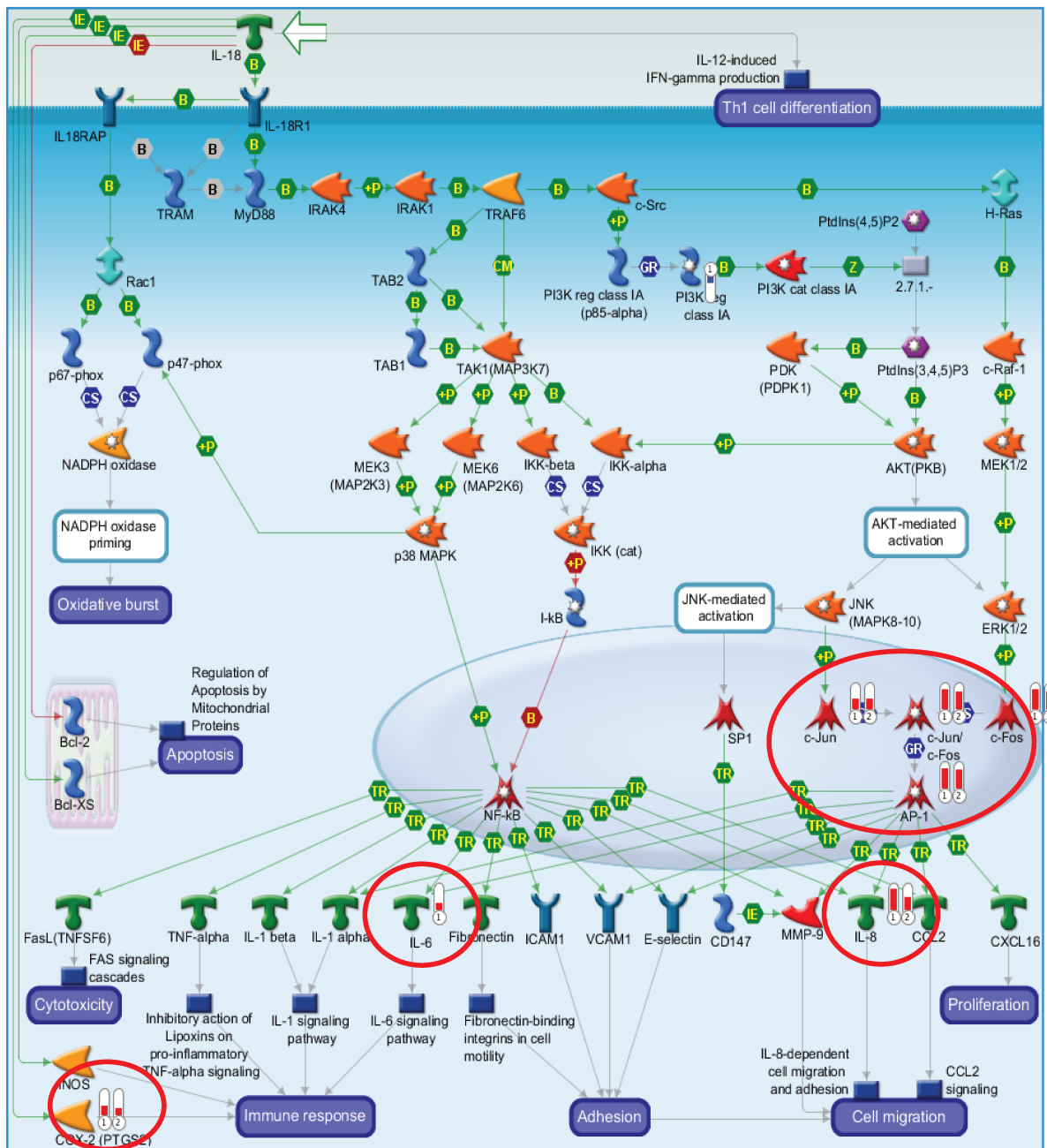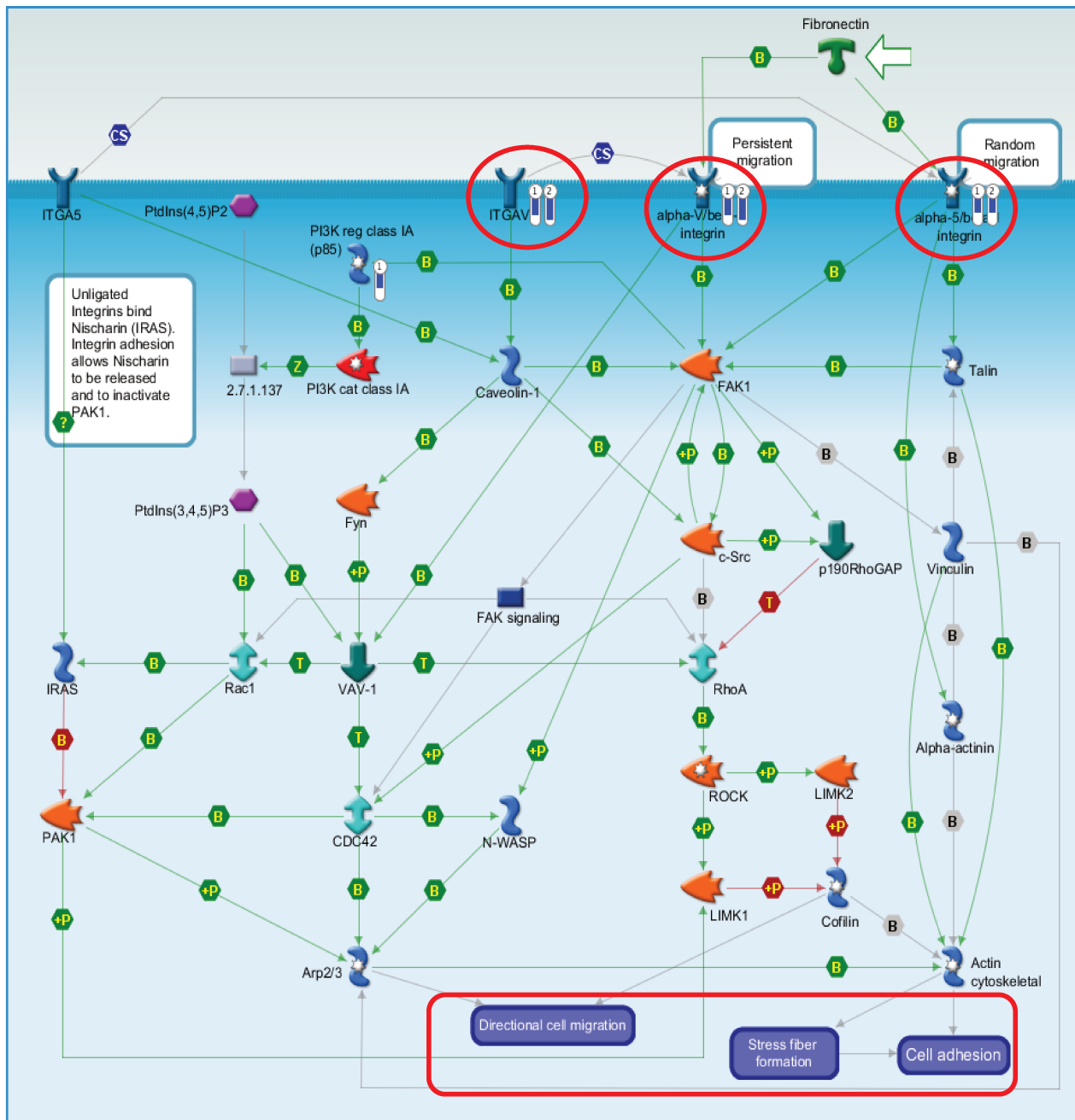
Figure 9. Representative map of the pathways whose genes involved were down-regulated by the OE (❶) and active fraction (❷) from *C. sativum*. The thermometer next to each component indicates the type (blue color means down-regulation) and the magnitude of the modulation of gene expression (MetaCore®, Thomson Reuters).

4. DISCUSSION

Due to recent advances in the exploration of *C. sativum* as a potential therapeutic agent against a number of diseases afflicting humans (Asgarpanah & Kazemivash, 2012), comprehensive research of its different properties has been encouraged. In this study, we investigated the effects of the chemically characterized EO from *C. sativum* leaves on the viability and adherence of *Candida albicans and non-albicans* strains, in planktonic and biofilm cultures. Furthermore, the EO was evaluated for its antifungal mode of action.

Although the amounts may vary, all the studies seem to confirm that the major components of coriander leaf EO are alcohols and aldehydes (Sahib *et al*., 2012), corroborating our results, as we found decanal, trans-2-decenal, 2-decen-1-ol and cyclodecane as major compounds. Most of these analytes have also been found as major constituents of coriander leaves samples from Kenya (Matasyoh *et al*., 2009), U.S.A. (Potter, 1996), Bangladesh (Bhuiyan, Begum & Sulatana, 2009), Fiji (Eyres *et al*., 2005) and Brazil (Begnami, Duarte & Furletti, 2010). So far, no study in the literature has demonstrated the effects of these alcohols and aldehydes tested alone against Candida *spp*.

The mono- and sesquiterpenes found in the leaves EO may be related to the antifungal activity observed. Natural products are considered strong inhibitors of microbial activity when MIC values are lower than 500 µg/ml (Duarte *et al*., 2007). Hence, the findings presented herein revealed that the EO from *C. sativum* has strong fungicidal effects against clinically relevant *Candida* species with low MIC and MFC values.

The fractionation process adopted in this bioguided study is well established (Jeon *et al*., 2011; Galvão *et al*., 2012). Nevertheless, the EO from *C. sativum* was proven to have a synergism of compounds with chemical complexity so its isolation into fractions may have led to a decreased antimicrobial activity, as confirmed by the higher MIC/MFC values found for the active fraction in relation to the crude oil, which is in agreement with other studies (Delaquis *et al*., 2002; Furletti *et al*., 2011; Galvão *et al*., 2012). It is worth noting that the mixture of numerous molecules in an EO play a role in defining the fragrance, density, color and above all, cell penetration (Cal, 2006), lipophilic or hydrophilic attraction and fixation on cell membranes, and cellular distribution. So, for biological purposes, it is more informative to study the crude oil rather than some of its components because the concept of synergism appears to be more meaningful (Bakkali *et al*., 2008). Allied to this fact are the poorer results observed in this study for the active fraction of *C. sativum* EO in comparison to the entire oil, leading only the EO to be selected for further microbiological testing.

The antifungal activity of EO depends fundamentally on their ability to pass through the cell wall and stay between fatty acid chains of the lipid bilayer, altering membrane fluidity and permeability, damaging membrane proteins, which leads to degradation of cytoplasmic membrane and cell death. As such, loss of cell homeostasis, leakage of cell contents and lysis are consequences of this inducted alterations in membrane structure and function (Pauli, 2006; Bakkali *et al.*, 2008). Notwithstanding the advances in the identification of the antifungal activity of *C. sativum* leaves EO, its modes of action on the viability and pathogenicity of yeasts remained poorly studied. Herein, we investigated two possible chief modes by which *C. sativum* leaves EO might have shown antifungal effects on *Candida* yeasts, namely: (i) blocking cell wall biosynthesis or (ii) increasing membrane ionic permeability. Our findings showed that the EO seems to bind to membrane ergosterol, rendering the cell membrane more permeable and resulting, ultimately, in cell death, as ergosterol is crucial to maintain cell integrity, viability, function and normal growth (Ghannoum & Rice, 1999). This same mechanism accounts for the antifungal activity of polyenes such as nystatin and amphotericin B (Pierce *et al.,* 2013). Tests of the effect of *C. sativum* EO on ergosterol biosynthesis (e.g. enzyme-targeting and analysis of 2-^{13}C-acetate incorporation) (Müller *et al.*, 2013) were not undertaken in our study, but are strongly recommended as other cell targets might also be involved in this EO antifungal activity.

Although with a rather different chemical composition, the EO from *C. sativum* seeds also has antimicrobial activity, whose primary mode of action is cell permeabilization and consequent DNA leakage in yeasts (Silva *et al.*, 2011a) and bacteria (Silva *et al.*, 2011b) based on flow cytometry analyses.

The study of antifungals' mode of action is an important strategy for limiting the emergence of resistance to the currently available agents, as well as for developing safer and more potent drugs against fungal infections (Lopes *et al.*, 2013). In addition to the primary mechanisms of action of antifungals (β-Glucans biosynthesis blocking and disturbance in ergosterol synthesis or function) (Pierce *et al.*, 2013), some agents can also have alternative effects on other targets, for instance, mitochondrial activity, which plays a role in generation and regulation of Reactive Oxygen Species (ROS), calcium homeostasis and ATP production, all related to cell viability (Sun *et al.*, 2013). Given this, we suggest the study of *C. sativum* EO upon other strategic targets associated with the regulation of metabolic processes in the fungal cell, in order to have its antifungal potential comprehensively elucidated.

From a clinical perspective, fungal biofilms formation leads to negative consequences to health, since they act as protected reservoirs of microorganisms (Ramage *et al.*, 2009) displaying properties that are extremely different from planktonic populations, mainly high-

level resistance to a number of antifungal agents (Ramage *et al*., 2012). It has been demonstrated an association between biofilm formation and increased virulence pattern and resistance in *Candida* species (Hasan *et al*., 2009; Ramage *et al.*, 2012). Hence, the study on the antifungal activity of natural products should prioritize biofilm growth models to reach greater reliability and closer approach to the human conditions. In our study, we used electron microscopy to assess the effect of *C. sativum* EO on oral *Candida* biofilm integrity. We demonstrated that the EO at relatively low concentrations (156.0 to 312.50 µg/ml) clearly disrupted yeast morphology. These findings corroborate those of the mode of action, as we observed that the cell wall structure seemed to be unaltered to some extent but did not the cell membrane, indicating that the EO possibly acts on membrane permeability rather than on cell wall biosynthesis. As it happens to gram-positive oral bacteria, the simple conformational change in *Candida* biofilms caused by the action of the EO could make them more susceptible and less virulent (Kajfasz *et al.*, 2010).

The initial stages of biofilm formation and adherence to a substrate material are mediated both by abiotic factors, such as surface hydrophobicity, and biotic factors, for instance, increased expression of adhesins and other cell-surface proteins (Ramage *et al*., 2005). Hence, the inhibition of adherence of yeast cells may constitute a promising target for disrupting the initial stages of biofilm formation in *Candida* spp. (Rane *et al.*, 2013). In this study, *C. sativum* EO was checked for its ability to prevent adherence of 72-h *Candida* biofilms onto a polystyrene substrate. We demonstrated that *C. sativum* EO accounted for 42-85 % inhibition of biofilm adherence at a concentration of 62.2 µg/ml on the tested strains, with statistically significant difference. In other study, anti-adherent activity was also found for the active fraction from *C. sativum* EO against *Streptococcus mutans*. Using the same method presented herein, the authors demonstrated that the selected fraction inhibited 95 % of biofilm adherence at a concentration of 31.2 µg/ml (Galvão *et al*., 2012).

The effect of *C. sativum* EO upon oral *C. albicans* biofilm development kinetics was previously established. A clear effect of the oil at 125 µg/ml on biofilm formation was observed after 48 h, characterized by an increase in the lag phase and a decrease in the biofilm growth (Furletti *et al*., 2011).

The inhibitory effect of the EO on proteases secreted by *C. albicans,* which play a major role in virulence*,* was evaluated using the agar diffusion method. Although with shortcomings related to sample liposolubility-dependent diffusion and lack of specificity, this technique has been reported by a number of studies to provide preliminary inhibition rates of the whole-proteolytic activity of microorganisms (Vermelho *et al*., 1996; Kantarcioglu & Yucel, 2002; Vaishali & Jain, 2012). *C. sativum* EO at MIC decreased about 7-8 % the proteolytic activity of *C.*

albicans with statistically significant difference, whereas sub-MIC concentrations had no effect compared to the control. As there is no study in the literature investigating this property for *C. sativum*, determination of extracellular proteinases activity in presence of *C. sativum* EO using spectrophotometric tests is now recommended.

Even though most of the EO are likely to be devoid of carcinogenicity – but not of cytotoxicity – some of them or rather some of their compounds may be considered as secondary carcinogens after metabolic activation (Guba, 2001; Bakkali *et al.*, 2008), what makes indispensable to carry out toxicological assays of whatever plant-derived material to be used in humans as a herbal formulation. In our study, we performed a preliminary pharmacogenomic analysis of the EO and active fraction from *C. sativum,* in order to establish how these samples would interfere with the human genome expression. Both the OE and its active fraction had relatively low cytotoxic activity, whose putative mechanisms affecting the genome expression involve the pathways of pro-inflammatory chemokines (e.g., IL-6, IL-8) and mitogen-activated protein kinase pathway, i.e., proliferation and apoptosis processes, as well as adhesion proteins (mostly integrins). This is the first translational study demonstrating the pharmacogenomic profile of *C. sativum* EO in the international literature. As such, our findings project genomic information for further *in vitro* and *in vivo* research.

C. sativum is approved for food uses by the Food and Drug Administration, which granted it the GRAS (Generally Regarded as Safe) status, and also by the Council of Europe (CoE) (Burdock & Carabin, 2009). Based on the history of consumption of *C. sativum* leaves without reported adverse effects, and lack of toxicity of the major constituent from the seeds EO (linalool)(Silva *et al.*, 2011a), the use of *C. sativum* as an additional food ingredient is considered safe (Burdock & Carabin, 2009). The median lethal dose (LD_{50}) of *C. sativum* seeds EO was determined as 2.257 ml/kg (Özbek *et al.*, 2006). Nevertheless, the LD_{50} of the leaves EO has not been reported in the literature and remains unclear, as chemical composition of the EO from the leaves differs substantially from that of the seeds, as aforementioned.

Given the above, numerous additional applications for this oil in either already existing medical accessories or other formulations for use in clinical practice to prevent *Candida* infections should be considered (Silva *et al.*, 2011a).

5. CONCLUSIONS

The EO from *C. sativum* leaves has strong antifungal and anti-adherent activity against *Candida* spp., as well as anti-proteolytic activity against *C. albicans*, and acts by increasing cell membrane ionic permeability rather than disturbing cell wall biosynthesis. All these properties

are probably due to the synergistic effect of the mono- and sesquiterpenes hydrocarbons identified. Furthermore, pharmacogenomic analyses revealed that *C. sativum* EO has relatively low cytotoxicity, and the putative mechanisms modulating the expression of the human genome affect the pathways of chemokines and mitogen-activated protein kinase (proliferation/apoptosis) as well as adhesion proteins.

Other toxicological bioassays and phase I and II clinical trials are now encouraged to further investigate the promising antifungal activity of *C. sativum* EO as an auspicious candidate to treat oral ailments such as denture-related candidiasis.

6. ACKNOWLEDGMENTS

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CONCLUSÃO

Com base nos resultados, pode-se concluir que:

1) O OE das folhas de *C. sativum* apresenta **forte atividade antifúngica**, com baixos valores de CIM e CFM, os quais foram menores que os de sua fração ativa.

2) Quanto ao modo de ação, verificou-se que o OE se liga ao ergosterol da membrana plasmática, **aumentando a permeabilidade iônica** e levando à **morte celular**, e não atua sobre vias relacionadas à biossíntese da parede celular.

3) O OE de *C. sativum* apresentou **atividade antiaderente significativa** contra biofilmes de *Candida* sobre todas as cepas testadas, indicando que o OE interfere em um dos fatores de virulência (aderência) essencial para a colonização do biofilme na superfície.

4) Também foi verificado que o OE apresentou **significativa atividade contra proteases** produzidas por *C. albicans*, as quais desempenham papel importante na virulência deste microrganismo, favorecendo a invasão tecidual.

5) Por fim, análise farmacogenômica revelou que o OE e sua fração ativa apresentam **citotoxicidade relativamente baixa** sobre células humanas, cujos alvos afetados no genoma humano foram: quimiocinas pró-inflamatórias relacionadas à quimiotaxia, proliferação e apoptose, bem como proteínas de adesão.

Outros bioensaios toxicológicos e clínicos de fase I e II são agora incentivados a fim de investigar a promissora atividade antifúngica do OE de *C. sativum* como candidato auspicioso para o tratamento de doenças estomatológicas, como candidíase oral associada ao uso de prótese.

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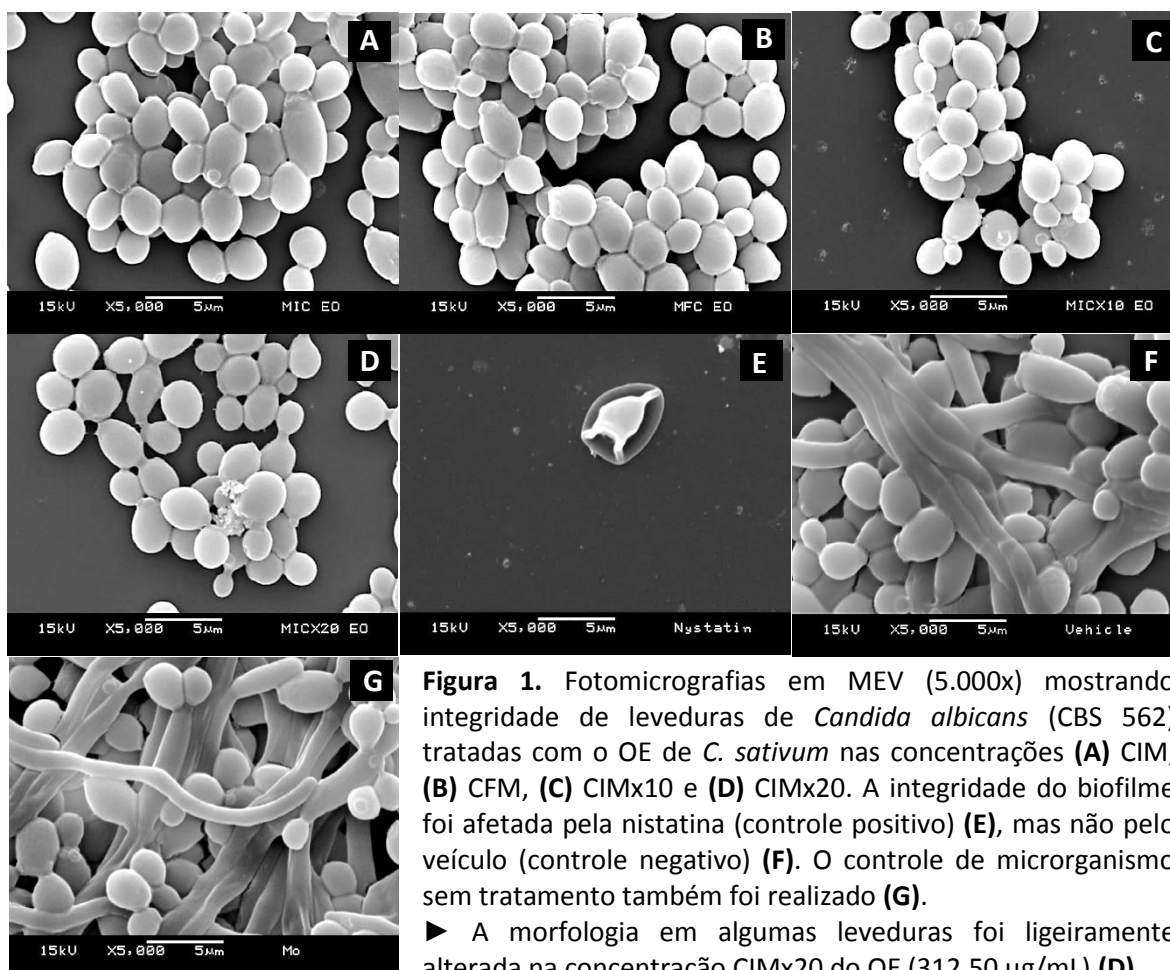
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APÊNDICE

Apêndice 1. Microscopia Eletrônica de Varredura

Seguem os resultados de Microscopia Eletrônica de Varredura incluindo várias concentrações do OE testadas (CIM, CFM, CIMx10 e CIMx20), controle positivo (nistatina, CIM), controle negativo (veículo) e controle de crescimento do microrganismo.

Figura 1. Fotomicrografias em MEV (5.000x) mostrando integridade de leveduras de *Candida albicans* (CBS 562) tratadas com o OE de *C. sativum* nas concentrações **(A)** CIM, **(B)** CFM, **(C)** CIMx10 e **(D)** CIMx20. A integridade do biofilme foi afetada pela nistatina (controle positivo) **(E)**, mas não pelo veículo (controle negativo) **(F)**. O controle de microrganismo sem tratamento também foi realizado **(G)**.
► A morfologia em algumas leveduras foi ligeiramente alterada na concentração CIMx20 do OE (312,50 μg/mL) **(D)**.

Figura 2. Fotomicrografias em MEV (5.000x) mostrando integridade de leveduras de *Candida tropicalis* (CBS 94) tratadas com o OE de *C. sativum* nas concentrações **(A)** CIM, **(B)** CFM, **(C)** CIMx10 e **(D)** CIMx20. A integridade do biofilme foi afetada pela nistatina (controle positivo) **(E)**, mas não pelo veículo (controle negativo) **(F)**. O controle de microrganismo sem tratamento também foi realizado **(G)**.
► A morfologia foi alterada a partir da concentração CFM do OE (62,25 µg/mL) **(B)**.

Figura 3. Fotomicrografias em MEV (5.000x) mostrando integridade de leveduras de *Candida krusei* (CBS 573) tratadas com o OE de *C. sativum* nas concentrações **(A)** CIM, **(B)** CFM, **(C)** CIMx10 e **(D)** CIMx20. A integridade do biofilme foi afetada pela nistatina (controle positivo) **(E)**, mas não pelo veículo (controle negativo) **(F)**. O controle de microrganismo sem tratamento também foi realizado **(G)**.

► A morfologia foi alterada a partir da concentração CFM do OE (31,25 µg/mL) **(B)**.

Figura 4. Fotomicrografias em MEV (5.000x) mostrando integridade de leveduras de *Candida dubliniensis* (CBS 7987) tratadas com o OE de *C. sativum* nas concentrações **(A)** CIM, **(B)** CFM, **(C)** CIMx10 e **(D)** CIMx20. A integridade do biofilme foi afetada pela nistatina (controle positivo) **(E)**, mas não pelo veículo (controle negativo) **(F)**. O controle de microrganismo sem tratamento também foi realizado **(G)**.

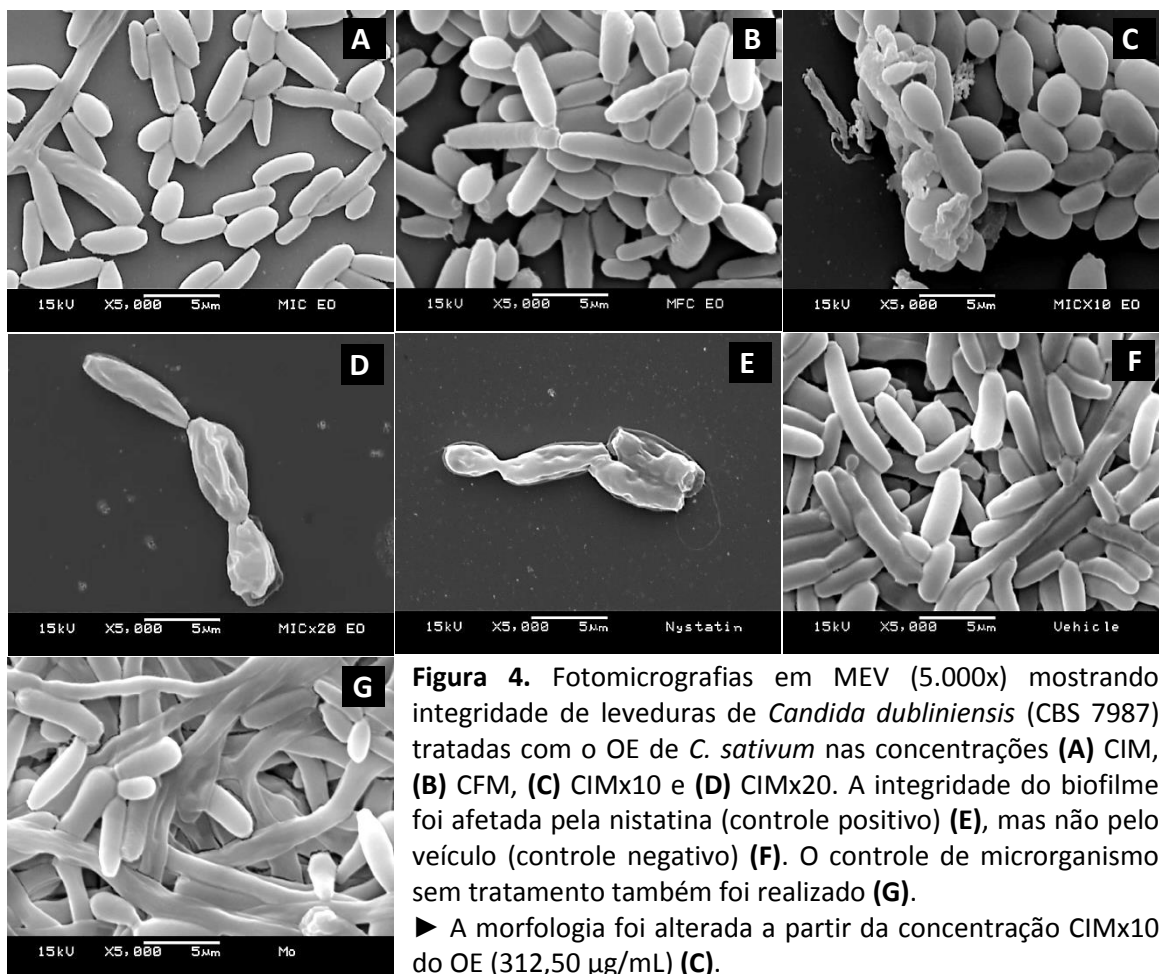► A morfologia foi alterada a partir da concentração CIMx10 do OE (312,50 µg/mL) **(C)**.

Figura 5. Fotomicrografias em MEV (5.000x) mostrando integridade de leveduras de *Candida rugosa* (CBS 12) tratadas com o OE de *C. sativum* nas concentrações **(A)** CIM, **(B)** CFM, **(C)** CIMx10 e **(D)** CIMx20. A integridade do biofilme foi afetada pela nistatina (controle positivo) **(E)**, mas não pelo veículo (controle negativo) **(F)**. O controle de microrganismo sem tratamento também foi realizado **(G)**.

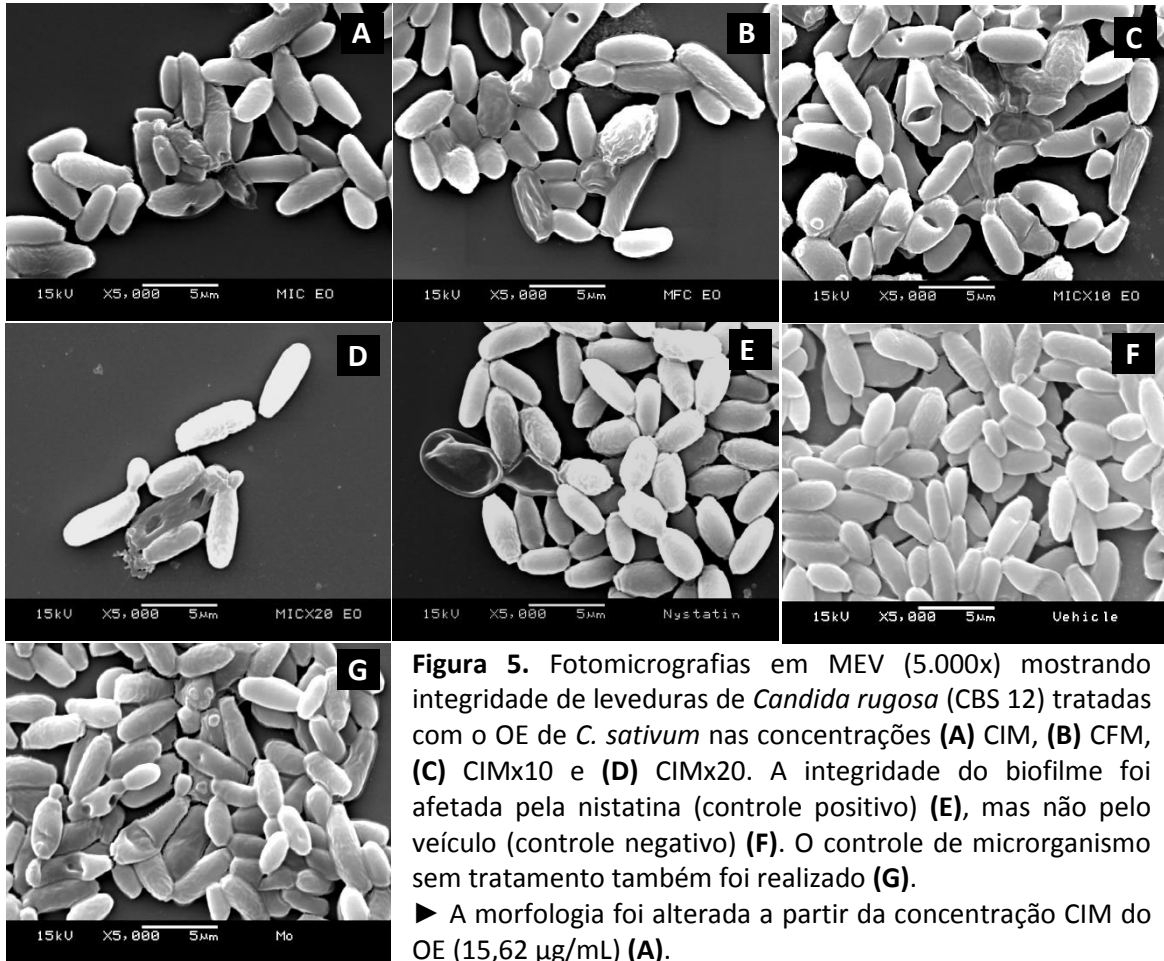► A morfologia foi alterada a partir da concentração CIM do OE (15,62 µg/mL) **(A)**.

Apêndice 2. Efeito do OE sobre a Atividade Proteolítica de *C. albicans*.

Abaixo, seguem fotos dos halos no meio de cultura YNB/BSA produzidos por proteases de *C. albicans* CBS 562. O intervalo de Pz foi calculado a partir da razão: diâmetro da colônia (dc) / diâmetro da colônia + halo (dch) e determinou a magnitude da atividade enzimática.

Intervalo de Pz	Atividade Enzimática
Pz = 1,0	Ausência de atividade enzimática
$1,0 > Pz \geq 0,64$	Atividade enzimática positiva
Pz < 0,64	Atividade enzimática fortemente positiva

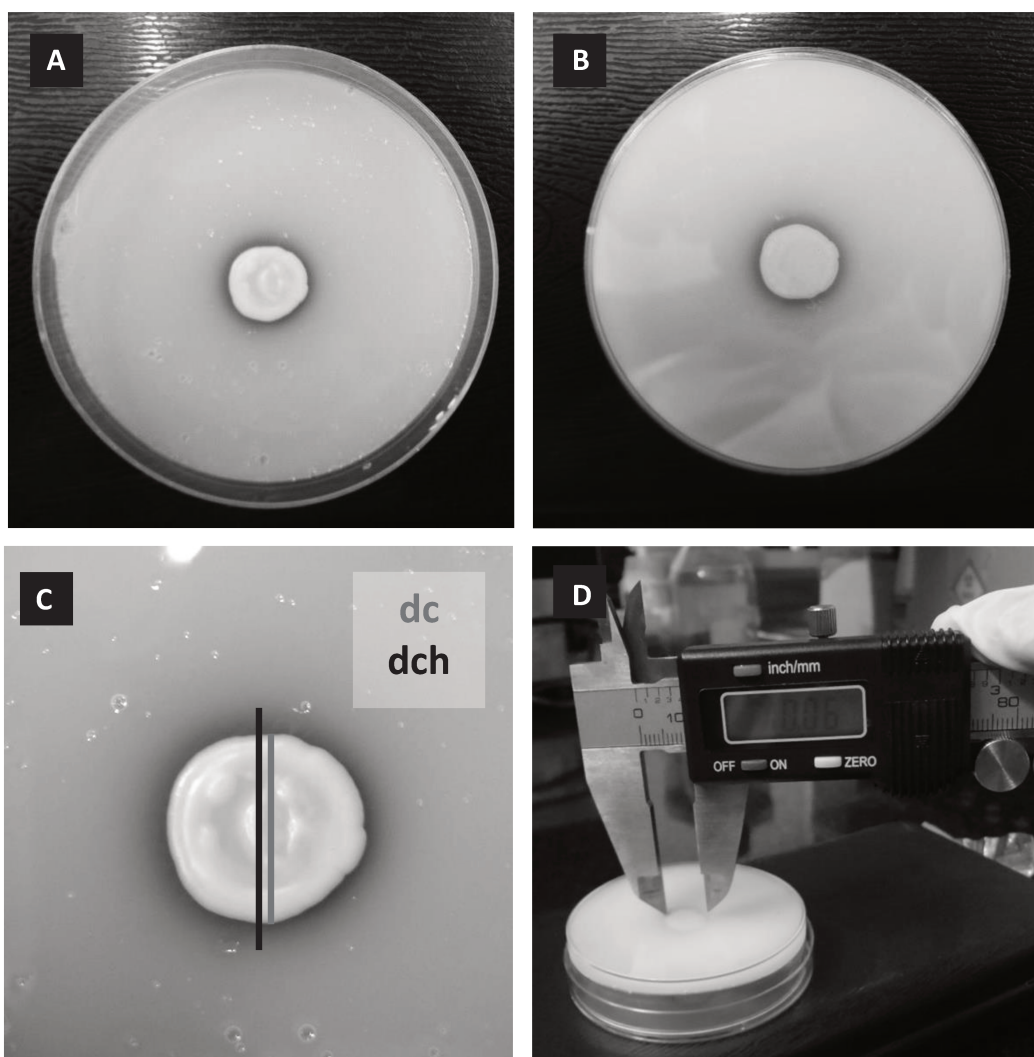**Figura 6.** Imagens representativas dos halos produzidos em meio de cultura YNB/BSA por proteases de *C. albicans* CBS 562. (A) Vista interior da placa; (B) Vista posterior da placa, sendo o lado escolhido para mensurações; (C) Cálculo de Pz: dc/dch (dc: diâmetro da colônia; dch: diâmetro da colônia + halo); (D) Mensurações das triplicatas usando paquímetro digital (Western DC-60, Resolução 0,01mm, China).

ANEXOS

Anexo 1. Informação CPG/01/2008. Trata do Formato Padrão das Dissertações de Mestrado e Teses de Doutorado da UNICAMP.

INFORMAÇÃO CCPG/01/2008

Tendo em vista a necessidade de revisão da regulamentação das normas sobre o formato e a impressão das dissertações de mestrado e teses de doutorado e com base no entendimento exarado no Parecer PG nº 1985/96, que trata da possibilidade do formato alternativo ao já estabelecido, a CCPG resolve:

Artigo 1º - O formato padrão das dissertações e teses de mestrado e doutorado da UNICAMP deverão obrigatoriamente conter:

I.   Capa com formato único ou em formato alternativo que deverá conter informações relativas ao nível (mestrado ou doutorado) e à Unidade de defesa, fazendo referência à Universidade Estadual de Campinas, sendo o projeto gráfico das capas definido pela PRPG.

II. Primeira folha interna dando visibilidade à Universidade, a Unidade de defesa, ao nome do autor, ao título do trabalho, ao número de volumes (quando houver mais de um), ao nível (mestrado ou doutorado), a área de concentração, ao nome do orientador e co-orientador, ao local (cidade) e ao ano de depósito. No seu verso deve constar a ficha catalográfica.

III. Folha de aprovação, dando visibilidade à Comissão Julgadora com as respectivas assinaturas.

IV. Resumo em português e em inglês (ambos com no máximo 500 palavras).

V.   Sumário.

VI. Corpo da dissertação ou tese dividido em tópicos estruturados de modo característico à área de conhecimento.

VII. Referências, formatadas segundo normas de referenciamento definidas pela CPG da Unidade ou por critério do orientador.

VIII. Todas as páginas deverão, obrigatoriamente, ser numeradas, inclusive páginas iniciais, divisões de capítulos, encartes, anexos, etc... As páginas iniciais poderão ser numeradas utilizando-se algarismos romanos em sua forma minúscula.

IX. Todas as páginas com numeração "impar" serão impressas como "frente" e todas as páginas com numeração "par" serão impressas como "verso".

§ 1º - A critério do autor e do orientador poderão ser incluídos: dedicatória; agradecimento; epígrafe; lista de: ilustrações, tabelas, abreviaturas e siglas, símbolos; glossário; apêndice; anexos.

§ 2º - A dissertação ou tese deverá ser apresentada na língua portuguesa, com exceção da possibilidade permitida no artigo 2º desta Informação.

§ 3º - As dissertações e teses cujo conteúdo versar sobre pesquisa envolvendo seres humanos, animais ou biossegurança, deverão apresentar anexos os respectivos documentos de aprovação.

Artigo 2º - A critério do orientador e com aprovação da CPG da Unidade, os capítulos e os apêndices poderão conter cópias de artigos de autoria ou de co-autoria do candidato, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.

§ único - O orientador e o candidato deverão verificar junto às editoras a possibilidade de inclusão dos artigos na dissertação ou tese, em atendimento à legislação que rege o direito autoral, obtendo, se necessária, a competente autorização, deverão assinar declaração de que não estão infringindo o direito autoral transferido à editora.

Artigo 3º - Dependendo da área do conhecimento, a critério do orientador e com aprovação da CPG da Unidade, a dissertação ou tese poderá ser apresentada em formato alternativo, desde que observados os incisos I, II, III, IV, V e VII do artigo 1º.

Artigo 4º - Para impressão, na gráfica da Unicamp, dos exemplares definitivos de dissertações e teses defendidas, deverão ser adotados os seguintes procedimentos:

§ 1º - A solicitação para impressão dos exemplares de dissertações e teses poderá ser encaminhada à gráfica da Unicamp pelas Unidades, que se responsabilizarão pelo pagamento correspondente.

§ 2º - Uma versão definitiva da dissertação ou tese **gravada em arquivo PDF**, deve ser encaminhada à gráfica da Unicamp acompanhada do formulário "Requisição de Serviços Gráficos", onde conste o número de exemplares solicitados.

§ 3º - A gráfica da Unicamp imprimirá os exemplares solicitados com capa padrão. Os exemplares solicitados serão encaminhados à Unidade em, no máximo, cinco dias úteis.

§ 4º - No formulário "Requisição de Serviços Gráficos" deverão estar indicadas as páginas cuja reprodução deva ser feita no padrão "cores" ou "foto", ficando entendido que as demais páginas devam ser reproduzidas no padrão preto/branco comum.

§ 5º - As dissertações e teses serão reproduzidas no padrão frente e verso, exceção feita às páginas iniciais e divisões de capítulos; dissertações e teses com até 100 páginas serão reproduzidas no padrão apenas frente, exceção feita à página que contém a ficha catalográfica.

§ 6º - As páginas fornecidas para inserção deverão ser impressas em sua forma definitiva, ou seja, apenas frente ou frente/verso.

§ 7º - O custo, em reais, de cada exemplar produzido pela gráfica será definido pela Administração Superior da Universidade.

Artigo 5º - É obrigatória a entrega de dois exemplares para homologação.

Artigo 6º - Esta Informação entrará em vigor na data de sua publicação, ficando revogadas as disposições em contrário, principalmente as Informações CCPG 001 e 002/98 e CCPG/001/00.

Campinas, 09 de abril de 2008

Profa. Dra. Teresa Dib Zambon Atvars
Presidente
Comissão Central de Pós-Graduação

Anexo 2. Comprovante de submissão do artigo científico ao periódico proposto.

PLOS ONE

Coriandrum sativum L. (coriander) Essential oil: antifungal activity and mode of action on Candida spp., and molecular targets affected in the human whole-genome expression
--Manuscript Draft--

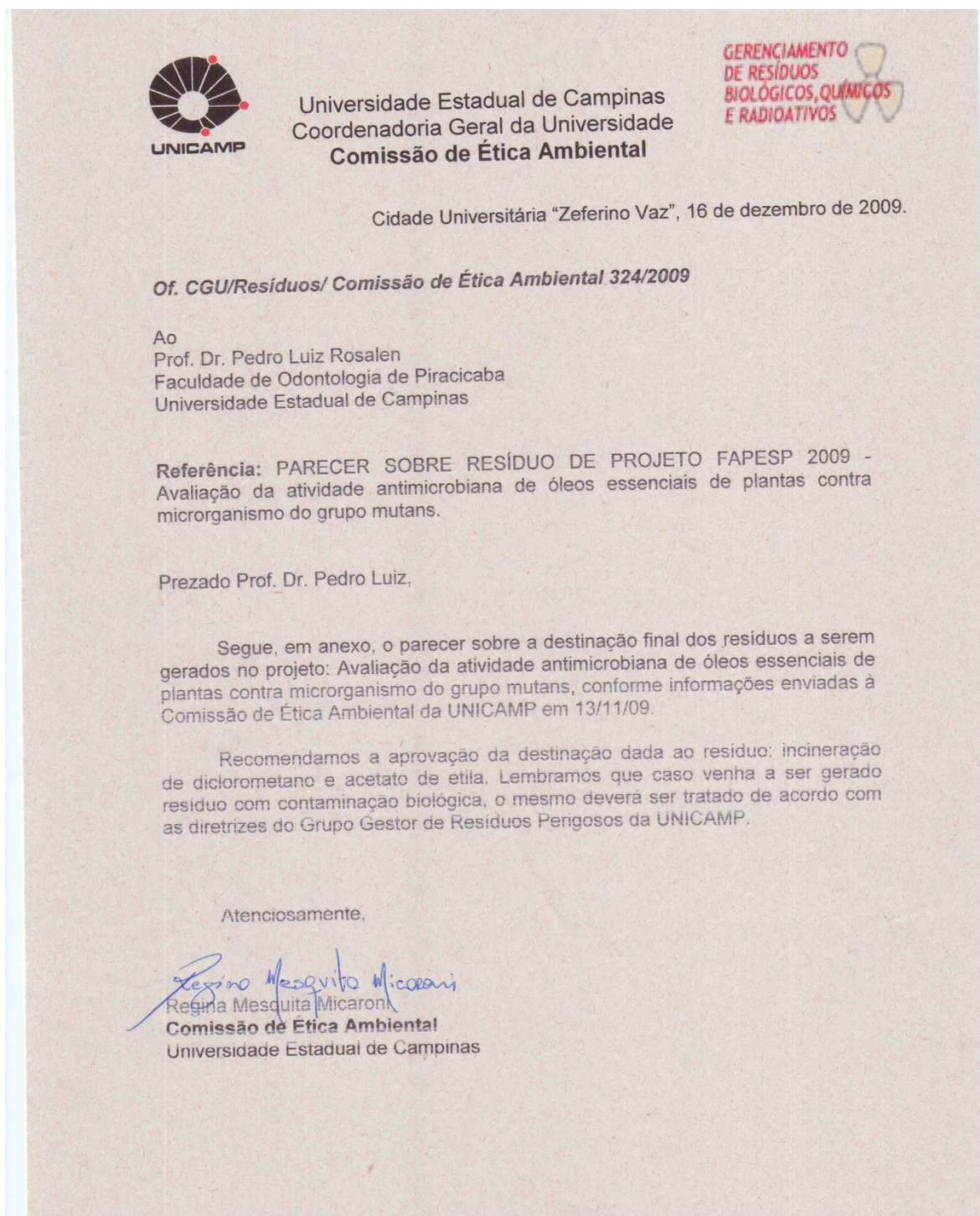|	
Manuscript Number:	PONE-D-13-54021
Article Type:	Research Article
Full Title:	Coriandrum sativum L. (coriander) Essential oil: antifungal activity and mode of action on Candida spp., and molecular targets affected in the human whole-genome expression
Short Title:	Antifungal activity of coriander essential oil
Corresponding Author:	Irlan de Almeida Freires, Ph.D. student Piracicaba Dental School, State University of Campinas ʹPiracicaba, Sao Paulo BRAZIL
Keywords:	Candida spp.; Essential Oils; Coriander; Antifungals; Pharmacogenomics.
Abstract:	Oral candidiasis is an opportunistic fungal infection of the oral cavity with increasingly worldwide prevalence and incidence rates. Novel specific-targeted strategies to manage this ailment have been proposed by using essential oils (EO) with antifungal properties. This work reports on the antifungal activity and mode of action of the EO from Coriandrum sativum L. (coriander) leaves on Candida spp. Also, the molecular targets affected in the whole-genome expression in human cells were determined. The EO phytochemical profile indicated monoterpenes and sesquiterpenes as major compounds, which are likely to account for the strong activity observed against yeasts viability. There seems to be a synergistic activity of the EO chemical compounds as their isolation into fractions led to a decreased antimicrobial power, confirmed by the higher inhibitory and fungicidal values found for the selected active fraction in relation to the entire oil. C. sativum EO may bind to membrane ergosterol, increasing ionic permeability and causing membrane damage to cell death, but it does not act on cell wall biosynthesis-related pathways. This mode of action is illustrated by photomicrographs showing disruption in biofilm integrity caused by the EO at varied concentrations. It was also found to inhibit Candida biofilm adherence onto a polystyrene substrate at low concentrations and to decrease the proteolytic activity of Candida albicans at minimum inhibitory concentration. Finally, the EO and its selected active fraction had low cytotoxicity on human cells, whose putative mechanisms affecting the whole-genome expression involve the pathways of chemokines and MAP-kinase (proliferation/apoptosis) as well as adhesion proteins. These findings highlight the auspicious antifungal activity of the EO from C. sativum leaves and encourage further toxicological and clinical research in this field.

Anexo 3. Certificado de aprovação do Comitê de Ética Ambiental – Universidade Estadual de Campinas/UNICAMP.

Salienta-se que o presente projeto é um recorte de um projeto maior com óleos essenciais financiado pela FAPESP (auxílio-pesquisa 2011/14757-0). Assim, a declaração abaixo contém o título original do projeto principal. Informamos que foram conduzidos os mesmos procedimentos de extração e descarte de solvente e material vegetal deferidos pela Comissão de Ética Ambiental.

Universidade Estadual de Campinas
Coordenadoria Geral da Universidade
Comissão de Ética Ambiental

Cidade Universitária "Zeferino Vaz", 16 de dezembro de 2009.

Of. CGU/Resíduos/ Comissão de Ética Ambiental 324/2009

Ao
Prof. Dr. Pedro Luiz Rosalen
Faculdade de Odontologia de Piracicaba
Universidade Estadual de Campinas

Referência: PARECER SOBRE RESÍDUO DE PROJETO FAPESP 2009 - Avaliação da atividade antimicrobiana de óleos essenciais de plantas contra microrganismo do grupo mutans.

Prezado Prof. Dr. Pedro Luiz,

Segue, em anexo, o parecer sobre a destinação final dos resíduos a serem gerados no projeto: Avaliação da atividade antimicrobiana de óleos essenciais de plantas contra microrganismo do grupo mutans, conforme informações enviadas à Comissão de Ética Ambiental da UNICAMP em 13/11/09.

Recomendamos a aprovação da destinação dada ao resíduo: incineração de diclorometano e acetato de etila. Lembramos que caso venha a ser gerado resíduo com contaminação biológica, o mesmo deverá ser tratado de acordo com as diretrizes do Grupo Gestor de Resíduos Perigosos da UNICAMP.

Atenciosamente,

Regina Mesquita Micaroni
Comissão de Ética Ambiental
Universidade Estadual de Campinas

Número do Processo na Comissão de Ética Ambiental da UNICAMP: 84/2009

IDENTIFICAÇÃO

1. Unidade: Faculdade de Odontologia de Piracicaba - FOP
2. Pesquisador responsável: Prof. Dr. Pedro Luiz Rosalen
3. Título do Projeto: Avaliação da atividade antimicrobiana de óleos essenciais de plantas contra microrganismo do grupo mutans.
4. Agência de Fomento: FAPESP

PARECER DO ASSESSOR

A natureza dos resíduos listados é condizente com as atividades previstas no projeto? Comente.

Sim, pois este projeto visa estudar a atividade antimicrobiana in vitro de óleos essenciais de espécimes da "Coleção de Plantas Medicinais e Aromáticas" do CPQBA/UNICAMP, contra Estreptococos do grupo mutans, principais agentes responsáveis pela cárie. Deste modo, está prevista a geração dos seguintes resíduos químicos: diclorometano e acetato de etila: Há a possibilidade de geração de resíduo biológico, visto que a atividade antimicrobiana será estudada.

A quantidade de resíduos estimada é condizente com as atividades do projeto? Comente.

Sim, uma vez que a quantidade estimada de resíduo químico gerado é de cerca de 20 kg, a qual é compatível com as atividades descritas no projeto. O custo previsto para o tratamento é de R$ 3,00/kg, ficando dentro da ordem de grandeza paga pela UNICAMP para incineração.

O(s) tratamento(s), a destinação final e o orçamento são adequados?

Sim, pois os compostos orgânicos: diclorometano e acetato de etila podem ser incinerados. Lembramos que caso venha a ser gerado resíduo com contaminação biológica, o mesmo deverá ser tratado de acordo com as diretrizes do Grupo Gestor de Resíduos.

Comentários, críticas e sugestões.

Recomendamos que os reagentes residuais cujas gerações sejam preestabelecidas devido ao emprego de reagentes específicos sejam descritos e as metodologias de tratamento indicadas.

Recomendamos a aprovação da destinação proposta para os resíduos de solventes. Os tratamentos dos reagentes residuais serão avaliados quando os mesmos puderem ser especificados.

Local: Campinas   Data: 16/12/09

Regina Mesquita Micaroni
Comissão de Ética Ambiental
Universidade Estadual de Campinas