UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Desenvolvimento de nanoemulsões catiônicas contendo benzofenonas da propolis vermelha brasileira visando ao tratamento de infecções fúngicas mucocutâneas

DANIEL FASOLO

Porto Alegre, maio de 2016

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Desenvolvimento de nanoemulsões catiônicas contendo benzofenonas da propolis vermelha brasileira visando ao tratamento de infecções fúngicas mucocutâneas

Tese apresentada por **Daniel Fasolo** para obtenção do TÍTULO DE DOUTOR em Ciências Farmacêuticas.

Orientador: Prof. Dr. Helder Ferreira Teixeira

Co-orientadora: Prof. Dra. Gilsane Lino von Poser

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"Antes de ser um excelente profissional seja um bom ser humano."

Autor desconhecido

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APRESENTAÇÃO

De acordo com as normas vigentes no Regimento do Programa de Pós-Graduação em Ciências Farmacêuticas, da Universidade Federal do Rio Grande do Sul, a presente tese de Doutorado foi redigida na forma de capítulos, com encarte de publicações. Assim, este exemplar encontra-se dividido da seguinte forma:

- Introdução;
- Objetivos (geral e específicos);
- Revisão do tema;
- Capítulo I: Activity of Brazilian red propolis extracts against non-*albicans Candida* species;
- Capítulo II: Determination of benzophenones in lipophilic extract of Brazilian red propolis, nanotechnology-based product and porcine skin and mucosa. Analytical and bioanalytical assays. Publicado em : *Journal of Pharmaceutical and Biomedical Analysis* 124 (2016) 57-66;
- Capítulo III: Topical delivery of antifungal Brazilian red propolis benzophenones-rich extract by means of cationic lipid nanoemulsions optimized by Box-Behnken Design;
- Discussão geral e perspectivas;
- Conclusões;
- Referências;
- Anexo: Artigo "Polyprenylated benzophenones enriched extracts obtained by supercritical carbon dioxide from the dry ethanolic extract of Brazilian red propolis"

RESUMO

Os compostos lipofílicos da própolis vermelha brasileira (PVB) têm recebido atenção especial devido a interessantes relatos referentes às suas atividades biológicas. Neste contexto, este estudo teve três etapas: a primeira objetivou investigar a atividade do extrato bruto etanólico da PVB e respectivas frações (obtidas com solventes de polaridade crescente, por meio de maceração em temperatura ambiente) contra cepas de *Candida* não-*albicans* (CNA) - *C. krusei, C. glabrata, C. tropicalis e C. parapsilosis*. A concentração inibidora mínima (CIM) foi determinada pelo método de microdiluição em caldo, com concentrações variando entre 1,9 a 500 µg/mL. Dentre as frações testadas, a fração hexânica (HEX) apresentou maior potencial antifúngico, alcançando valor de CIM de 1,95 µg/mL contra espécies de *C. parapsilosis*. Para cepas de *C. glabrata, C. krusei* e *C. tropicalis*, os valores de CIM obtidos foram variáveis (1,95-250 µg/mL). Ensaio colorimétrico de MTT foi utilizado para confirmar o dano celular e os resultados variaram de 80,66 a 94,44%, com extensa morte celular causada pela HEX, incluindo efeitos contra cepas resistentes de CNA, principalmente *C. glabrata* e *C. parapsilosis*. Esta potencial capacidade antifúngica está relacionada com compostos lipofílicos, provavalmente benzofenonas polipreniladas (BPPs), anteriormente descritas para PVB. A segunda etapa teve como objetivo avaliar a composição química do extrato hexânico da PVB (HEXred) por CLUE-DAD-EM. A investigação química resultou, principalmente, na identificação de BPPs (oblongifolina A, gutiferona E e/ou xantochimol). Após, um método de CLAE-UV isocrático foi validado para a determinação do teor total de BPPs (a 260 nm) expresso como garcinol, um diasterorisômero da gutiferona E disponível comercialmente. O método mostrou ser específico, exato, preciso e linear (0,1 a 10 µg/mL) para a determinação de BPPS na HEXred e na nanoemulsão (NE) contendo HEXred, bem como em amostras de pele e mucosa suínas, após estudos de permeação/retenção. O efeito matriz foi determinado para todas as matrizes complexas, demonstrando baixo efeito durante a análise. A estabilidade do método foi verificada por meio da exposição da HEXred às condições de estresse ácida, alcalina, oxidativa e térmica. Não houve interferência dos produtos de degradação durante a análise, indicando que o método

analítico/bioanalítico proposto provou ser simples e confiável para a determinação de BPPs na presença de diferentes matrizes. Como terceiro passo nós buscamos otimizar a incorporação de BPPs em NE destinada ao tratamento de infecções fúngicas mucocutâneas. A otimização da NE foi realizada por meio de experimento de Box-Behnken, que permitiu avaliar simultaneamente a influência das concentrações do fosfolipídeo da lecitina de gema de ovo, do lípido catiónico DOTAP e das BPPs nas propriedades físico-químicas da NE, bem como na eficiência de associação (EA) das BPPs. Utilizando o software Mini-Tab®, a formulação ótima foi selecionada com base no menor tamanho de gotícula e maiores potencial zeta e eficiência de associação, exibindo um tamanho médio de 140,56 ± 5,22 nm, potencial zeta de + 60,72 ± 3,07 e 99,55 ± 1,09% de EA. Células de difusão do tipo Franz foram usadas para avaliar a distribuição das BPPs através da pele e mucosa suínas, sendo encontradas BPPs nas camadas de ambos os tecidos (principalmente na derme). Uma maior quantidade de BPPs (até 3 vezes) foi detectada na pele e mucosa lesadas o que demonstra o efeito da integridade do tecido na distribuição das BPPs, como sugerido por imagens de microscopia confocal de fluorescência. As BPPs foram detectadas no fluido receptor apenas quando a mucosa esofágica foi lesada. A atividade antifúngica das formulações foi investigada contra espécies de CNA - *C. krusei, C. glabrata, C. tropicalis e C. parapsilosis*. Os valores de CIM variaram de 0,654 a 2,617 µg/mL, com dano celular superior a 78% como verificado por ensaio de MTT. Tais resultados sugerem que a NE otimizada possui potencial promissor para ser utilizada topicamente para o tratamento de infecções fúngicas mucocutâneas causadas por espécies de CNA.

Palavras-chave: própolis vermelha brasileira, benzofenonas polipreniladas, atividade antifúngica, *Candida* não-*albicans*, validação de método, nanoemulsão tópica.

ABSTRACT

Lipophilic compounds of Brazilian red propolis (BRP) have received increasing attention due to some interesting findings regarding their biological activities. This study had three steps: first we aimed to investigate the activity of BRP crude ethanolic extract and their fractions (obtained with increasing polarity solvents, through maceration at room temperature) against non-*albicans Candida* (NAC) strains. Minimal inhibitory concentration (MIC) was determined by the broth microdilution method, with concentrations ranging from 1.9 to 500 µg/mL. Among the tested fractions, *n*-hexane fraction (HEX) showed higher antifungal potential, achieving MIC values of 1.95 µg/mL against *C. parapsilosis* strains. On *C. glabrata, C. krusei* and *C. tropicalis* strains, variable MIC values were obtained (1.95 to 250 µg/mL). MTT colorimetric assay was employed to confirm the cell damage and the results ranged from 80.66 to 94.44%, with extensive cell death caused by HEX, including the effects against NAC resistant strains, mainly *C. glabrata* and *C. parapsilosis*. This potential antifungal capacity showed by HEX is related to the lipophilic compounds, probably polyprenylated benzophenones (PPBs), previous described for BRP. In the second step we aimed to evaluate the chemical composition of BRP *n*-hexane extract (HEXred) by UPLC-PDA-MS. Chemical investigation mainly resulted in the identification of PPBs in this extract, named oblongifolin A, guttiferone E, and/or xanthochymol. After that, an isocratic HPLC-UV method was validated for the determination of total content of PPBs (at 260 nm) expressed as garcinol, a commercially available guttiferone E diastereoisomer. The method showed to be specific, precise, accurate, and linear (0.1 to 10 µg/mL) for the determination of PPBs in HEXred, BRP-loaded nanoemulsions (NE), as well as, in porcine skin and mucosa samples after permeation/retention studies. The matrix effect was determined for all complex matrices, demonstrating low effect during the analysis. The stability-indicating method was verified by submitting HEXred to acidic, alkaline, oxidative, and thermal stress conditions. No interference of degradation products was detected during analysis, indicating that the proposed analytical/bioanalytical method proved to be simple and reliable for the determination of PPBs in the presence of different matrices. As third step we aimed to optimize the

incorporation of PPBs into NE intended for the treatment of mucocutaneous fungal infections. The optimization of NE was performed by means of a Box-Behnken Design, which allowed evaluating simultaneously the influence of the phospholipid egg-lecithin, the cationic lipid DOTAP and PPBs concentrations on the physicochemical properties of NE, as well as on the association efficiency (AE) of PPBs. By using the Mini Tab® software, the optimal formulation was selected based on the smallest droplet size and highest zeta potential and AE, exhibited a mean average size of 140.56±5.22 nm, zeta potential of +60.72±3.07 and AE of 99.55±1.09%. Franz-type diffusion cells were used to evaluate PPBs distribution through porcine skin and mucosa being PPBs found in both mucosa and skin layers (mainly in the dermis). A higher amount of PPBs (up to 3-fold) was detected in impaired skin and mucosa demonstrating the effect of the integrity of the tissue on PPBs distribution, as suggested by confocal fluorescence microscopy images. PPBs were detected in the receptor fluid only when esophageal mucosa was impaired. The antifungal activity of the formulations was investigated against NAC species – *C. krusei, C. glabrata, C. tropicalis* and *C. parapsilosis*. MIC values varied from 0.654 to 2.617 µg/mL, with cell damage higher than 78 % as verified by MTT assay. Such results suggest that the optimized NE have promising potential to be used topically for the treatment of mucocutaneous fungal infections caused by NAC strains.

Keywords: Brazilian red propolis, polyprenylated benzophenones, antifungal activity, non-*albicans Candida*, method validation, topical nanoemulsion.

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BPP/BPPs	Benzofenona poliprenilada/ Benzofenonas polipreniladas
CFM	Concentração Fungicida Mínima
CNA	*Candida* não-*albicans*
CIM	Concentração Inibitória Mínima
CLAE	Cromatografia Líquida de Alta Eficiência
CLUE-DAD-EM	Cromatografia Líquida de Ultra Eficiência acoplada a Detector de Arranjo de Diodos e Espectrômetro de Massas
CLAE-UV	Cromatografia Líquida de Alta Eficiência acoplada a Detector de Ultravioleta
DOTAP	1,2-dioleoil,3-trimetilamôniopropano
HEX	Fração *n*-hexano
HEXred	Extrato hexânico da própolis vermelha brasileira
MTT	Brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio
NE/NEs	Nanoemulsão/Nanoemulsões
ODD	Octildodecanol
PVB	Própolis vermelha brasileira
TCM	Triglicerídeos de cadeia média
UV	Ultravioleta

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ANEXO 169

1. INTRODUÇÃO

As infecções causadas por fungos patogênicos são muito comuns e vêm se tornando mais frequentes nos últimos anos. A candidíase invasiva (infecção sistêmica) é uma das principais causas de infecções hospitalares ligadas a uma série de fatores de risco, tais como a terapia antimicrobiana preventiva, terapias imunossupressoras, cateteres urinários e venosos (QUINDÓS, 2014; DELALOYE e CALANDRA, 2014). As candidíases mucocutâneas (infecção superficial) como orofaríngea, vulvovaginal e cutânea também têm sido correntemente relatadas. Sabe-se que o agente mais frequente de candidíase é a *C. albicans*. Entretanto, observa-se nas duas últimas décadas um aumento da taxa de infecções causadas por espécies de *Candida* não-*albicans* (CNA), especialmente *C. tropicalis, C. parapsilosis, C. glabrata* e *C. krusei* (GIOLO e SVIDZINSKI, 2010; PFALLER *et al.,* 2011; ORASCH, 2014).

A terapia antifúngica para o tratamento das candidíases inclui polienos, azóis e equinocandinas. Contudo, os efeitos adversos, a toxicidade, as interações medicamentosas e a resistência a estes fármacos limitam seu uso, principalmente em pacientes com insuficiência hepática ou renal (COLOMBO *et al.*, 2006; ZARAGOZA e PEMÁN, 2012; SARDI *et al.*, 2013). Neste cenário, tem se observado um aumento de casos de infecção oportunista causada por *Candida,* tornando-se necessárias novas opções terapêuticas (SARDI *et al.*, 2013).

Desde os primórdios, recursos naturais são utilizados como ferramentas para a medicina popular, a qual, por sua vez, serve como fonte para o desenvolvimento de pesquisas científicas. A biodiversidade oferece uma variedade de compostos ativos que podem se tornar úteis no tratamento das mais diversas doenças existentes, sejam eles na forma de substâncias isoladas de plantas ou sinteticamente modificadas a partir deste protótipo natural (BAKER *et al.,* 1995; CORDELL, 1995). STUFFNESS e DOUROS, no ano de 1982, já haviam relatado que acima de 50% dos fármacos usados na clínica possuíam sua origem a partir de produtos naturais e, segundo NEWMANN e colaboradores (2003), somente na área de doenças infecciosas, entre os anos de 1981 a 2002, 75% dos novos fármacos foram originados de recursos naturais. Entretanto, de acordo com BEDOYA e colaboradores (2009), apenas cerca de 10% da biodiversidade mundial já foi testada para atividade biológica, ou seja, existe um enorme potencial de compostos naturais aguardando ser descoberto.

Dentre as famílias de plantas, uma em especial, Clusiaceae, tem provado ser uma excelente fonte de compostos ativos. Esta possui mais de 1000 espécies especialmente distribuída nos trópicos (BENNETT e LEE, 1989), sendo as espécies dos gêneros *Garcinia* e *Clusia* ricas em metabólitos secundários. Estudos fitoquímicos com frutos, cascas dos caules, flores, sementes, folhas e raízes de plantas destes gêneros contribuíram para o isolamento de diversas moléculas, incluindo xantonas (HO, HUANG e CHEN, 2002; RUKACHAISIRIKUL *et al.,* 2003; HAY *et al.,* 2004) e benzofenonas preniladas, conhecidas como polipreniladas ou poli-isopreniladas (CUESTA-RUBIO *et al.,* 2001; LAKSHMI, KUMAR e DENNIS, 2002; ITO *et al.,* 2003; ABE *et al.,* 2004; BAGGETT *et al.*, 2005; HERATH *et al.,* 2005; MONZOTE *et al.*, 2011). As benzofenonas polipreniladas (BPPs) têm sido foco de diversos estudos devido às suas propriedades biológicas. Cabe salientar que os gêneros *Garcinia* e *Clusia* têm sido reportados como as principais fontes de BPPs, porém outros gêneros também apresentam estes compostos como *Vismia, Allanblackia, Moronobea, Symphonia, Hypericum, Tovomita, Tovomiptosis Ochrocarpus* (ACUNA, JANCOVSKI e KENNELLY, 2009). Esta classe de substâncias ocorre, principalmente, em frutos, galhos, pericarpo, caules, folhas e casca do caule de plantas do gênero *Garcinia* (figura 1).

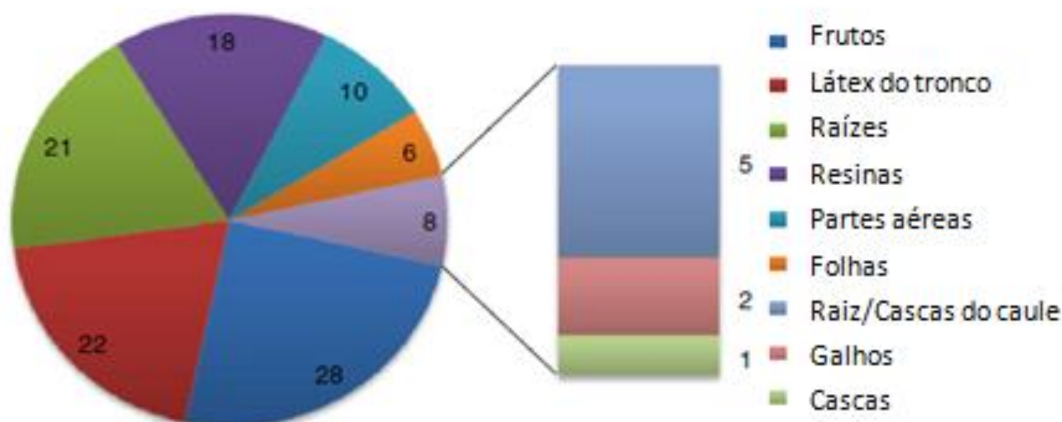

Figura 1. Percentual de BPPs preniladas nas diferentes partes das plantas (adaptado de KUMAR, SHARMA e CHATTOPADHYAY, 2013).

As resinas constituem uma fonte importante de BPPs, principalmente pelo fato do uso destas pelas abelhas para a produção de própolis, a qual é extensamente utilizada na medicina popular em virtude de sua gama de atividades, as quais incluem antifúngica, anti-inflamatória, antioxidante, antitumoral, imunomoduladora, entre outras (TRUSHEVA *et al.*, 2004). A própolis é um material resinoso produzido pelas abelhas a partir de exsudato de ramos das plantas misturado com cera e enzimas presentes na saliva destes insetos, cuja composição depende da origem geográfica e da espécie de abelha (DUTRA *et al.,* 2008). A própolis brasileira foi classificada por PARK e colaboradores (2000) em doze grupos, de acordo com a sua composição e origem geográfica. Mais recentemente, a própolis vermelha (denominação decorrente da sua intensa cor vermelha), pertencente a um novo grupo de própolis, tem despertado grande interesse devido às diversas atividades farmacológicas, incluindo antimicrobiana e citotóxica, geralmente sendo relatadas para extratos etanólicos e hidroetanólicos (ALENCAR *et al.*, 2007; RIGHI *et al.*, 2011).

A análise química (qualitativa e quantitativa) da própolis é complexa, uma vez que se trata de uma mistura de diferentes substâncias, sendo que os mais citados são os compostos fenólicos, como flavonoides e benzofenonas (BANKOVA *et al.,* 2000; MELLIOU e CHINOU, 2004; TRUSHEVA *et al.,* 2004; LUSTOSA *et al.*, 2008; POPOVA *et al.,* 2010). Em termos de análise quantitativa não há, até o momento, monografia específica disponível para a própolis em virtude da variação química apresentada por este produto em função da vegetação no local de coleta e da sazonalidade (SIMÕES-AMBROSIO *et al.*, 2010; GUO *et al.*, 2011; PICCINELLI *et al*, 2011; SFORCIN e BANKOVA, 2011). Assim, o desenvolvimento de metodologia analítica, etapa fundamental na descoberta de produtos, torna-se também uma tarefa desafiadora. A composição química de extratos de origem natural é geralmente expressa em um marcador químico ou uma classe de compostos. As agências reguladoras determinam que um método analítico deve ser validado em termos de linearidade, seletividade, precisão, exatidão e robustez (ICH, 2005; FDA, 2014) e, além disso, segundo NIESSEN e colaboradores (2006), o efeito de matriz (grau de interferência de cada matriz presente no estudo) deve ser avaliado para demonstrar a versatilidade e confiabilidade deste método.

Nanoemulsões têm sido investigadas como potenciais carreadores para extratos e/ou frações de compostos bioativos oriundos de produtos de origem natural (SARAF, 2010). Os compostos de menor polaridade encontram-se preferencialmente incorporados no núcleo oleoso e/ou adsorvido na interface óleo/água, enquanto que os solúveis permanecem na fase aquosa externa. Esses sistemas são constituídos de nanogotículas de óleo dispersas em água através do uso de um sistema tensoativo adequado (GUPTA *et al.*, 2016). O desenvolvimento de nanoemulsões lipídicas de uso tópico contendo compostos bioativos de origem natural, especialmente de reduzida hidrossolubilidade, tem sido objeto de estudo em nosso grupo de pesquisa (FASOLO, BASSANI e TEIXEIRA, 2009; SILVA *et al.,* 2009; ARGENTA *et al.,* 2011; VARGAS *et al.,* 2012, ARGENTA *et al.*, 2014). Até o momento, o conjunto dos resultados obtidos, indica um baixo fluxo de permeação dos compostos de interesse através da mucosa e/ou pele de orelha suína, apontando para uma potencial aplicação destes produtos para o tratamento local.

Considerando os relatos recentes de atividade antifúngica de extratos da própolis vermelha brasileira frente a espécies emergentes de CNA, a presente tese de doutorado visa otimizar a composição de nanoemulsões lipídicas contendo um extrato de própolis vermelha brasileira com atividade frente a cepas de CNA, com ênfase no desenvolvimento de produtos para o tratamento local (de pele e de mucosas) de infecções fúngicas, bem como avaliar *in vitro* a atividade dessas formulações.

2. OBJETIVOS

2.1. Objetivo Geral

Desenvolver nanoemulsões lipídicas contendo fração lipofílica da própolis vermelha brasileira visando o tratamento local de infecções fúngicas causadas por espécies de *Candida* não-*albicans.*

2.2. Objetivos Específicos

- Avaliar a atividade antifúngica *in vitro* do extrato bruto da própolis vermelha brasileira e respectivas frações obtidas através de maceração e selecionar as mais promissoras;
- Caracterizar quimicamente extratos e frações de própolis vermelha brasileira mais promissoras em relação a atividade antifúngica;
- Otimizar, através de desenho experimental, uma nanoemulsão contendo a fração lipofílica da própolis vermelha brasileira que apresente menor diâmetro de gotícula e maior potencial zeta e eficiência de encapsulação;
- Desenvolver metodologia analítica por cromatografia líquida de alta eficiência para a quantificação de composto(s) majoritário(s) antes e após a incorporação nas nanoemulsões e na presença de matrizes biológicas (pele e mucosa suína).
- Avaliar a permeação/retenção dos compostos ativos contidos na nanoemulsão otimizada na pele e mucosa de origem suína, íntegras e lesadas;
- Avaliar a atividade antifúngica da nanoemulsão otimizada frente às espécies de *Candida* não-*albicans*.

3. REVISÃO DO TEMA

3.1. Própolis

A própolis é um produto resinoso que contém cera, enzimas, açúcar e exsudatos de plantas coletadas por abelhas *Apis mellifera* a partir de diversas fontes vegetais, dentre elas pinheiro (*Araucaria angustifolia*), salgueiro (*Salix alba*), alecrim-do-campo (*Baccharis dracunculifolia)* e rabo-de-bugio (*Dalbergia ecastophyllum)*. A palavra "própolis" possui origem grega e significa "*pro* = a favor, em frente, na entrada" e "*polis* = comunidade ou cidade", ou seja, "a favor/em defesa da colmeia" (CASTALDO e CAPASSO, 2002; PARK *et al.*, 2004a; DAUGSCH *et al.*, 2008). A própolis é utilizada pelo homem desde os tempos antigos em rituais de mumificação, medicina popular e, mais recentemente, na indústria de alimentos e bebidas, cosméticos, anti-sépticos bucais e cremes dentais (WAGH, 2013). A composição química é complexa e variável sendo altamente influenciável pela vegetação no local de coleta (DUTRA *et al.*, 2008; SILVA *et al.* 2008; GUO *et al.*, 2011; PICCINELLI *et al*, 2011; SFORCIN e BANKOVA, 2011; ATHIKOMKULCHAI *et al.*, 2013) e pela sazonalidade, o que pode interferir em suas propriedades biológicas (SIMÕES-AMBROSIO *et al.*, 2010). Neste contexto, pode-se facilmente associar a composição química de cada tipo de própolis com sua origem geográfica e respectiva flora, como descrito na Tabela 1.

Tabela 1. Comparativo geral de alguns tipos de própolis mundiais.

Origem	Composição	Fontes	Referência
Europa	Flavonoides; Ésteres de ácidos fenólicos	Ramos de *Populus* spp.	Popova *et al.*, 2007
Brasil (própolis verde)	Derivados prenilados do ácido *p*-cumárico	Ramos apicais de *Baccharis dracunculifolia*	Salatino *et al.*, 2005
Venezuela/ Cuba	BPPs	Flores de *Clusia major* e *Clusia minor* (Venezuela) e *Clusia nemorosa* (Cuba)	Trusheva *et al.*, 2004; Hernández *et al.*, 2005

Treze tipos de própolis brasileira já foram caracterizados, segundo o perfil químico (PARK *et al.,* 2000; SILVA *et al.,* 2008). Como exemplo, têm-se as própolis cujas origens botânicas são: Sul, Sudeste e Nordeste do país, sendo relacionadas as resinas como: da *Populus* sp., da *Hyptis divaricata* e de *Dalbergia ecastophillum*. A classificação da própolis brasileira, de acordo com as características físico-químicas e respectiva origem, está apresentada na Tabela 2.

Tabela 2. Classificação da própolis brasileira de acordo com as características físico-químicas e origem (Adaptado de TORETI *et al.*, 2013).

Grupo	Cor	Origem
1	Amarela	Sul
2	Marrom	Sul
3	Marrom escura	Sul
4	Marrom	Sul
5	Marrom esverdeada	Sul
6	Marrom avermelhada	Nordeste
7	Marrom esverdeada	Nordeste
8	Marrom escura	Nordeste
9	Amarela	Nordeste
10	Amarela escura	Nordeste
11	Amarela	Nordeste
12	Verde ou marrom esverdeada	Sudeste
13	Vermelha	Nordeste

As própolis dos Grupos 3 e 6 apresentam-se como promissores agentes anticárie e antiplaca, principalmente devido à sua propriedade antimicrobiana e capacidade de reduzir os fatores de virulência associados aos micro-organismos

causadores de cárie dental, bem como a produção de ácidos, a síntese de glucanos solúveis e insolúveis em água, entre outros (PARK *et al.,* 1998a, 1998b; KOO *et al.,* 2000a, 2000b, 2003; DUARTE *et al.,* 2003; HAYACIBARA *et al.,* 2005; DUARTE *et al.,* 2006).

A própolis encontrada na região sul do país (Grupo 3) é composta, prevalentemente, por flavonoides, terpenoides e ácidos fenólicos que são considerados os componentes bioativos da própolis (GHISALBERTI, 1979; BANKOVA *et al.,* 1982; IKENO *et al.,* 1991; BANKOVA, 1996; KOO *et al.,* 1999; BANKOVA *et al.*, 2005). Já a própolis do Grupo 6, tem um perfil químico diferente dos outros tipos de própolis, caracterizado pela ausência de flavonoides e derivados de ácido cinâmico e pela presença de uma grande quantidade de compostos apolares de cadeia longa, bem como, presença de benzofenona prenilada (plukenetiona H), que está relacionada à atividade antimicrobiana reportada para esse tipo de própolis (CASTRO *et al.,* 2009).

A própolis brasileira do Grupo 13 é majoritariamente composta por isoflavonoides, uma subclasse dos flavonoides, capaz de prevenir problemas de saúde relacionados à menoupausa, osteoporose, doenças cardiovasculares, câncer de próstata e cólon de útero (GREENWOOD *et al.,* 2000; LUTHRIA *et al.,* 2007). Recebeu este nome devido à sua cor vermelha intensa devido a presença de retusapurpurinas (ALENCAR *et al.*, 2007) e diversas propriedades biológicas e farmacológicas têm sido descritas, incluindo antimicrobiana e citotóxica (TRUSHEVA *et al.*, 2006; ALENCAR *et al.*, 2007; RIGHI *et al.*, 2011; FROZZA *et al.*, 2013).

A composição química da própolis vem sendo estudada e compostos fenólicos, como flavonoides e derivados do ácido cinâmico são os mais citados. Ácidos diterpênicos, diterpenos e benzofenonas também foram relatados para os diferentes tipos de própolis (BANKOVA *et al.,* 2000; MELLIOU e CHINOU, 2004; TRUSHEVA *et al.,* 2004; LUSTOSA *et al.*, 2008; POPOVA *et al.,* 2010). De acordo com SALATINO e colaboradores (2005), muitos compostos estão envolvidos na atividade biológica da própolis. As quantidades de ácido cafeico, flavonoides e ésteres fenólicos parecem ser os principais responsáveis pela atividade biológica do extrato ao qual pertencem, todavia, também foi evidenciada a existência de efeito sinérgico entre

estes e outros componentes, como terpenoides, esteroides e aminoácidos (KARTAL, YILDIZ e KAYA, 2003). Já para própolis vermelha, além dos isoflavonoides, foi observada a presença de benzofenonas (TRUSHEVA *et al.,* 2006), diterpenos e triterpenos (SAWAYA *et al.,* 2006; SAWAYA *et al.,* 2007).

Diversas atividades, incluindo antimicrobiana, antioxidante, antidiabética, antiparasitária, antitumoral, anti-herpética, anti-inflamatória e analgésica já foram avaliadas para diferentes tipos de própolis (BANKOVA *et al.,* 1995; BANSKOTA *et al.,* 2000; VYNOGRAD, VYNOGRAD e SOSNOWSKI, 2000; MARCUCCI *et al.,* 2001; SAWAYA *et al.,* 2002; CUNHA *et al.,* 2004; SAWAYA *et al.,* 2004; TRUSHEVA *et al.,* 2006; FISCHER *et al.,* 2007; ZAMAMI *et al.,* 2007; PAULINO *et al.,* 2008; SALOMÃO *et al.,* 2008; BOUKRAA e SULAIMAN, 2009; BURIOL *et al.,* 2009; SAWAYA *et al.,* 2009).

Ainda, efeito imunomodulador (PARK *et al.,* 2004b; ORSATTI *et al.,* 2010), capacidade de aumento da resistência a infecções (TYLER, 1987), propriedade antiviral (BURDOCK, 1998), bem como, ação inibitória sobre a divisão celular (ISHIHARA *et al.,* 2009), já foram relatados na literatura para esse produto. Em estudo com própolis do Paraná, a mesma foi considerada não-tóxica, apresentando DL_{50} maior que 5 g/kg de peso corporal dos ratos avaliados (ARAUJO *et al.,* 2002).

3.2. Benzofenonas

Benzofenonas naturais constituem uma classe contendo algumas centenas de produtos identificados, com grande diversidade estrutural, porém possuem um esqueleto comum fenil-carbonil-fenil **(1)** (Figura 1), o qual é composto por dois anéis: anel A (derivado da via do ácido chiquímico) é geralmente um anel de benzeno contendo 0, 1, ou 2 substituintes e anel B (derivado da via de acetato-malonato) sofre prenilação e ciclização produzindo uma ampla variedade de compostos (BAGGET *et al.*, 2005; CUESTA-RUBIO, PICCINELLI e RASTRELLI, 2005; ACUNA, JANCOVSKI e KENNELLY, 2009; KUMAR e SHARMA, 2013), como pode ser observado na Figura 1.

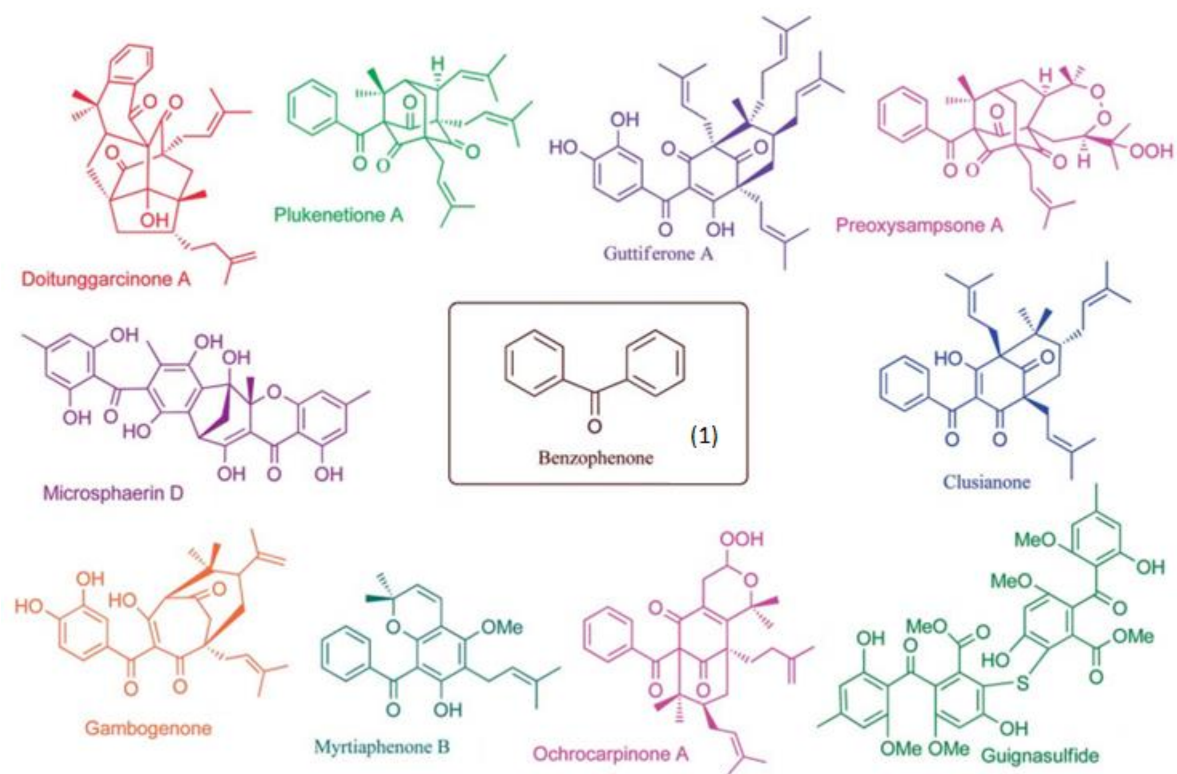

Figura 1. Exemplos de estruturas químicas das benzofenonas naturais (Fonte: WU, LONG e KENNELLY, 2014).

As benzofenonas polipreniladas (BPPs) representam uma subclasse importante das benzofenonas devido aos seus substituintes variáveis e sistemas complexos de anéis, sendo o sistema biciclo nonano o mais comumente encontrado, o qual pode ser observado em destaque na estrutura do garcinol (Figura 2).

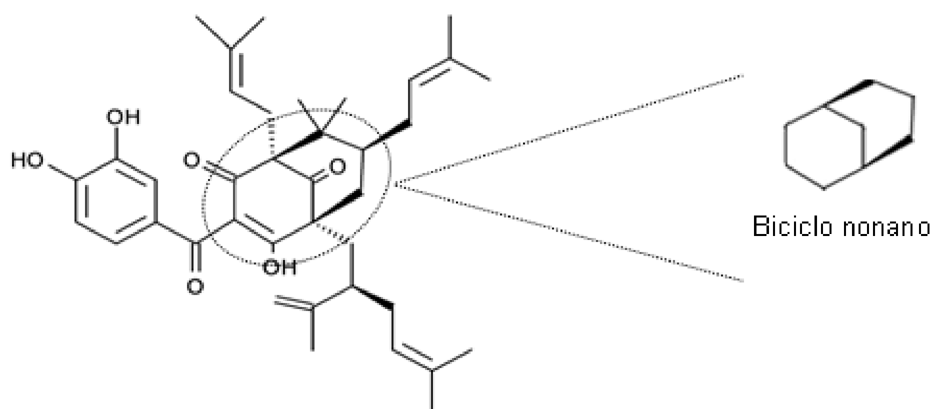

Figura 2. Fórmula estrutural do garcinol.

O garcinol ($C_{38}H_{50}O_6$) é um dos principais representantes das BPPs para as espécies do gênero *Garcinia*, com relatos de extração e isolamento a partir dos frutos, pericarpo e casca do caule de *G. purpurea, G. indica, G. pedunculata, G. cambogia* e *G. assigu* (SAHU, DAS e CCHATTERJEE, 1989; ITO *et al.*, 2003; CIOCHINA e GROSMANN, 2006; MASULLO *et al.*, 2008). Estudos realizados demonstraram que este composto possui propriedades anti-inflamatória, anticâncer, entre outras (SANG *et al.*, 2002). Esta substância também foi relatada por MARTI e colaboradores (2009) no látex do tronco de *Moronobea coccinea* (Clusiaceae), além de outros compostos relacionados como coccinona A–H, 14-deoxigarcinol, 7-epi-isogarcinol, 7-epi-garcinol, isogarcinol e cicloxantochimol.

3.3. Terapia antifúngica

Infecções causadas por fungos estão associados a altas taxas de morbidade e mortalidade, especialmente em pacientes imunocomprometidos e gravemente enfermos. Os agentes mais importantes de micoses oportunistas são *Candida albicans* e espécies de *Candida* não-*albicans* (CNA) como *C. glabrata, C. krusei, C. parapsilosis* e *C. tropicalis*, que são responsáveis nos últimos 20-30 anos por mais de 95% das candidíases invasivas (PEMÁN e SALAVERT, 2012; YAPAR, 2014). O maior estudo multicêntrico realizado na América Latina relatou uma incidência de candidemia em hospitais terciários brasileiros de 2,49 casos por mil internações hospitalares e taxa de mortalidade de 54%. Estes valores são 2-10 vezes superiores aos registrados em hospitais dos Estados Unidos e da Europa (COLOMBO *et al.*, 2006).

As terapias antifúngicas para o tratamento das candidíases invasiva ou mucocutânea incluem polienos, azóis e equinocandinas. Contudo, os efeitos adversos, toxicidade, interações medicamentosas e resistências crescentes limitam o uso desses fármacos, principalmente em pacientes com insuficiência hepática ou renal (COLOMBO *et al.*, 2006; ZARAGOZA e PEMÁN, 2012; SARDI *et al.*, 2013). A administração profilática e empírica de antifúngicos, em particular de azóis, devido a sua disponibilidade e por possuir amplo espectro, tem contribuído para o desenvolvimento de resistência a fungos (ALMIRANTE *et al.*, 2005). Neste cenário,

tem se observado um aumento de casos de infecção oportunista causada por *Candida* tornado-se necessárias novas opções terapêuticas (SARDI *et al.*, 2013).

Muitos relatos são encontrados na literatura que demonstram a eficácia de produtos naturais como fontes ricas de agentes potencialmente novos e seletivos para o tratamento de doenças importantes (NEWMANN e CRAGG, 2012). A própolis, um produto natural com vários efeitos farmacológicos, pode ser uma alternativa interessante na descoberta de novos compostos antimicrobianos, uma vez que atua como defesa da colmeia contra bactérias invasoras e fungos (BANSKOTA *et al.*, 2001).

3.4. Nanoemulsões e a administração tópica de fármacos

Do ponto de vista farmacêutico, nanoemulsões (NEs) podem ser definidas como sistemas constituídos por gotículas de óleo de diâmetro nanométrico uniformemente dispersas em uma fase aquosa, estabilizadas por um tensoativo. Possuem aparência leitosa, baixa viscosidade, sendo consideradas como carreadores para compostos bioativos que podem estar dispersos na fase oleosa e/ou adsorvidos na interface óleo/água das nanoestruturas (TADROS *et al.*, 2004, ANTON e VANDAMME, 2011; KOTTA *et al.*, 2014). Neste sentido, os óleos de origem vegetal e/ou semissintética (constituídos, principalmente, de triglicerídeos de cadeia média) são correntemente utilizados para compor a fase interna das NEs (YILMAZ e BORCHERT, 2006) enquanto as lecitinas (constituídas por misturas de fosfolipídeos extraídos da gema de ovo ou da soja, cujo componente majoritário é a fosfatidilcolina) são tensoativos bastante empregados (KIBBE, 2000; KOMMURU *et al.*, 2001, BIDONE *et al.* 2014). Os óleos e tensoativos formadores do núcleo oleoso correspondem de 5 a 20% da formulação.

O uso de lecitinas na obtenção de NEs tem sido relatado devido ao seu efeito na estabilização destas, uma vez que essas conferem uma carga negativa às nanogotículas auxiliando na prevenção da coalescência (PIEMI *et al.*, 1999; ZHOU *et al.,* 2010). Além disso, devido ao fato de que a composição heterogênea das lecitinas

pode resultar em uma variabilidade significativa da estabilidade destas formulações, a utilização da combinação de lecitinas com tensoativos sintéticos (co-tensoativos) como, por exemplo, polissorbato 80 e poloxâmero na preparação de NEs se torna interessante para evitar este problema (YILMAZ e BORCHERT, 2006; PRIMO *et al.,* 2007). Estudo realizado por ALVES, GUTERRES e POHLMANN (2005) demonstrou este tipo de associação, no qual os autores prepararam NE contendo nimesulida utilizando polissorbato 80 e monoestearato de sorbitano como sistema estabilizante. Outra característica importante em relação aos fosfolipídeos (que compões as lecitinas) é a propriedade hidratante da pele atribuída a estes (ZHOU *et al.,* 2010).

Dentre os métodos de formação das NEs, a técnica da emulsificação espontânea merece destaque pela vantagem de ser simples e rápida, possuir baixo custo e poder ser realizada a temperatura ambiente. Esta técnica consiste em verter a fase oleosa solubilizada em um solvente orgânico apropriado, contínua e lentamente, sobre a fase aquosa, sob agitação constante e moderada. Neste estágio, formam-se gotículas de óleo dispersas em água, sendo o solvente orgânico removido por evaporação sob pressão reduzida (VANDAMME e ANTON, 2010). Um fator importante neste tipo de técnica é o preparo à temperatura ambiente para evitar a degradação por hidrólise dos fosfolipídeos, pois em estudo realizado por RABINOVICH-GUILATT e colaboradores (2005), no qual uma emulsão contendo fosfolipídeos foi submetida à autoclavagem e condições térmicas aceleradas a uma temperatura de 50 °C, a ocorrência deste fenômeno foi observada.

A influência das propriedades físico-químicas da nanoestrutura, diâmetro de partícula, carga de superfície e viscosidade, na interação da NE com a pele (e na velocidade e extensão da permeação das substâncias veiculadas neste sistema) encontra-se bem relatada na literatura (BENITA, 1999; TADROS *et al.*, 2004). Segundo BOUCHEMAL e colaboradores (2004) e SONNEVILLE-AUBRUN e colaboradores (2004), o reduzido diâmetro de gotícula permite a aplicação de um depósito uniforme na pele, conferindo uma elevada superfície de contato do sistema com este órgão, sendo uma das principais vantagens das NEs em comparação aos sistemas convencionais para uso tópico. Além disso, os autores enfatizam que estas

formulações também podem promover a penetração dos compostos de interesse através da fluidização natural da pele na presença de óleos e tensoativos.

A pele, maior órgão do corpo humano, divide-se em três camadas distintas (epiderme, derme e hipoderme) e possui como função principal a proteção contra a perda de água e a penetração de agentes externos, exercendo também importante papel na termorregulação e na excreção de várias substâncias (WALTERS e ROBERT, 2002). A camada mais externa da epiderme, o estrato córneo, é constituída pelos corneócitos (células mortas ricas em queratina) localizados em uma matriz intercelular constituída por bicamadas lipídicas contendo ácidos graxos, ceramidas, fosfolipídeos, triglicerídeos e colesterol (SCHAEFER, REDELMEIER e BENECH-KIEFFER, 1999). Assim, no interior desta matriz, observa-se a presença de regiões hidrofílicas e lipofílicas, o que confere ao estrato córneo a função de barreira da pele, constituindo o principal obstáculo para a liberação tópica de fármacos: a baixa taxa de difusão do fármaco através deste (BOUWSTRA e HONEYWELL-NGUYEN, 2002). Devido a esta condição, o uso de NEs para administração tópica de fármacos pode modular a difusão transepidermal de compostos bioativos, podendo alterar a farmacocinética e a biodistribuição destes através da pele (CEVC, 2004).

Em estudo realizado por nosso grupo, SILVA (2006) utilizou octildodecanol (ODD) e triglicerídeos de cadeia média (TCM) no desenvolvimento de duas NEs lipídicas contendo o isoflavonoide genisteína. Estudo de permeação cutânea *in vitro* da genisteína foi realizado a partir da sua dispersão em ODD e TCM, bem como das NEs obtidas com os diferentes óleos. O total de genisteína permeada a partir dos óleos ODD e TCM (34,76 e 21,36 $\mu g/cm^2$, respectivamente) e das NEs com ODD e TCM (20,55 e 15,64 $\mu g/cm^2$, respectivamente) foi significativamente inferior em relação ao fluxo intrínseco (58,25 $\mu g/cm^2$), sugerindo uma maior afinidade da genisteína pelos veículos empregados. Isto pode levar a um maior tempo de retenção do isoflavonoide nas primeiras camadas da pele, pois modula a sua absorção e favorece a sua ação quando se deseja um efeito tópico.

Para a avaliação da permeação de substâncias através de mucosas, dentre diversos modelos, tanto a mucosa bucal quanto a mucosa esofágica, ambas de origem

suína, são utilizadas para mimetizar o tecido humano (HOOGSTRAATE *et al.,* 1996). Ambas as mucosas possuem um epitélio escamoso estratificado, escamoso e não queratinizado (sustentado por fibras de tecido conectivo), porém a diferença entre elas é que a esofágica possui uma camada de fibras musculares dispostas longitudinalmente (SQUIER e KREMER, 2001). Além disso, segundo CONSUELO e colaboradores (2005a), a composição lipídica destes dois epitélios é qualitativamente e quantitativamente similar, onde a alta quantidade de glicosilceramidas e o baixo teor de ceramidas evidenciam um epitélio não queratinizado. Este mesmo grupo de pesquisa comparou a permeabilidade do citrato de fentanil em ambas as mucosas, encontrando uma correlação positiva (CONSUELO *et al.,* 2005b).

3.5. Extrato de Própolis e Atividade Antifúngica

Relatos da atividade antifúngica para o extrato de própolis são evidenciados na literatura desde 1988, estudo no qual foi descrita atividade desse derivado vegetal frente a 23 cepas de isolados clínicos do gênero *Candida*, sendo fungistático na concentração de 550 µg/mL (ROJAS e LUGO, 1988). FERNANDES e colaboradores (1995) avaliaram o potencial antimicrobiano do extrato etanólico de própolis frente a diferentes fungos. Dentre as espécies testadas, *C. albicans* e *C. tropicalis* apresentaram maior sensibilidade, quando comparadas a *C. parapsilosis* e *C. guilliermondii.* KUJUMGIEV e colaboradores (1999) evidenciaram efeito antifúngico, frente a *C. albicans,* de própolis obtidas de diferentes regiões do mundo, incluindo amostras brasileiras. Os autores verificaram atividades semelhantes para própolis com composições químicas diferentes, sugerindo que a atividade pode estar relacionada a uma combinação de diferentes substâncias presentes nesses produtos, evidenciando o valor farmacológico desta mistura natural.

A comparação do potencial antimicrobiano *in vitro* de própolis coletadas nas quatro estações do ano, frente a *C. albicans* e *C. tropicalis* isoladas clinicamente, foi realizada e os resultados demonstraram que ambos os fungos foram suscetíveis a baixas concentrações de própolis. Ainda, não foram encontradas diferenças na

concentração inibitória mínima em relação à sazonalidade de coleta das amostras de própolis (SFORCIN *et al.,* 2001).

OTA e colaboradores (2001) verificaram atividade fungicida do extrato etanólico de própolis, obtido no estado de São Paulo, demonstrando a seguinte ordem de sensibilidade: *C. albicans* > *C. tropicalis* > *C. krusei* > *C. guilliermondii*. No trabalho desenvolvido por OLIVEIRA e colaboradores (2006), os autores evidenciaram que cepas de *C. parapsilosis* foram as mais sensíveis à atividade antifúngica exibida pelo extrato etanólico de própolis, obtida no estado do Paraná. Ainda, neste trabalho, o extrato foi avaliado frente à 67 isolados fúngicos pertencentes às espécies: *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. kefyr*, *C. guilliermondii*, *C. lusitanea*, *C. glabrata*, *C. stellatoidea, Trichosporon sp., Geotrichum candidum* e *Saccharomyces cerevisiae,* apresentando valores de CIM na faixa de 3,2 a 50 μg/mL.

O efeito antifúngico *in vitro* do extrato etanólico de própolis, frente a 15 cepas de micro-organismos causadores de micoses superficiais (*C. glabrata, C. albicans, Trichosporum* sp. e *Rhodotorula* sp.), foi relatado por SILICI e KOC (2006). Como resultados, o extrato apresentou forte inibição do crescimento dos fungos, com concentração inibitória mínima (CIM) entre 0,01 e 1,65 μg/mL, sendo *Rhodotorula* sp. a cepa mais sensível. Por outro lado, *C. glabrata* foi a cepa mais resistente (CIM para a própolis: 1,65 μg/mL).

A comparação entre atividade antifúngica exibida por diferentes extratos etanólicos de própolis foi avaliada por MORA e colaboradores (2008), utilizando quatro amostras provenientes de diferentes regiões do México, bem como quatro extratos comerciais. *C. albicans* foi o micro-organismo alvo, sendo empregada uma cepa de referência (ATCC 10231) e 36 isolados clínicos. O extrato obtido da amostra proveniente da cidade de Cuautitlán Izcalli (México) apresentou a maior atividade biológica, inibindo 94,4% dos isolados clínicos na concentração de 0,8 mg/mL, já a cepa referência foi inibida a uma concentração de 0,6 mg/mL.

Os extratos hidroalcoólicos (etanol 80%) das própolis vermelha (Pernambuco) e verde (Minas Gerais) brasileiras foram comparados em relação às suas atividades antifúngicas frente a sete cepas de *Candida: C. albicans, C. luzitaniae, C.*

dublinienses, C. krusei, C. tropicalis, C. parapsilosis e *C. guilliermondii*. As espécies mais sensíveis foram *C. albicans* e *C. guilliermondii,* sendo o extrato da própolis vermelha mais ativo em relação à própolis verde (ABREU, 2008).

Estudo comparativo também foi desenvolvido por DOS SANTOS (2009). O autor avaliou oito extratos etanólicos de própolis, obtidos de regiões distintas do estado do Rio Grande do Sul, frente a 20 cepas de *Fonsecaea pedrosoi*, concluindo que o derivado com melhor efeito antifúngico foi aquele obtido na cidade de Santo Antônio da Patrulha, apresentando valor de CIM igual a 625 μg/mL.

DOTA e colaboradores (2011) relataram atividade do extrato etanólico de própolis frente a micro-organismos desencadeadores de candidíase vulvovaginal (*C. albicans, C. glabrata, C. parapsilosis, C. guilliermondii* e *C. tropicalis*). Nesse estudo, 96,63% dos isolados foram inibidos pelo extrato de própolis, apresentando atividade frente a *C. albicans* e CNA. Controversamente, a tintura de própolis obtida nos estudos de DE ALMEIDA e colaboradores (2012) apresentou atividade frente às leveduras: *C. krusei* e *C. tropicalis*, mas não foi capaz de inibir o crescimento de *C. albicans*. Assim, fatores de virulência distintos entre cepas da mesma espécie, mas com diferentes especificações, podem explicar essas discrepâncias nos resultados.

Em estudo mais recente, desenvolvido por REIDEL (2014), foi observada atividade antifúngica do extrato etanólico de própolis nativa do Rio Grande do Sul, sendo as cepas de *C. krusei* e *C. parapsilosis* mais susceptíveis à ação do derivado, apresentando valores de CIM $\leq$ 250 e $\leq$ 125 μg/mL, respectivamente. Ainda, os extratos etanólico e hexano de própolis vermelha foram efetivos na inibição do crescimento de *C. glabrata*, apresentando valores de CIM $\leq$ 31,25 μg/mL, sendo mais ativos que o controle positivo utilizado nos experimentos: fluconazol (CIM: 32 μg/mL).

Os resultados distintos observados para os extratos de própolis, obtidos nos estudos acima, sugerem que as composições química geral e flavonoídica, em específico, são qualitativa e quantitativamente variáveis, dependendo das diferenças regionais. Ainda, a quantidade e os componentes encontrados na própolis também variam de acordo com a espécie de abelha que coleta a própolis, mesmo tratando-se da

mesma região (KOO e PARK, 1997). Portanto, amostras provenientes de diferentes regiões geográficas, bem como a época de coleta, o gênero e/ou a espécie de abelha e o método de preparo do derivado são fatores que influenciam na composição do produto obtido (SFORCIN *et al.,* 2000; BANKOVA *et al.,* 2002; KOSALEC *et al.,* 2005; SALATINO *et al.,* 2011; TAGLIACOLLO e ORSI, 2011).

4. CAPÍTULO I: Activity of Brazilian red propolis extracts against non-*albicans* *Candida* species

Activity of Brazilian red propolis extracts against non-*albicans Candida* species

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ABSTRACT

The aim of this study was to investigate the activity of Brazilian Red Propolis (BRP) against non-*albicans Candida* strains. BRP fractions were obtained with increasing polarity solvents, through maceration at room temperature. The antifungal assay was conducted using a total of nine opportunistic yeasts strains. Minimal inhibitory concentration (MIC) of the fractions was determined by the broth microdilution method, with concentrations ranging from 1.9 to 500 μg/mL. MTT colorimetric assay was employed to confirm the cell damage of *n*-hexane (HEX) fraction, using also other resistant strains. Among the tested fractions, HEX showed higher antifungal potential, achieving MIC values of 1.95 μg/mL against *C. parapsilosis* strains. On *C. glabrata, C. krusei* and *C. tropicalis* strains, variable MIC values were obtained (1.95 to 250 μg/mL). Yeast damages (MTT assay) ranged from 80.66 to 94.44%, with extensive cell death caused by BRP HEX fraction. We also highlight the effects against *Candida* resistant strains, mainly *C. glabrata* and *C. parapsilosis*. The potential antifungal capacity showed by HEX fraction is related to the lipophilic compounds, probably polyprenylated benzophenones (PPBs), previous described in BRP. Therefore, the BRP HEX fraction represents a valuable natural product for development of new therapeutic alternative in the treatment of non-*albicans Candida* infections, even those caused by resistant yeasts.

Keywords: Brazilian red propolis, *Candida*, antifungal, resistant strains.

1 INTRODUCTION

Infections caused by fungi are still associated with high morbidity and mortality mainly in immunocompromised and severely ill patients. The most important agents of opportunistic mycoses are *Candida albicans* and the *non-albicans Candida* species (NACs) as *C. glabrata, C. krusei, C. parapsilosis and C. tropicalis* which are responsible in the last 20–30 years for more than 95% of invasive candidiasis (PEMÁN and SALAVERT, 2012; YAPAR, 2014). The largest multicentric study conducted in Latin America reported an incidence of candidemia in Brazilian tertiary-care hospitals of 2.49 cases per 1000 hospital admissions and mortality rates of 54%. These values are two to fifteen times higher to those recorded in the Northern Hemisphere (COLOMBO *et al.*, 2006).

Therapeutic antifungal agents for the treatment of invasive or mucocutaneous candidiasis include the polyenes, azoles and echinocandins. Nevertheless, adverse effects, toxicity, drug interactions and increasing resistances limit the use of these drugs, mainly in patients with hepatic or renal impairment (COLOMBO *et al.*, 2006; ZARAGOZA and PEMÁN, 2012; SARDI *et al.*, 2013). The recent increase of opportunistic *Candida* infection has occurred in the last years and there is a critical need for new therapeutic options (SARDI *et al.*, 2013).

Many reports are found in literature demonstrating the effectiveness of natural products as potentially rich sources of novel and selective agents for the treatment of important diseases (NEWMAN and CRAGG, 2012). Propolis, a natural material with multiple pharmacological effects, could be an interesting alternative in the discovery of new antimicrobial compounds since it acts defending the hive against invasive bacteria and fungi (BANSKOTA, TEZUKA and KADOTA, 2001). This resinous product contains beeswax, enzymes, sugar and plant exudates collected by *Apis mellifera* bees from various plant sources. The chemical composition is complex and variable being highly influenceable by the vegetation at the place of collection (PICCINELLI *et al.*, 2011; SFORCIN and BANKOVA, 2011; ATHIKOMKULCHAI

et al., 2013) and by the seasonality, which may interfere in its biological properties (SIMÕES-AMBROSIO *et al.*, 2010).

It is remarkable that even with variable composition, the different propolis samples present similar biological activities. Regarding the antimicrobial activity, the effect against bacterial and fungal pathogens, including *Candida* species, have been reported (OTA *et al.*, 2001; SANTOS *et al.*, 2005; QUINTERO-MORA *et al.*, 2008; CASTRO *et al.*, 2009; PETROVA *et al.*, 2010; MASSARO *et al.*, 2014).

Brazilian propolis was classified by Park and coworkers (2002) in twelve major groups according to physicochemical properties and geographic locations. Recent attention has been given to a new group, classified as Group 13, named *red propolis* due to its intense red color (ALENCAR *et al.*, 2007). For this propolis, biological and pharmacological properties have been described, including antimicrobial, cytotoxic, among others (TRUSHEVA *et al.*, 2006; ALENCAR *et al.*, 2007; RIGHI *et al.*, 2011; FROZZA *et al.*, 2013).

Most propolis formulations are prepared using ethanolic extracts which present a wide array of compounds. In this study the Brazilian red propolis (BRP) was partitioned in increasing polarity solvent and the fractions, including *n*-hexane, dichloromethane, ethyl acetate and ethanol were investigated against NACs isolates. Minimal inhibitory concentration (MIC) and MTT assay were performed in order to determinate anti-*Candida* potential of BRP samples and estimate yeast damages caused.

2 MATERIALS AND METHODS

2.1 Propolis extraction

BRP (Natucentro®, Minas Gerais, Brazil) was cut into small pieces and 50 g were extracted with ethanol by maceration at room temperature (3x24 h). Further, the extracts were filtered and concentrated in a rotaevaporator at 50 °C, yielding 80.6% of

a crude ethanolic extract (CEE). Other portion (50 g) was extracted successively with *n*-hexane (HEX), dichloromethane (DIC), ethyl acetate (ETA) and ethanol (ETH), (drug/solvent ratio=1:10 w/v) by maceration (3x24 h), yielding, after vacuum evaporation, 50.90%, 32.46%, 5.68% and 1.46%, respectively. The *n*-hexane fraction was treated with acetone to remove waxes yielding 36.60% of HEX, in relation to the starting material.

2.2 Antifungal assay

A total of nine strains (clinical isolates and reference strains) of opportunistic yeasts were tested for the antifungal susceptibility: *Candida glabrata* (CG40039, CG03), *C. krusei* (ATCC6258, CK04), *C. parapsilosis* (CP04, CP08, CP07,) and *C. tropicalis* (ATCC750, CT08). MIC values of the fractions were determined by the broth microdilution method according to M27-A3 documents determined by the Clinical Laboratory and Standards Institute (CLSI, 2008) with RPMI-MOPS (RPMI 1640 medium containing l-glutamine, without sodium bicarbonate and buffered to pH 7.0 with 0.165 mol/L MOPS buffer) (Sigma–Aldrich Co., St. Louis, USA). The concentrations of the samples ranged from 1.95 to 500 µg/mL and the MIC was defined as the lowest concentration of compounds at which the microorganism tested did not demonstrate visible growth after incubation at 32 °C for 48 h. Amphotericin B kindly supplied by Cristalia® (Brazil) was used as positive control, in the same concentration range of the samples. The experiments were carried out in duplicate.

A more detailed analysis of susceptibility testing, based in a colorimetric assay using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co. St. Louis, Mo.), was employed to estimate the cell damage caused by BRP fraction with the lowest MIC values, including fluconazole resistant strains and other clinical isolates (CG RL34, CG RL34m, CK 03, CP RL27, CP RL27m, CP RL27m, CT 72A). The fraction was submitted to the same conditions to determine MIC values and, after 48 h of incubation at 32 °C, the supernatant was discarded and then the cells were incubated with a 150 µL of MTT

solution (0.05 μg/mL in RPMI) for another 4 h at 32 °C. After, the MTT solution was removed and MTT formazan crystals were extracted with 150 μL of isopropanol. Absorbances (A) were measured on a multiplate reader (SpectraMax®) in two wavelengths (570 and 690 nm) to be adjusted for nonspecific absorption (Mosmann, 1983). Yeast damage (%) was calculated by comparing the absorbance of samples treated with the selected fraction and non-treated samples. The experiments were carried out in triplicate.

3 RESULTS AND DISCUSSION

Candida albicans is the most prevalent species involved in invasive fungal infections. Nevertheless, the frequency of infections caused by NACs is increasing. These yeasts are responsible for 35-65% of all candidaemias and occur more frequently in patients with hematologic malignancies, preterm neonates and recipients of marrow transplant. They can also cause *Candida* bloodstream infections in intensive care unit and surgical patients as well as in HIV-positive patients. As stated before, several studies have demonstrated the effect of propolis against *Candida* species but, as far as we known, the activity of BRP against NACs has been little explored. Thus, in this study, besides the crude ethanolic extract, the fractions obtained with solvents in increasing polarity were investigated for the activity against *C. parapsilosis*, *C. tropicalis*, *C. krusei* and *C. glabrata*, the most frequent NACs associated with diseases (SARDI *et al.*, 2013).

Table 1 shows the MIC of BRP ethanol extract and fractions obtained with increasing polarity solvents against NAC strains. As a general rule, HEX fraction was more active against all the tested strains in comparison with fractions obtained with more polar solvents, suggesting a higher antifungal effect of the lipophilic compounds. The highest activity of HEX fraction was detected against *C. parapsilosis* strains (MIC value of 1.95 μg/mL for all strains). This MIC value is similar to those reported for reference drugs such as echinocandin, azoles or polyenes (ANDES *et al.*, 2010; BRAUTASET *et al.*, 2011; MELETIADIS *et al.*, 2012). On the other hand, strains of

C. glabrata, *C. krusei* and *C. tropicalis* showed a variable susceptibility to HEX fraction (1.95 to 250 μg/mL). Such variability has been described for emerging fungi such as non-*albicans Candida* (YASHAVANT *et al.*, 2013). Some reports have described the antifungal activity of BRP against yeasts and dermatophytes and MIC values can vary greatly. To date, Righi and coworkers (2011) reported a MIC value of 256 μg/mL for BRP methanol extract against *C. albicans*. Siqueira and coworkers (2009) obtained MIC values from 64 to 128 μg/mL for BRP ethanol extract against *Trichophyton* spp. Amphotericin B, the positive control, was active even at the lowest tested concentration against all strains.

Table 1. Minimum inhibitory concentration (MIC; μg/mL) of crude ethanol extract (CEE) and hexane (HEX), dichloromethane (DIC), ethylacetate (ETA) and ethanol (ETH) fractions of BRP and amphotericin B (AMP) against NACs

Strain	**CEE**	**Fractions**				**AMP**
		HEX	**DIC**	**ETA**	**ETH**	
C. glabrata CG40039	15.62	1.95	15.62	31.25	31.25	1.95
C. glabrata CG03	31.25	31.25	15.62	62.50	125.00	1.95
C. krusei ATCC6258	31.25	31.25	62.50	125.00	125.00	1.95
C. krusei CK04	62.50	62.50	62.50	250.00	125.00	1.95
C. parapsilosis CP04	15.62	1.95	125.00	250.00	62.50	1.95
C. parapsilosis CP08	15.62	1.95	62.50	125.00	125.00	1.95
C. parapsilosis CP07	15.62	1.95	62.50	125.00	62.50	1.95
C. tropicalis ATCC750	250.00	250.00	125.00	500.00	250.00	1.95
C. tropicalis CT08	125.00	62.50	62.50	500.00	125.00	1.95

In this scope, it is relevant to highlight the observed increasing number of infections caused by *Candida* spp resistant to antifungal agents, including fluconazole, especially in hospital environment. Such resistance leads to high failure rates in the therapy against these fungi, morbidity and mortality (ZOMORODIAN *et al.*, 2011).

Thus, alternative therapies capable to overcome this fungi resistance have been proposed and include the antifungal agents association, by synergic effects, and also the discovery of new natural products with potential against such resistant pathogenic fungi (ALVES *et al.*, 2012). Pippi and coworkers (2015) have already demonstrated the antifungal effect of BRP hexane extract on fluconazole resistant *Candida* strains and against polymicrobial cultures composed by multiple resistant isolates. The extract also demonstrated to improve the fluconazole activity against *Candida* sp, with synergic effects between BRP hexane extract and this widely used antifungal agent.

Thus, considering the strong antifungal responses of the HEX fraction, we performed, after MIC determination, the MTT assay in order to confirm the yeast damage caused by this BRP fraction (Table 2). By this method, we also evaluated samples against other clinical isolates and fluconazole resistant *Candida* strains. According to the results, cell damages ranging from 80.66 to 94.44% were verified, showing strong yeast death caused by BRP HEX fraction. MIC determination was confirmed, once *C. glabrata* CG40039 and *C. parapsilosis* CP04 were the most susceptible yeasts (BRP HEX fraction MIC: 1.95 μg/mL). We also highlight the effect of this BRP fraction against resistant *Candida* strains, mainly on *C. glabrata* RL34m and *C. parapsilosis* RL27m.

Table 2. Minimum inhibitory concentration (MIC; µg/mL) and cell damage (%) of HEX fraction of BRP against selected NACs

Strain	MIC	Cell damage (MTT)[a]
C. glabrata CG RL34	7.813	83.41±1.19
**C. glabrata* CG RL34m	7.813	90.08±1.02
C. glabrata CG40039	1.95	89.33±2.74
**C. krusei* CK 03	250	88.72±1.93
C. krusei CK ATCC 6258	31.25	84.02±2.04
C. parapsilosis CP RL27	15.625	81.79±1.64
**C. parapsilosis* CP RL27m	15.625	80.66±1.76
C. parapsilosis CP04	1.95	90.38±1.94
**C. tropicalis* CT 72A	31.25	94.44±1.11
C. tropicalis CT ATCC 750	250	91.57±1.35
C. tropicalis CT05	62.5	92.30±1.23

*Fluconazole resistant *Candida* strains;
[a]Percentage of yeast damage in the corresponding MIC values of BRP HEX fraction.

In our study, the low MIC values obtained against non-*albicans Candida* were related to the lipophilic compounds present in the HEX fraction. Brazilian red propolis presents various classes of substances in its composition. The main compounds are isoflavonoids, pterocarpans, chalcones, flavonoids and PPBs (PICCINELLI *et al.*, 2011; LÓPEZ *et al.*, 2014). Taking into account that PPBs are the most lipophilic substances among the above cited compounds, probably they are responsible for the verified antifungal activity of HEX extract.

4 CONCLUSIONS

The HEX fraction obtained from the Brazilian red propolis may represent an alternative for the treatment of non-albicans Candida infections, also those caused by resistant strains. As next challenges, the technological development of a formulation based on these bioactive compounds and *in vitro/in vivo* assays are necessary to make feasible the use of this natural material in the treatment of infections caused by these opportunistic microorganisms.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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4. CAPÍTULO II: Determination of benzophenones in lipophilic extract of Brazilian red propolis, nanotechnology-based product and porcine skin and mucosa. Analytical and bioanalytical assays

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Determination of benzophenones in lipophilic extract of Brazilian red propolis, nanotechnology-based product and porcine skin and mucosa. Analytical and bioanalytical assays

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ABSTRACT

Lipophilic compounds of Brazilian Red Propolis (BRP) have received increasing attention due to some interesting findings regarding their biological activities. This study was first aimed at evaluating the chemical composition of BRP *n*-hexane extract (HEXred) by UPLC-PDA-MS. Chemical investigation mainly resulted in the identification of polyprenylated benzophenones (PPBs) in this extract, named oblongifolin A, guttiferone E, and/or xanthochymol. After that, an isocratic HPLC-UV method was validated for the determination of total content of PPBs (at 260 nm) expressed as garcinol, a commercially available guttiferone E diastereoisomer. The method showed to be specific, precise, accurate, and linear (0.1 to 10 μg/mL) for the determination of PPBs in HEXred, BRP-loaded nanoemulsions, as well as, in porcine skin and mucosa samples after permeation/retention studies. The matrix effect was determined for all complex matrices, demonstrating low effect during the analysis. The stability-indicating method was verified by submitting HEXred to acidic, alkaline, oxidative, and thermal stress conditions. No interference of degradation products was detected during analysis. Therefore, the proposed analytical and bioanalytical methods proved to be simple and reliable for the determination of PPBs in the presence of different matrices.

Keywords: Brazilian Red Propolis, polyprenylated benzophenones, HPLC-UV, validation, garcinol.

1 INTRODUCTION

Propolis is a resinous product that contains beeswax, enzymes, sugar, and plant exudates collected by *Apis mellifera* bees from different plant sources. Various parameters may have effect on the chemical composition of propolis being the vegetation at the place of collection the most relevant (PICCINELLI *et al.*, 2011; SFORCIN and BANKOVA, 2011; ATHIKOMKULCHAI *et al.*, 2013). Brazilian propolis was classified by Park, Alencar and Aguiar (2002) in twelve major groups according to the physicochemical properties and the geographic locations. Brazilian Red propolis (BRP), a new group classified as Group 13, received this name due to its strong red color (ALENCAR *et al.*, 2007).

Various biological and pharmacological properties have been described for BRP (TRUSHEVA *et al.*, 2006; ALENCAR *et al.*, 2007; RIGHI *et al.*, 2011; FROZZA *et al.*, 2013). A well-documented literature has demonstrated the antimicrobial activity of this propolis, usually as ethanol preparations, against bacterial and fungal pathogens, including *Candida* species (OTA *et al.*, 2001; SANTOS *et al.*, 2005; QUINTERO-MORA *et al.*, 2008; CASTRO *et al.*, 2009; PETROVA *et al.*, 2010; MASSARO *et al.*, 2014). Recently, a pronounced antifungal activity of the *n*-hexane extract of the BRP against non-*albicans Candida* (NAC) species – *C. krusei, C. glabrata, C. tropicalis* and *C. parapsilosis* – was demonstrated (PIPPI *et al.*, 2015). The yeast damage of this extract was also confirmed by MTT assay.

Previous literature has demonstrated the presence of the polyprenylated benzophenones (PPBs) guttiferone E, xanthochymol and oblongifolin A in the lipophilic extract (*n*-hexane) of BRP. The chemical structures of these PPBs are shown in the Figure 1. However, the complete separation of these PPBs was not achieved even by high-throughput ultra-fast liquid chromatography. In fact, the isomers guttiferone E and xanthochymol have been considered as an inseparable mixture (TRUSHEVA *et al.*, 2006; PICCINELLI *et al.*, 2011; LÓPEZ *et al.*, 2014). Gustafson and coworkers (1992) had already reported the extraction of guttiferone E and xanthochymol from *Garcinia* and *Clusia* species as a mixture.

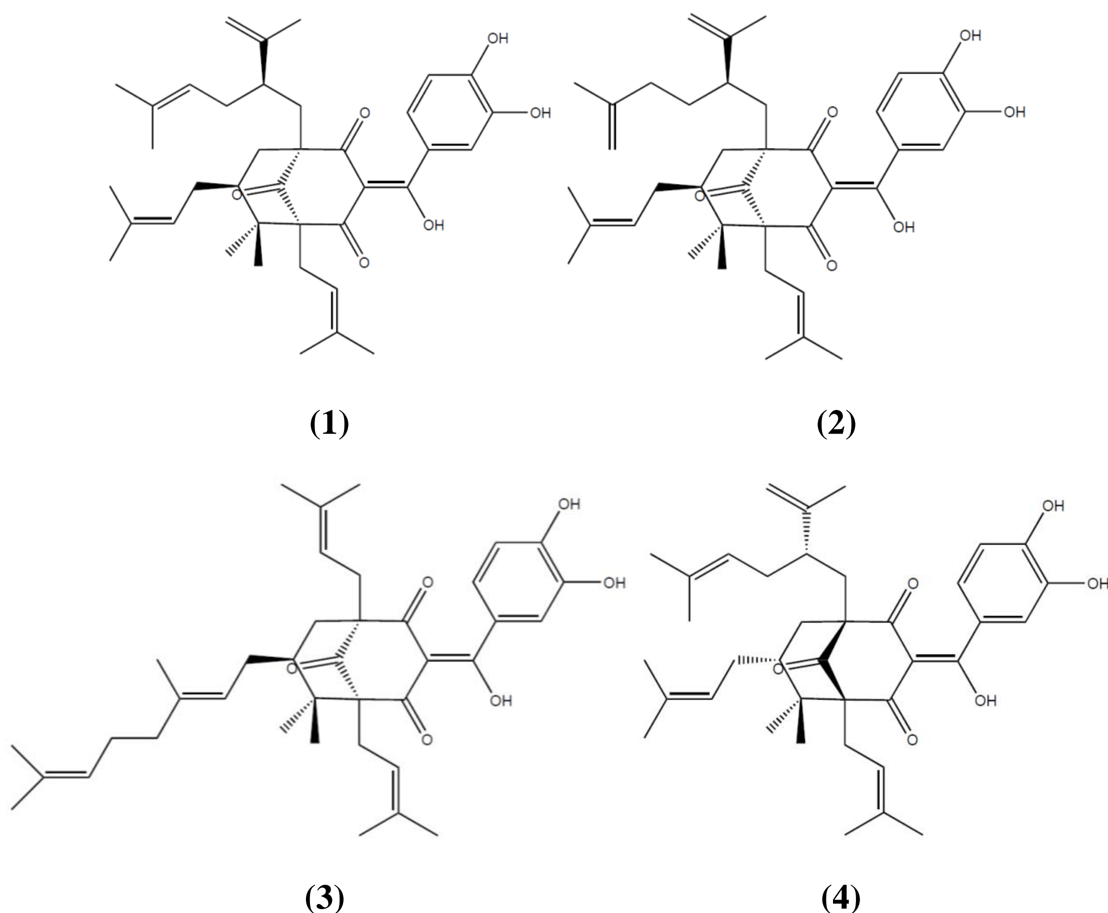

Figure 1. Chemical structure of guttiferone E (1), xanthochymol (2), oblongifolin A (3) and garcinol (4).

Despite the promising results concerning the biological activities of lipophilic compounds from BRP preparations, analytical studies have yet to be performed. Pharmaceutical analysis of propolis is a difficult task once it is often composed of a complex mixture of compounds. In this study, we first aimed to characterize the chemical composition of the HEXred by UPLC-PDA-MS. After that, a stability-indicating HPLC/UV method to determine the PPBs content in HEXred, nanotechnology-based product and porcine skin/mucosa samples of permeation/retention studies was validated. PPBs content was expressed as garcinol, a commercially available PPB guttiferone E *diastereoisomer*. The matrix effect for all of these applications was assessed to demonstrate the versatility and reliability of the method.

2 MATERIAL AND METHODS

2.1 Materials

Garcinol (purity ≥ 95%) was purchased from Cayman Chemical (Michigan, USA). Methanol and acetonitrile were HPLC grade and all other reagents used were analytical grade. Red propolis was purchased from Natucentro® (Minas Gerais/Brazil). Porcine skin and porcine esophageal mucosa were purchased from Cooperativa Ouro do Sul (Rio Grande do Sul/Brazil). Egg-lecithin (Lipoid E-80) and medium chain triglycerides (MCT) were purchased from Lipoid GmbH (Ludwigshafen, Germany).

2.2 Preparation of *n*-hexane BRP extract (HEXred)

The BRP was ground and the powdered material was successively extracted by maceration with *n*-hexane over 72 h (3 times) and sample:solvent ratio of 1:10 (w/v). The extracts were combined and evaporated to dryness under reduced pressure using a rotary evaporator at 40 ^{o}C. The residue was treated with cold acetone to obtain an insoluble fatty residue that was filtered through paper filter. The acetone-soluble fraction was evaporated to dryness and used in the further experiments.

2.3 UPLC-PDA-MS analysis of HEXred

The HEXred was analyzed by UPLC-PDA-MS. A C_{18} reversed-phase column (Hypersil Gold 10 mm x 2.1 mm, 1.8 µm, Thermo Scientific, Massachusetts, USA) was used, operating at 45 ºC. The analyses were performed using positive ion mode electrospray ionization mass spectrometry (ESI(+)-MS). The optimized system parameters were as follows: capillary (V) 3000, sample cone (V) 30, extraction cone (V) 3.3, temperatures of source (120 ºC) and desolvation (300 ºC) and collision energy

4 V. Nitrogen was used as nebulizer gas and argon as collision gas. Samples were eluted using a linear gradient system and the mobile phases consisted of a mixture of water:formic acid (100:0.1, v/v) (A) and methanol (Tedia® HPLC grade, Ohio, USA) (B). The gradient profile was: 0.0–1.54 min from 40 to 65% of B, 1.54-4.38 min from 65 to 70% of B, 4.38-7.21 min from 70 to 75% of B, 7.21-8.63 min from 75 to 80% B, 8.63-11.00 min 80% of B, 11.00-11.46 min from 80 to 85% of B, 11.46-12.88 min from 85 to 88% of B, 12.88-14.29 min from 88 to 90% of B, 14.29-15.71 min from 90 to 40% of B and finally, to restore the initial conditions, 15.71-16.00 min 40% of B. The flow-rate was 0.4 mL/min and the injection volume was 2 μL. The detection was at 260 nm in the photodiode array detector. The UV spectra were recorded with a 200-400 nm range.

2.4 HPLC/UV analysis of PPBs

The total content of PPBs was expressed as garcinol. A stock solution of garcinol was prepared in methanol due to its solubility in this solvent. Stock solutions of each matrix (HEXred, nanoemulsion, porcine skin, porcine esophageal mucosa, adhesive tape, and receptor fluid) were prepared by extraction for 30 minutes in methanol. The chromatographic column used was a reversed phase C_{18} (Shim-pack ODS, Shimadzu, Kyoto, Japan) 250 x 4.6 mm, 5 μm. The separation was carried out under isocratic elution with methanol:acetonitrile (80:20, v/v) during 10 min at room temperature. The optimized system parameters were as follows: flow rate 1 mL/min, injection volume 20 μL, and detection wavelength at 260 nm.

2.5 Validation of HPLC/UV method for PPBs

The HPLC-UV method was validated in terms of linearity, selectivity, precision, accuracy and robustness according to ICH (2005) and FDA (2014) guidelines for the matrices evaluated: HEXred, nanoemulsion, porcine skin and

esophageal mucosa, adhesive tape, and receptor fluid (30% ethanol in phosphate buffered saline – PBS – pH 7.4).

2.5.1 Linearity, limits of detection and quantification and matrix effect

Seven linearity curves of garcinol were constructed on three consecutive days by diluting the garcinol stock solution with the solution of each matrix, comprising six points on the concentration of 0.1, 0.5, 1, 2, 4, 6, 8 and 10 µg/mL. Statistical analysis of the regression line was performed and the respective equation and determination coefficient (R^2) were determined for each matrix studied. Limits of detection (LOD) and quantification (LOQ) were determined based on the standard deviation of the response and the slope, using the calibration curve data. The low limit of quantification (LLOQ) was determined by assaying five independent samples of garcinol and calculated the relative standard deviation (RSD). LLOQ was the lowest concentration of garcinol that could be determined with precision and accuracy. The matrix effect was calculated based on the comparison of the slopes of the calibration curves of garcinol in the solvent (methanol) and in the different matrices. The equation used to calculate the matrix effect was ME% = 100 x $[1-(S_m/S_s)]$, where S_m = slope of the calibration curves of garcinol in the solvent and S_s = slopes of the calibration curves of garcinol in the matrices (NIESSEN, MANINI and ANDREOLI, 2006).

2.5.2 Specificity

The specificity of the method was evaluated by analyzing excipient mixtures of formulations, as well as forced degraded, skin and mucosa samples in the presence of garcinol methanol solutions. The system response was examined through the presence of interference or overlaps with garcinol response.

2.5.3 Forced degradation study

For this study, garcinol standard solution (500 µL) was diluted with 500 µL of each stressing solution (hydrochloric acid, sodium hydroxide and hydrogen peroxide). For light and heat stressing conditions, stock solution was diluted with 500 µL of water. Analyses were performed every 15 minutes during the first hour and after every hour until 6 hours. After each analysis time was completed, 500 µL of mobile phase were added to the conditions hydrogen peroxide (3% and 30%), heat and light. For the acidic and alkaline conditions, 500 µL of the solution with opposite pH were added, in order to neutralize the medium and stop the degradation. Evaluation of the forced degraded solutions by peak purity analysis using a photodiode array detector/mass spectral evaluation was performed to confirm that the active peak does not co-elute with any degradation product generated as a result of the forced degradation.

2.5.4 Precision and accuracy

The precision and accuracy of the method for the quantification of garcinol was determined by examining the intraday and interday reproducibility. Six replicates of garcinol solution spiked into each matrix stock solution at three concentration levels were analyzed (1, 2 and 4 µg/mL). The interday reproducibility was tested on three different days (n = 18 for each concentration level). Precision was expressed in terms of the relative standard deviation (RSD) and accuracy was expressed in percent (with respective RSD).

2.5.5 Accuracy in biological matrices (recovery)

To estimate accuracy, garcinol standard solution was added at three concentration levels (1, 2 and 4 µg/mL) in porcine epidermis/dermis, porcine stratum

corneum (after tape stripping process) and porcine esophageal mucosa. For each concentration, six replicates were evaluated. After solvent evaporation, garcinol was extracted with methanol for 30 min in ultrasound bath, filtered, and measured by HPLC. The recovery percentages of the samples were calculated from the areas which were compared with the standard areas at the equivalent concentrations.

2.5.6 Robustness

Robustness is a measure for the susceptibility of a method to small changes that might occur during routine analysis. In order to test the robustness of the method, the Plackett-Burman experimental design was used, in which the HPLC parameters deliberately varied were wavelength (±2 nm), flow rate (±0.2 mL/min), and percent of methanol in the mobile phase (±3 %) (VANDER HEYDEN *et al.*, 2001).

2.5.7 System Suitability

According to the FDA (2014), the system suitability comprises confirmatory test procedures and parameters to ensure that the chromatographic system will function correctly as an integrated system for the intended analysis. The parameters evaluated were retention time, theoretical plates, tailing factor, and resolution between the peak of interest (garcinol) and the closest potential interfering peak.

2.5.8 Garcinol stability in matrices

The stability of garcinol spiked matrices was evaluated by assaying it after 48 h of storage at room temperature, and compared with freshly prepared solutions.

2.6 Method application

The determination of PPBs in real samples – HEXred and nanoemulsion (NE) – was performed by the validated HPLC/UV method. Nanoemulsions were prepared according to Bidone and coworkers (2014). The PPBs permeation/retention was evaluated on Franz type diffusion cells (surface area = 1.77 cm^2 and receptor volume = 10.0 mL). The excised circular porcine ear skin and esophageal mucosa were placed between the donor and receptor compartments. The inner face was positioned up in the donor compartment of the Franz cells and these were maintained at a constant temperature of 32 ± 1.0 ºC during the experiment, while being stirred on a magnetic stirrer at 650 rpm. The donor compartment was filled with 500 µL of a nanoemulsion containing HEXred (NE_{HEXred}), maintaining the sink conditions for the assay.

After 8 h, an aliquot of the receptor fluid was withdrawn for analysis and the skin or mucosa was removed from the cell and cleaned using a cotton swab. The tape stripping process made the separation of stratum corneum, where the first stripped adhesive tape was discarded and the following 14 tapes were used for the stratum corneum analysis. The epidermis was separated from dermis by scraping, using a scalpel, and the dermis was reduced to tiny pieces. All samples were placed separately in test tubes. To extract the PPBs from skin layers, 5 mL of methanol were added and samples were maintained in an ultrasound bath, during 30 minutes. The porcine esophageal mucosa, after 8 h, was removed from the cell and cleaned using a cotton swab. Then, it was cut into tiny pieces, placed in test tubes and submitted to the same extraction technique. Aliquots of each sample were filtered and analyzed.

3 RESULTS AND DISCUSSION

3.1 Chemical characterization of HEXred

Figure 2 shows the chromatogram of HEXred. The chromatogram revealed the presence of two main peaks at 9.59 and 9.79 min. UV data of both peaks (λ_{max} at 250 and 355 nm) suggested the presence of polyprenylated benzophenones (Figure 3). These peaks showed protonated molecules [M+H] at m/z 603.3, indicating the presence of guttiferone E and/or xanthochymol (peak at 9.59 min) and oblongifolin A (peak at 9.79 min). The fragmentation of the latter compound presented an ion at m/z 399, attributed to oblongifolin A (Figure 3). Taken together, our results follow in line with previous literature (TRUSHEVA *et al.*, 2006; PICCINELLI *et al.*, 2011; LÓPEZ *et al.*, 2014), that has reported the presence of PPBs in lipophilic extracts of BRP. Such a result indicates a similar composition of this raw material, even when coming from different batches and origins.

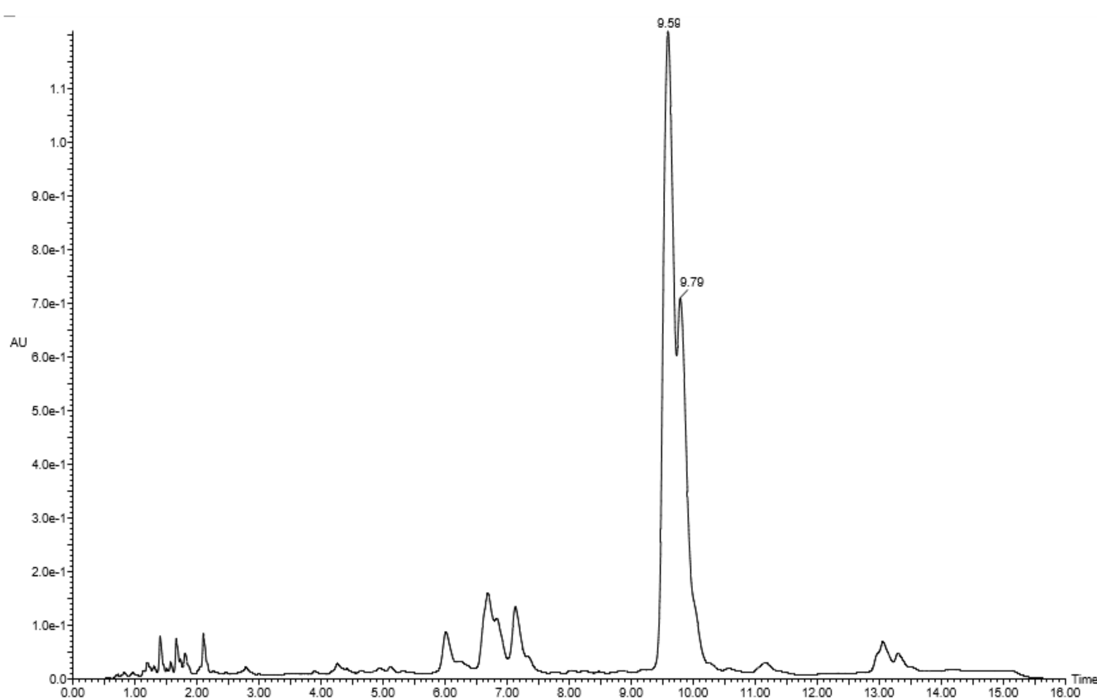

Figure 2. Chromatographic profile of the HEXred by UPLC method/MS.

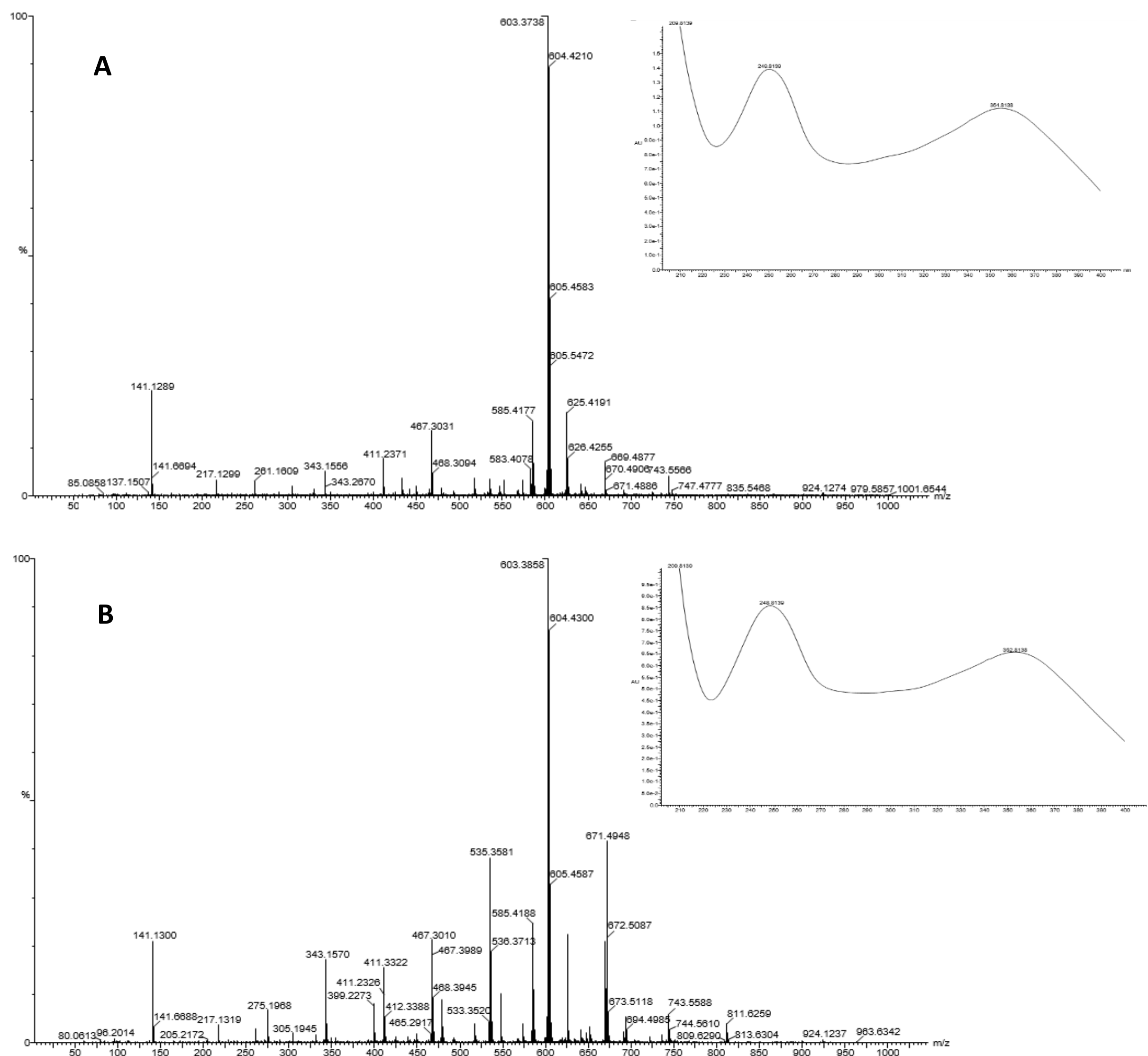

Figure 3. ESI/MS and UV spectra of peaks at 9.59 min (A), and 9.79 min (B) from HEXred by UPLC/MS.

3.2 Development and validation of an HPLC/UV method

As stated before, HEXred is mainly composed by PPBs. In a preliminary set of experiments, different chromatographic parameters have been tested as suitable conditions to separate PPBs of BRP. However, in most cases, the base peak was broad with a low peak purity and the separation of these compounds with satisfactory resolution was not achieved. Besides that, the separation of isomers such as the PPBs is a very difficult task. Thus, we decided to validate an HPLC/UV method to

determine the total content of PPBs intended to be used in the development of topical products containing HEXred. The total content of PPBs was expressed as garcinol, a commercially available PPB. Garcinol presented identical ^{1}H and ^{13}C NMR spectra of guttiferone E and its optical rotation $[\alpha]_d = +101°$ was opposite in sign to that reported for garcinol $[\alpha]_d = -125°$. Therefore, guttiferone E is a diastereoisomer of garcinol and a double bond isomer of xanthochymol. The use of patterns with similar structure to the analyte in the validation of analytical methods is a viable alternative when there is no standard availability of the substance to be measured (VIANNA *et al.*, 2011).

In a first set of experiments, the specificity of the method was evaluated. This is a key parameter once it shows if the components of different matrices or products of degradation could interfere in the determination of garcinol. The chromatographic separation at 260 nm, with respective analyses by mass detector and photodiode array detector, is shown in the Figure 4. The retention time of garcinol was 6.9 min, with λ_{max} at 252/355 and MS spectra with characteristic $[M+H]^+$ ion m/z 603.3, in accordance to previous literature (BHARATE *et al.*, 2014). Then, all matrices stock solutions (blank matrices) were analyzed in comparison with each garcinol spiked matrix, evaluating the peak purity of them (Figure 5). No interference was detected for the matrices on the results, indicating that co-eluting matrix components with the standard did not occur.

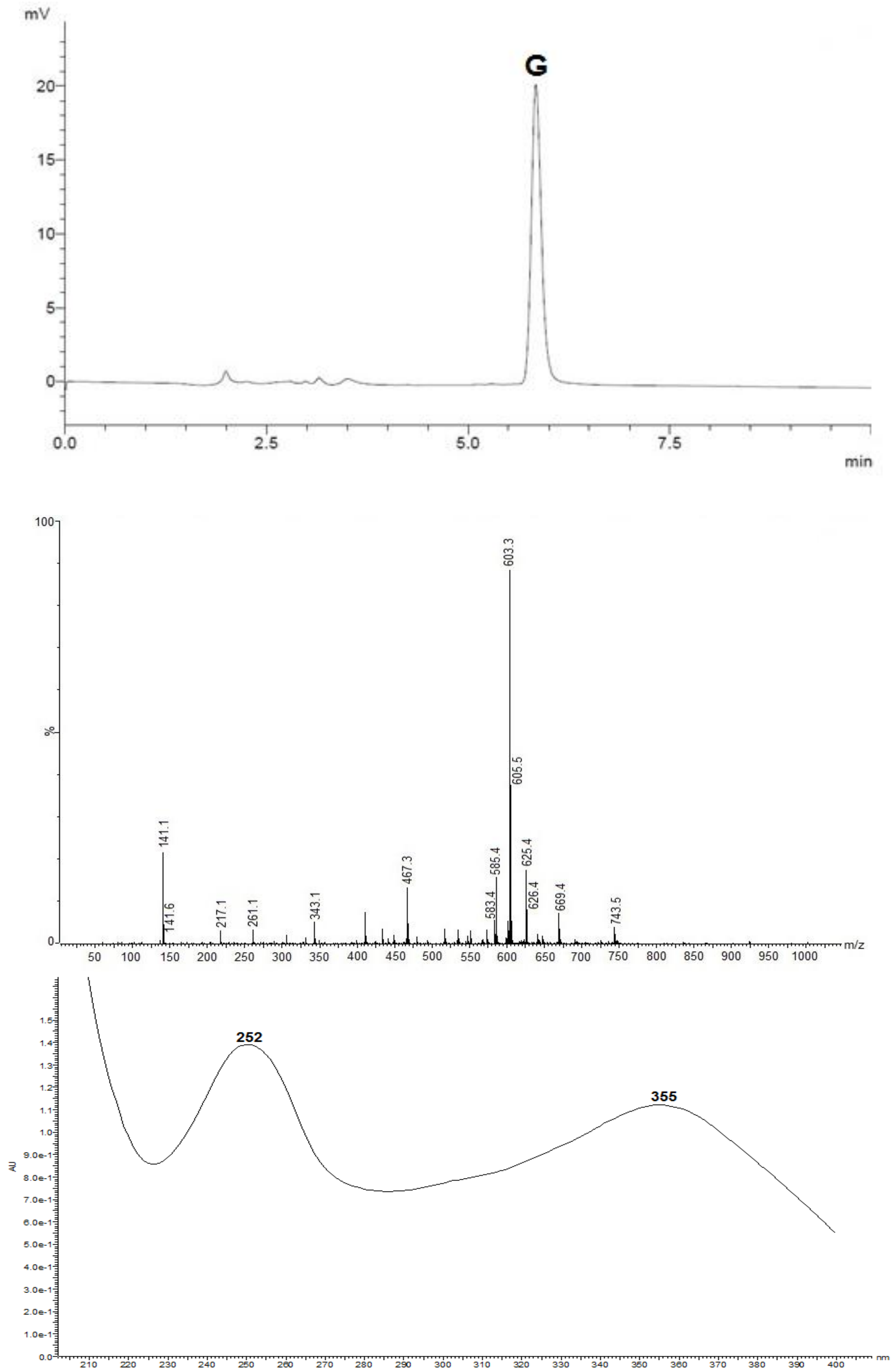

Figure 4. Chromatographic separation at 260 nm, mass spectra and UV-VIS spectra of garcinol (G).

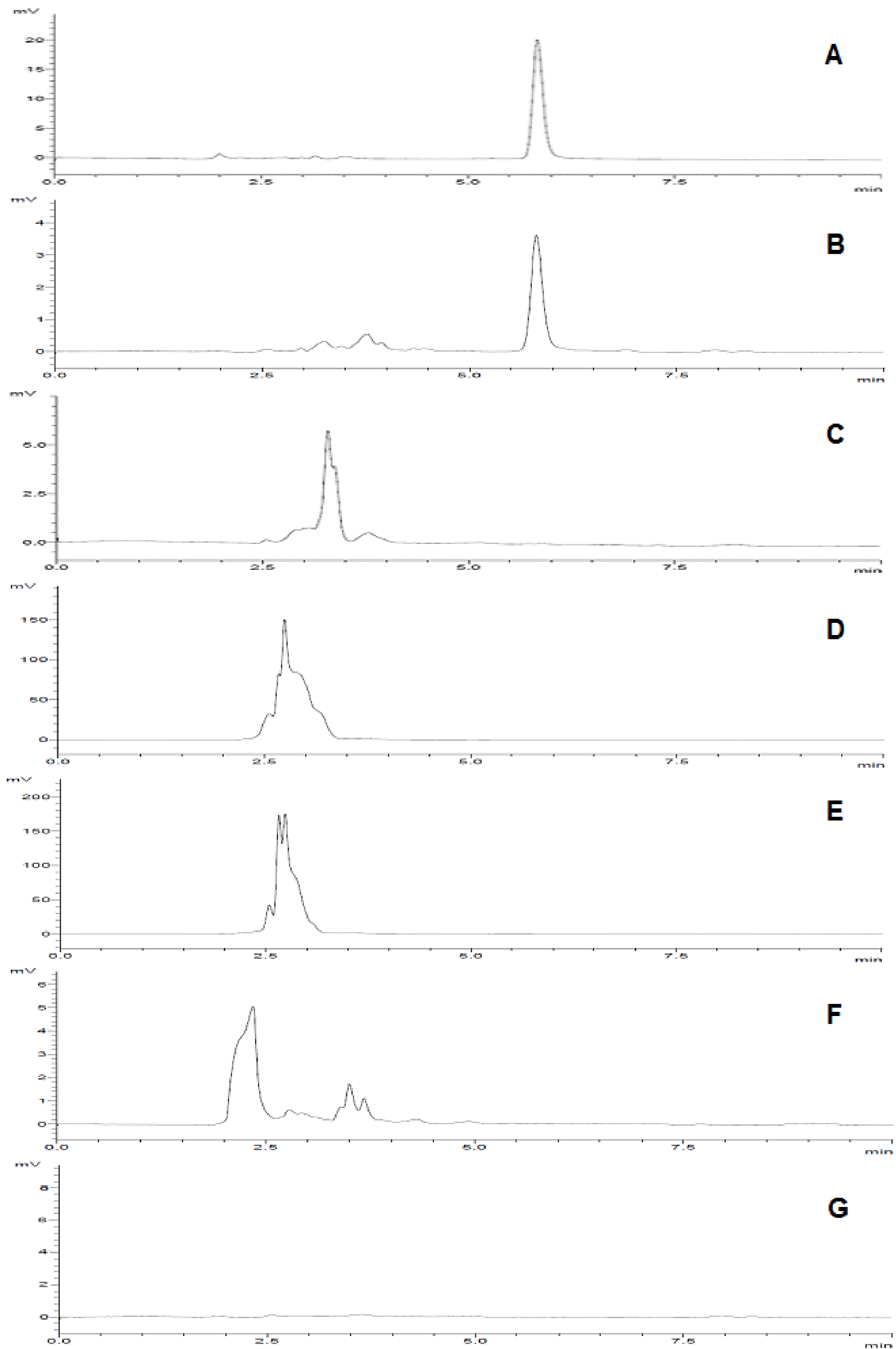

Figure 5. Specificity for the different matrices evaluated (A=Garcinol standard solution, B=HEXred, C=Nanoemulsion, D=Porcine skin, E=Porcine esophageal mucosa, F=Adhesive tape, G=Receptor fluid).

As next step, the standard was subjected to different stress conditions. Samples were submitted to different stressing agents: light, heat, hydrogen peroxide 3% and 30%, hydrochloric acid 0.1 M and 1 M and sodium hydroxide 0.1 M and 1 M. No interference of degradation products in the quantification of garcinol was observed, as can be seen in Figure 6, for the most degrading condition among all evaluated (data from the remaining conditions are not presented, but also showed no interference). The peak purity of the standard was also evaluated after 6 hours at different conditions and it has remained unchanged.

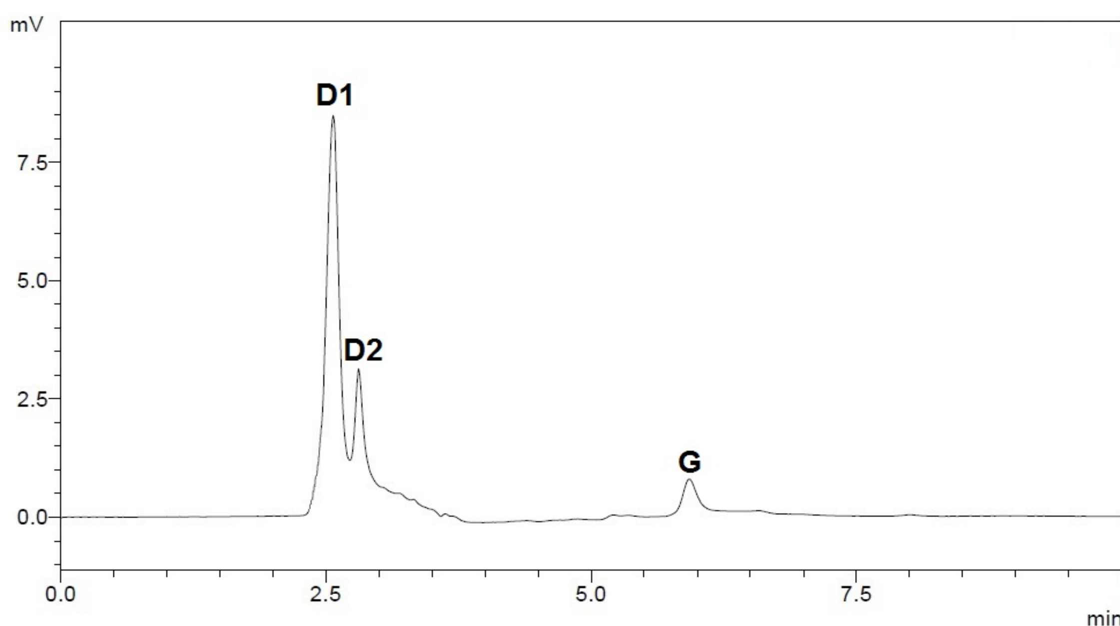

Figure 6. Chromatographic separation of degradation products (D1 and D2) of garcinol (G).

The non-interference in quantification of the compound of interest is a prerequisite and can be measured by comparing the response obtained from a standard solution with that from a spiked pre-treated sample. The analytical curves were constructed in order to evaluate whether results obtained by the developed method were directly proportional to the analyte concentration in the samples. The equation, R^2, LLOQ, LOD, and LOQ were determined. Based on the data obtained in equations, the matrix effect of each sample was also calculated. According to FDA (2014), matrix effect is the direct or indirect alteration or interference in response due to the presence

of unintended analytes or other interfering substances in the sample. All these results are shown in Table 1. The LLOQ was the first concentration of linear range 0.1 µg/mL. The data revealed that developed method is linear in the quantification range proposed and, according to Niessen, Manini and Andreoli (2006), the evaluated matrices resented low matrix effect (-20%<ME%<20%).

Table 1. Linearity of the standard (garcinol) and the matrix effect (ME) of each matrix evaluated.

Matrix	Equation	R^2	LOD (µg/mL)	LOQ (µg/mL)	ME (%)
Standard	y = 10067x + 164.73	0.9996	0.047	0.141	-
HEXred	y = 10317x + 333.05	0.9989	0.077	0.233	2.42
Nanoemulsion	y = 10238x + 197.47	0.9992	0.068	0.206	1.67
Skin	y = 10664x + 286.42	0.9979	0.089	0.270	5.59
Mucosa	y = 10238x + 359.84	0.9987	0.087	0.263	1.67
Tape	y = 10377x + 304.72	0.9990	0.073	0.222	2.98
Receptor fluid	y = 10204x + 896.20	0.9995	0.051	0.154	1.34

The data of intraday and interday precision and accuracy for the garcinol spiked matrices are shown in Table 2. The intraday precision data for all samples showed a relative standard deviation (RSD) value between 0.84-3.84 for HEXred, nanoemulsion, and receptor fluid and between 1.10-4.49 for porcine skin, porcine esophageal mucosa, and tape adhesive. The interday precision data showed a RSD value between 1.56-4.70 for HEXred, nanoemulsion, and receptor fluid and 2.20-6.70 for porcine skin, porcine esophageal mucosa, and tape adhesive. The accuracy results for garcinol in all matrices were within 96% to 104% range. The evaluation of accuracy by recovery of the garcinol added in biological matrices was also performed and results are in Table 3. The recovery data for garcinol previously added in biological matrices were within FDA recommendations (RSD < 4.07%) to

bioanalytical method validation (FDA, 2014) and the extraction time (30 minutes) was adequate for complete garcinol recovery from all evaluated matrices. No interference of the matrix components was observed.

Table 2. Intraday and interday precision and accuracy for garcinol spiked matrices.

Matrix	Amount added*	Intraday* (RSD)[a]	Interday* (RSD)[b]	Accuracy % (RSD)[a]
	1	1.04 (2.48)	1.01 (4.70)	103.97 (2.48)
HEXred	2	1.96 (2.98)	1.98 (2.58)	98.00 (2.98)
	4	4.15 (2.23)	4.02 (4.60)	103.64 (2.23)
	1	1.02 (2.60)	1.02 (1.99)	101.90 (2.60)
Nanoemulsion	2	2.05 (0.84)	2.08 (3.24)	102.00 (0.84)
	4	3.84 (1.04)	3.85 (1.56)	96.00 (1.04)
	1	0.97 (3.89)	1.00 (4.86)	96.50 (3.89)
Skin	2	2.06 (1.10)	1.98 (5.53)	103.00 (1.10)
	4	3.94 (1.77)	3.92 (4.71)	98.41 (1.77)
	1	1.00 (3.21)	0.99 (2.56)	99.52 (3.21)
Mucosa	2	1.96 (3.96)	2.04 (6.70)	98.00 (3.96)
	4	3.92 (3.31)	3.92 (2.23)	98.10 (3.31)
	1	1.01 (1.37)	0.98 (4.43)	100.51 (1.37)
Tape	2	2.05 (4.49)	2.07 (3.48)	102.00 (4.49)
	4	4.12 (2.28)	4.10 (2.20)	103.00 (2.28)
	1	0.99 (1.77)	1.02 (3.00)	99.57 (1.77)
Receptor fluid	2	2.08 (3.84)	2.08 (2.70)	104.00 (3.84)
	4	3.94 (2.42)	3.95 (1.74)	98.59 (2.42)

* µg/mL; [a] n = 6; [b] n = 18; RSD = relative standard deviation.

Table 3. Recovery of the garcinol added in biological matrices.

Matrix	Amount added*	Recovery % (RSD)[a]
	1	97.31 (3.60)
Skin (epidermis/dermis)	2	99.88 (2.14)
	4	99.01 (2.72)
	1	95.12 (3.89)
Skin (Stratum Corneum**)	2	96.08 (3.10)
	4	96.94 (1.77)
	1	100.60 (4.07)
Mucosa	2	101.96 (3.64)
	4	98.79 (2.29)

* µg/mL; **After tape stripping process; [a] n = 6; RSD = relative standard deviation.

Figure 7 shows the results for robustness of the method evaluated by Plackett-Burman experimental design for the different matrices. Results provided by experimental design correspond to the percentage of garcinol in the samples compared to the standard solution, in each experiment. No significant changes were observed for the various factors evaluated, since the $t_{calculated}$ values were below the $t_{critical}$ values ($\alpha = 0.05$). This demonstrates robustness of the developed method since changes introduced in the experimental conditions did not result in significant variations in the obtained results.

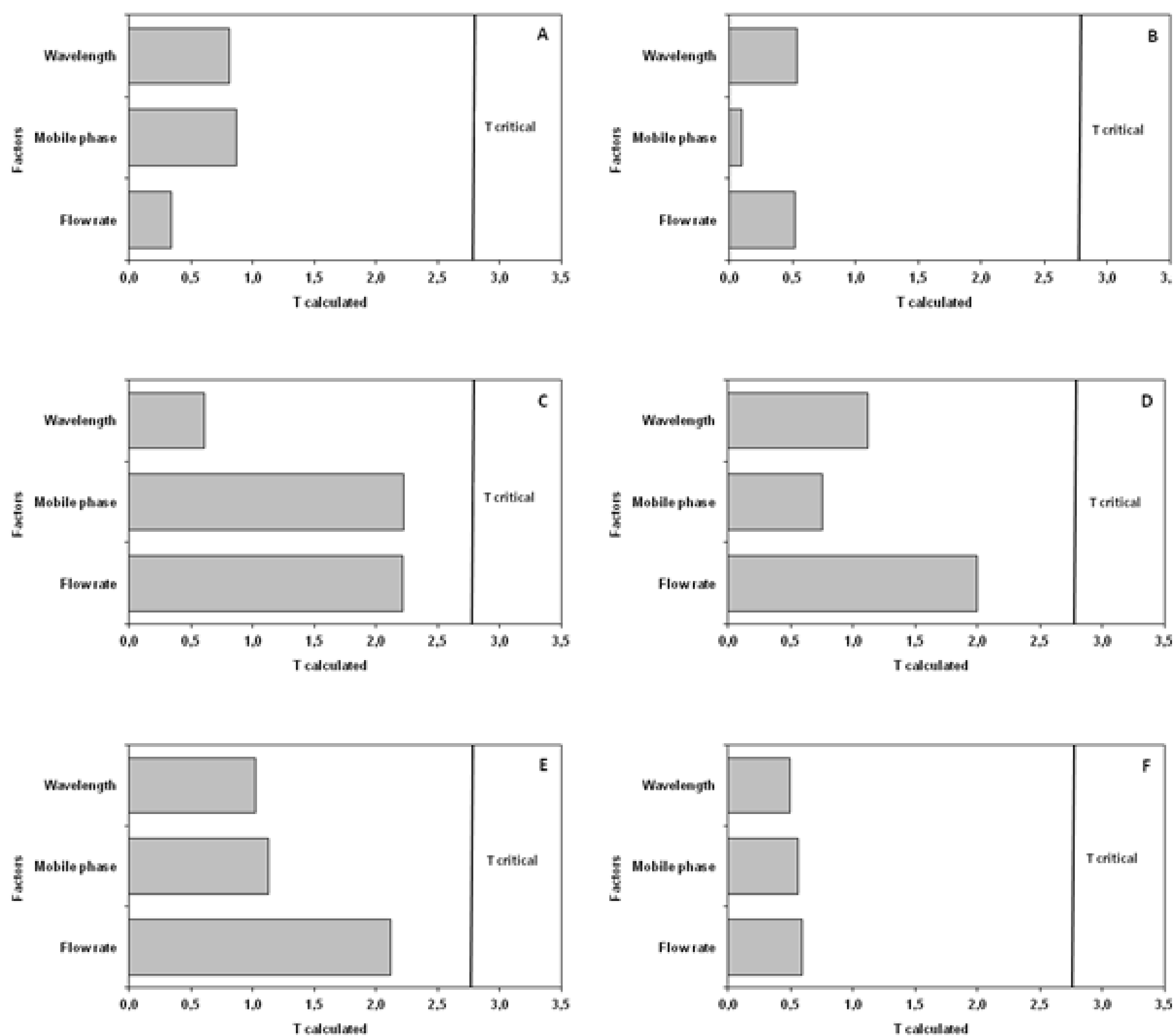

Figure 7. Robustness by Plackett-Burman experimental design for the different matrices (A=HEXred; B=nanoemulsion; C=porcine skin; D=porcine esophageal mucosa; E=adhesive tape; F=receptor fluid).

Concerning the system suitability, results for garcinol parameters evaluated were retention time 5.9 min, theoretical plates 2228, tailing factor 1.5, and resolution 4.2. The stability of garcinol spiked matrices was also evaluated. No change in the content of solutions stored at room temperature (48 h) was detected, and the values found in all matrices were in the range of 98.76 - 100.12%.

3.3 Application of the method

The determination of PPBs in real samples using the validated HPLC/UV method proposed in this study was finally performed in HEXred (PPBs represent approximately 70% of the chemical composition), nanoemulsion containing HEXred (PPBs concentration = 1 mg/mL) and samples from permeation/retention study (i.e. receptor fluid, skin and mucosa layers). Results showed the precision of the method (for analytical assays RSD < 2.11% and for bioanalytical assays RSD < 12.62%) (Table 4).

Table 4. Determination of PPBs in real samples by the validated HPLC method.

Sample	Mean of six replicates (RSD)
HEXred (mg/mg)	0.68 (1.87)
NE_{HEXred} (mg/mL)	0.97 (2.11)
Skin retention	
Stratum corneum (μg/cm^2)	<LLOQ*
Epidermis (μg/cm^2)	0.62 (10.59)
Dermis (μg/cm^2)	0.96 (12.62)
Receptor fluid (μg/mL)	LLQ
Mucosa permeation	
Esophageal mucosa (μg/cm^2)	0.15 (9.33)
Receptor fluid (μg/mL)	0.41 (10.91)

*Lower than limit of quantification; RSD = relative standard deviation.

About the *in vitro* skin permeation/retention study using Franz cells, no PPBs could be detected in the receptor fluid and stratum corneum after 8 h, although they could be quantified with adequate precision in the epidermis and dermis layers. For

evaluation of permeation through the mucosa, the method proved to be suitable for quantifying PPBs in both tissue and receptor fluid. This is an important finding, considering that these substances have shown antifungal activities (PIPPI *et al.*, 2015), requiring application on skin/mucosa to carry out their properties. In the literature, no report was found concerning the validation of a method for quantification of PPBs after skin/mucosa permeation, being this study the first, fast and validated method for this purpose. In this context, the method led us to estimate PPBs in skin and mucosa samples from permeation/retention assay with satisfactory precision.

4 CONCLUSIONS

Our results indicate that HEXred is mainly composed by PPBs. The proposed HPLC/UV method proved to be simple and reliable for the determination of total content of PPBs, expressed as garcinol, in the HEXred and their nanotechnology-based products as well as in the presence of different matrices, including biological matrices, which was confirmed by the low matrix effect. Moreover, the method proved to be specific, linear, precise, accurate, and robust for the determination of PPBs in different samples. Overall, determination of these compounds in lipophilic extracts of BRP can be considered as useful strategy for further studies of standardized preparations and development of products from this natural resource.

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6. CAPÍTULO III. Topical delivery of antifungal Brazilian red propolis benzophenones-rich extract by means of cationic lipid nanoemulsions optimized by Box-Behnken Design

Topical delivery of antifungal Brazilian red propolis benzophenones-rich extract by means of cationic lipid nanoemulsions optimized by Box-Behnken Design

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ABSTRACT

Mucocutaneous fungal infections are usually treated with topical application of medication. Recent studies have demonstrated the antifungal activity of a benzophenones-rich extract (BZP) from Brazilian red propolis (BRP) against non-*albicans Candida* strains. This study aimed to optimize the incorporation of BZP into nanoemulsions (NE) intended for the treatment of mucocutaneous fungal infections. The optimization of NE was performed by means of a Box-Behnken Design, which allowed evaluating simultaneously the influence of the phospholipid egg-lecithin, the cationic lipid DOTAP and BZP concentrations on the physicochemical properties of NE, as well as on the association efficiency of BZP. By using the Mini Tab® software, the optimal formulation selected, based on the smallest droplet size and highest zeta potential and association efficiency, exhibited a mean average size of 140.56±5.22 nm, zeta potential of +60.72±3.07 and association efficiency (AE) of 99.55±1.09%. Franz-type diffusion cells were used to evaluate BZP distribution through porcine skin and mucosa. BZP were found in both mucosa and skin layers (mainly in the dermis). A higher amount of BZP (up to 3-fold) was detected in impaired skin and mucosa demonstrating the effect of the integrity of the tissue on BZP distribution, as suggested by confocal fluorescence microscopy images. BZP were detected in the receptor fluid only when esophageal mucosa was impaired. The antifungal activity of the formulations was investigated against non-*albicans Candida* species – *C. krusei*, *C. glabrata*, *C. tropicalis* and *C. parapsilosis*. MIC values varied from 0.654 to 2.617 µg/mL, with cell damage higher than 78 % as verified by MTT assay. Such results suggest that the optimized NE have promising potential to be used topically for the treatment of mucocutaneous fungal infections.

1 INTRODUCTION

Candida infections are opportunistic mycoses that can manifest as surface or invasive conditions, ranging from superficial oral thrush, vaginitis, nail and skin diseases (mucocutaneous) to systemic, which are mostly fatal and commonly referred to as candidemia (SPAMPINATO and LEONARDI, 2013). Although *C. albicans* is still considered the most common pathogen associated with *Candida* infections, over the last few years, the incidence of cases with non-*albicans Candida* (NAC) species, such as *C. krusei, C. glabrata, C. tropicalis* and *C. parapsilosis* have been increasingly reported (JAMES *et al.*, 2006; PEMÁN and SALAVERT, 2012; YAPAR, 2014).

Anticandidal drugs include the polyenes, azoles and, echinocandins. Nevertheless, adverse effects, toxicity, drug interactions and increasing resistances limit the use of these drugs, mainly in candidemic patients with hepatic or renal impairment (COLOMBO *et al.*, 2006; ZARAGOZA and PEMÁN, 2012; SARDI *et al.*, 2013). Although amphotericin have become the standard treatment for serious fungal infections for approximately five decades, adverse effects prompted the continued search for effective but less toxic options. Fluconazole emerged as an alternative.

However, certain species of NACs are less susceptible to fluconazole. To date, *C. tropicalis* is less susceptible to fluconazole than *C. albicans*, *C. tropicalis* and *C. parapsilosis* are both generally susceptible to azoles, *C. glabrata* is intrinsically more resistant to antifungal agents, particularly to fluconazole, and *C. krusei* is inherently resistant to fluconazole (CRUCIANI and SERPELLONI, 2008; ECKERT and LENTZ, 2012, SMEEKENS *et al.*, 2013). Since clinical management of fungal infections is compromised by the emergence of antifungal drug resistance, there is a critical need for new therapeutic options (SARDI *et al.*, 2013).

Many reports have stated the importance of natural products as potential sources of novel and selective agents for the treatment of different diseases (NEWMANN and CRAGG, 2012). Propolis, a natural product with multiple

pharmacological effects, has been considered to the discovery of new antimicrobial compounds since it acts defending the hive against invasive bacteria and fungi (BANSKOTA, TEZUKA and KADOTA, 2001). This resinous product contains beeswax, enzymes, sugar, and plant exudates collected by *Apis mellifera* bees from various plant sources. The chemical composition is complex and variable being related to the local vegetation which determines its chemical diversity (PICCINELLI *et al.*, 2011; SFORCIN and BANKOVA, 2011). Regarding the antimicrobial activity, its effect against bacterial and fungal pathogens has been reported (OTA *et al.*, 2001; SANTOS *et al.*, 2005; QUINTERO-MORA *et al.*, 2008; CASTRO *et al.*, 2009). Brazilian propolis was classified by PARK and coworkers (2002) in twelve major groups according to physicochemical properties and geographic locations. Recent attention has been given to a new group, classified as Group 13, named red propolis due to its intense red color (ALENCAR *et al.*, 2007). Brazilian red propolis (BRP) has been reported to have several activities, including antimicrobial and most studies have been conducted with ethanolic and hydroethanolic extracts (TRUSHEVA *et al.*, 2006; ALENCAR *et al.*, 2007; RIGHI *et al.*, 2011; FROZZA *et al.*, 2013; DE MENDONÇA *et al.*, 2015). Preliminary results of our research group showed a remarkable antifungal activity of the *n*-hexane extract of BRP that is mainly composed by polyprenylated benzophenones (BZP), named oblongifolin A, guttiferone E, and/or xanthochymol (Figure 1) (PICCINELLI *et al.*, 2011; LOPEZ *et al.*, 2014; PIPPI *et al.*, 2015; FASOLO *et al.*, 2016).

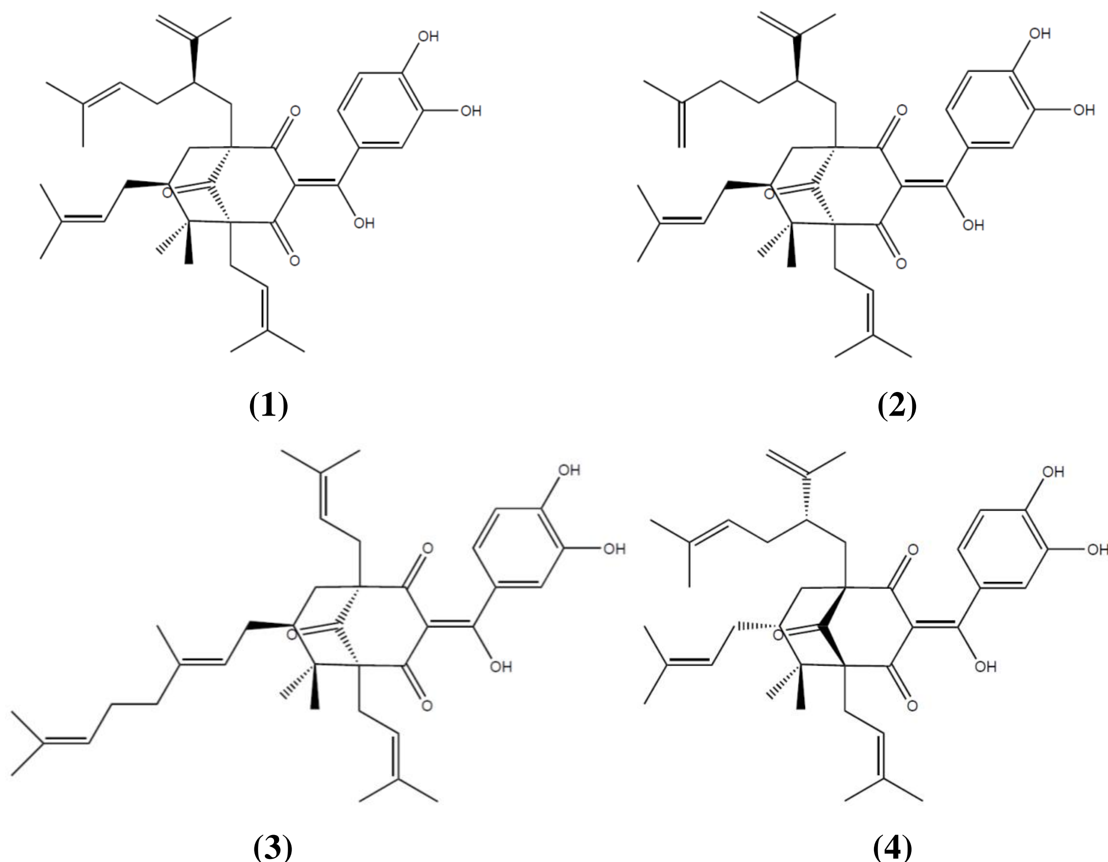

Figure 1. Chemical structure of guttiferone E (1), xanthochymol (2), oblongifolin A (3) and garcinol (4).

Recently, the incorporation of extracts, fractions, or isolated bioactive compounds from extracts into nanoemulsions (NE) had been described in the literature especially for poorly soluble compounds (FASOLO *et al.*, 2009; BIDONE *et al.*, 2014; NEMITZ *et al.*, 2015). The composition of such nanosystems may play a role in the physicochemical properties of nanoemulsions, association efficiency as well as on the permeation/retention of bioactive compounds through the skin. Experimental designs are a relevant tool to optimization of formulation experiments. The great advantage of these designs is an organized approach, where the researcher is guided to perform a set of experiments adequate for the selected objective, i.e., the analyst can control the factors that he believes to be relevant in the response variables of the experiment. Among the objectives of the use of experimental designs, the optimization should be highlighted because it leads to the prediction of response values for all possible combinations of independent variables and also to the identification of the best

experimental point. Response surface methodology has become the standard approach for the optimization of conditions and processes, both in the laboratory and industry. These designs allowed us to estimate interaction and even quadratic effects, and therefore give us an idea of the shape of the response surface we are investigating. One of the Response surface methodology, the Box-Behnken Design (BBD) requires factors at only three levels, avoids treatment combinations that are extreme and the variance of the predicted response at any point depends only on its distance from the design center point (ERIKSSON, 2008; HONARY, EBRAHIMI and HADIANAMREI, 2014).

In this study, we applied the BBD to optimize the composition of topical NE containing BZP from BRP. Our first aim was to evaluate simultaneously the effect the phospholipid lecithin, the cationic lipid DOTAP, and the BZP concentration on the physicochemical properties of nanoemulsions, and association efficiency of BZP. Next, the distribution of BZP in skin and mucosa was investigated for the optimized formulations, as well as their *in vitro* antifungal activity against non-*albicans Candida* species.

2 MATERIAL AND METHODS

2.1 Material

BRP was purchased from Natucentro® (Minas Gerais/Brazil). Porcine skin and porcine esophageal mucosa were purchased from Cooperativa Ouro do Sul (Rio Grande do Sul/Brazil). Egg-lecithin (Lipoid E-80), DOTAP and medium chain triglycerides (MCT) were purchased from Lipoid GmbH (Ludwigshafen, Germany). Octyldodecanol and isopropyl myristate were purchased from Delaware® (Rio Grande do Sul/Brazil). Garcinol (purity ≥ 95%) was purchased from Cayman Chemical (Michigan, USA).

2.2 Preparation of BZP

The BRP was ground and the powdered material was successively extracted by maceration with *n*-hexane over 72 h (3 times) and sample:solvent ratio of 1:10 (w/v). The extracts were combined and evaporated to dryness under reduced pressure using a rotary evaporator at 40 ^{o}C. The residue was treated with cold acetone to obtain an insoluble fatty residue that was filtered through paper filter and evaporated to dryness.

2.3 High-performance liquid chromatography analysis

The quantification of BZP in different samples (oils, formulations, and skin/mucosa layers) was assessed by high-performance liquid chromatography (HPLC) using previously validated conditions (FASOLO *et al.*, 2016). The total content of BZP was expressed as garcinol (Figure 1), a guttiferone E diastereoisomer. In brief, the chromatographic column used was a reversed phase C_{18} (Shim-pack ODS, Shimadzu, Kyoto, Japan) 250 x 4.6 mm, 5 µm. The separation was carried out by isocratic elution with methanol:acetonitrile (80:20, v/v) for 10 min at room temperature. The optimized system parameters were as follows: flow rate 1 mL/min, injection volume 20 µL, and detection wavelength at 260 nm.

2.4 Solubility of BZP in different oils

To select the oil core of NE, the solubility of garcinol was determined by adding an excess amount of this compound to different oils (medium chain triglycerides, isopropyl myristate and octyldodecanol). The mixtures were kept under constant magnetic stirring for 24 h at 20 °C ± 1 ºC to reach equilibrium. After, the samples were centrifuged at 15,000 rpm for 15 min, and an aliquot of the supernatant

of each sample was weighed and diluted. The garcinol content was determined by HPLC as described above. Analysis was carried out in triplicate.

2.5 Preparation of the NE

The preparation of the NE was made by spontaneous emulsification procedure. The oil core components (isopropyl myristate, egg-lecithin and DOTAP) were dissolved in ethanol (28 mL). BZP was previously dissolved in acetone (2 mL) and combined with the other components of the oil phase. Then, this organic phase was poured into the water phase composed of 60 mL of water under moderate and constant magnetic stirring. After 15 min, the formulation was concentrated to 10 mL by evaporation under reduced pressure at 40 °C. NE without BZP (blank NE) was prepared as control formulation.

2.6 Physicochemical characterization of the NE

Mean droplet size and polydispersity index (PI) of the NE were measured by photon correlation spectroscopy (PCS) and zeta potential by electrophoretic mobility, both at 25 °C, using a Malvern Zetasizer® Nano-ZS 90 (Malvern Instruments, Malvern, UK). Before the measurements the samples were diluted 1:1,000 with ultrapure water (droplet size and PI analysis) and 1.0 mM NaCl solution (zeta potential). pH was determined using a digital potentiometer (Digimed, São Paulo, Brazil).

The association efficiency (AE) was determined by adding NE_{BZP} to ultrafiltration membranes (100,000 Da cutoff, Ultrafree; Merck Millipore) and centrifuged at 10,000 rpm for 50 min. Then, free BZP was quantified in the ultrafiltrate as described above and the AE (%) was estimated by the difference between the total

and free-BZP concentrations. All analyzes were carried out in triplicate just after the preparation and after 15 and 30 days of storage.

2.7 Optimization of formulation by Response Surface Methodology (RSM)

The Box-Behnken Design, a RSM, was applied in order to study the influence of three components in the physicochemical properties of the final NE. Three independent variables were chosen: lecithin (mg; X_1), DOTAP (mg; X_2) and BZP (mg; X_3) amounts (Table 1). As response factor, or dependent variables, it was chosen the mean average size (nm), the zeta potential (mV) and the AE (%). A total of 15 randomized runs involving the three independent variables were performed and with these results it was possible to determine the best concentrations of the independent variables to obtain an optimal formulation (with smaller particle size and higher zeta potential and AE).

Table 1. Independent variables used in the Box-Behnken Design

Independent variables	**Symbol**	**Levels**		
		-1	**0**	**1**
Lecithin (mg)	X_1	100	200	300
DOTAP (mg)	X_2	6,98	13,97	20,96
BZP (mg)	X_3	5	10	15

Multiple linear regression and further statistical analysis were performed using the Minitab® 16 software. Experimental data were fitted by the following second-order polynomial model according to the following equation:

$$y = A_0 + \sum_{i=1}^{k} A_i X_i + \sum_{i=1}^{k} A_{ii} X_i X_i + \sum_{i=1}^{k-1} \sum_{j=1+1}^{k} A_{ij} X_i X_j$$

where A_0, A_i, A_{ii}, and A_{ij} represent the regression coefficients of constant, linear, quadratic, and interactions terms, respectively, while X_i, X_j, and X_k represent the independent variables, and k is the number of variables. Linear, quadratic, and interaction coefficients were determined by the least square regression followed by ANOVA. Values with $p < 0.05$ were considered significant.

2.8 BZP permeation/retention through intact and impaired porcine skin and mucosa

The permeation/retention of BZP through intact and impaired porcine ear skin and esophageal mucosa was carried out by using Franz-type diffusion cells. The system was kept at a controlled temperature (32 ± 1 °C) and constant stirring (650 rpm). Skin or esophageal pieces were set between the donor and receptor compartments of the Franz cell, on a surface area of 1.77 cm^2. The receptor compartment was supplied with a mixture of phosphate buffered solution pH 7.0 (PBS):ethanol (70 : 30) and NE_{BZP} (500 µL) that was placed in the donor compartment. After 8 h, an aliquot of the fluid receptor was withdrawn and the skin or mucosa was removed from the cell. Samples were cleaned using PBS pH 7.0 and a cotton swab.

To evaluate the distribution of BZP into the skin layers fromNE_{BZP}, BZP retention was determined in the stratum corneum, epidermis, and dermis. The tape stripping process was used to separate the stratum corneum (Scotch Tape 750; 3M, St Paul, MN, USA). Briefly, the first stripped tape was discarded, while the following 14 tapes were used for BZP assay (stratum corneum). The epidermis was separated from the dermis using a scalpel and the layers were weighed. After, the dermis was reduced to tiny pieces and all samples were placed separately in test tubes. To extract the BZP from skin layers, 5 mL of methanol were added and samples were maintained in an ultrasound bath, during 30 minutes. In similar experimental conditions, porcine esophageal mucosa was cut into tiny pieces, placed in test tubes and submitted to the

same extraction technique. Samples were filtered through 0.45 µm Millipore membranes and analyzed by HPLC validated method as previously described (FASOLO *et al.*, 2016). For the evaluation of impaired skin, tape stripping was performed by using 60 tapes before the experiment and the impaired mucosa was obtained by removing the superficial epithelium layer with a scalpel. The results were expressed as mean ± standard deviation (n=6) of BZP per skin weight (µg/g).

2.9 Histological analysis

After 8 h of permeation/retention study, histological analyzes were performed in order to observe skin/mucosa morphology changes after treatment with NE_{BZP}. Skin and mucosa were cleaned with a swab, immersed in formaldehyde solution, dehydrated, paraffin embedded, sectioned (6 µm thick) and colored by hematoxylin-eosin. The pieces were observed under optical microscopy using 40x magnification.

2.10 Confocal fluorescence microscopy

To visualize the distribution of fluorescent NE_{BZP} through the skin/mucosa layers a confocal fluorescence microscopy was used. Nile red was added (0.05%) to the organic phase during NE preparation. After 8 h of permeation/retention study, the skin/mucosa samples were mounted with Tissue-tec O.C.T.® (Sakura Finetechnical, Tokyo, Japan) onto a metal sample holder and frozen at -20 °C. Vertical 40 µm thick slices were obtained with a cryostat (Leica CM 1850) and the slides were evaluated using a confocal microscopic Olympus FluoView™ 1000. The images were taken at a 40X optical zoom.

2.11 Antifungal activity

2.11.1 Fungal Strains

Eight non-*albicans Candida* (NAC) strains, clinical isolates and references from American Type Culture Collection (ATCC), including *C. glabrata* (CG), *C. parapsilosis* (CP), *C. krusei* (CK) and *C. tropicalis* (CT), belonging to the mycology collection of the Applied Mycology Laboratory of Universidade Federal do Rio Grande do Sul (UFRGS), Brazil, were used in this study (Table 2).

Table 2. Non-*albicans Candida* strains

Strains	Sensitive strain	Resistant strain*
CG RL34	X	
CG RL34m		X
CP RL27	X	
CP RL27m		X
CK ATCC 6258	X	
CK 03		X
CT ATCC 750	X	
CT 72A		X

* To fluconazole, in accordance with CLSI (2012).

2.11.2 Antifungal susceptibility test

The minimum inhibitory concentration (MIC) of BZP and NE_{BZP} was determined by the broth microdilution method according to the M27-A3 protocol

(CLSI, 2008) taking into account the updates of the M27-S4 document (CLSI, 2012). MICs values were defined as the lowest concentration of compounds at which the micro-organisms tested did not demonstrate visible growth at 32 °C in 48 h. Prior to experiments, the BZP was dissolved in dimethyl sulfoxide (DMSO; Synth) and diluted with Roswell Park Memorial Institute (RPMI) 1640 broth medium without sodium bicarbonate (Sigma-Aldrich Co., St. Louis, USA) and pH 7.0 with morpholinepropansulfonic acid (MOPS) (Sigma-Aldrich Co., St. Louis, USA). NE_{BZP} was diluted by the same procedure, excluding the step with DMSO. The BZP concentrations of the samples ranged from 0.082 to 41.875 µg/mL. Amphotericin B kindly supplied by Cristalia® (Brazil) was used as positive control in the same concentration range of the samples. The experiments were carried out in quadruplicate.

2.11.3 MTT assay

A more detailed analysis of susceptibility test, based on a colorimetric assay using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich Co., St. Louis, USA), was used to confirm the cell damage of BZP and NE_{BZP} (Mosmann, 1983). After 48 h of MIC study, the supernatant was discarded and then the cells were incubated with a 150 µL of MTT solution (0.05 µg/mL in RPMI) for another 4 h at 32 °C. Next, the MTT solution was removed and the MTT formazan crystals were extracted with 150 µL of isopropanol. Absorbances (A) were measured on a multiplate reader (SpectraMax®) in two wavelengths (570 and 690 nm) to be adjusted for nonspecific absorption. Yeast damage (%) was calculated by comparing the absorbance of samples treated with BZP and NE_{BZP} and non-treated samples. The experiments were carried out in quadruplicate.

2.12 Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate differences between mean values and were considered as statistically significant when $p < 0.05$.

3 RESULTS AND DISCUSSION

Propolis-based preparations have been widely used to treat mucocutaneous infections by bacterial, fungal and virus (SFORCIN and BANKOVA, 2011). Our research group has recently demonstrated antifungal effects of an *n*-hexane extract of BRP against NAC strains (PIPPI *et al.*, 2015). This extract is a benzophnones-rich extract (approximately 70%) mainly composed of a mixture of the BZP guttiferone E and/or xanthochymol and oblongifolin A (FASOLO *et al.*, 2016). Due to the low water solubility of BZP, we proposed here its incorporation into nanoemulsions, suitable for skin/mucosa topical applications.

3.1 Optimization of formulation

The oil represents one of the most important excipients in the NE formulation because it can solubilize lipophilic drugs. The selection of the oil core was based on the solubility of BZP. Isopropyl myristate, medium chain triglycerides and octyldodecanol were selected once they are widely used in pharmaceutical and cosmetic emulsions especially due to its ability to solubilize marked amount of lipophilic drugs (ROWE *et al.*, 2012; ARGENTA *et al.*, 2015; LI *et al.*, 2015; ZHAO *et al.*, 2015). Among them, isopropyl myristate was chosen for further studies due to the highest ability to solubilize BZP (5.1±0.36 mg/g) in comparison with medium chain triglycerides (3.4±0.22 mg/g) and octyldodecanol (3.1±0.28 mg/g).

NEs are composed of an oil core very often stabilized by a combination of surfactants. In this study, after setting the isopropyl myristate as oil core, egg-lecithin and DOTAP were selected as surfactants. Egg-lecithin is a complex mixture of phospholipids (combined with other substances such as carbohydrates, fatty acids, and triglycerides) that have been used in the formulation of skin care products due to its penetration enhancer properties and biocompatibility. DOTAP is a positively charged lipid that may have an effect on the stability of nanoemulsions and their interactions with negatively-charged outer epithelial cells, that could lead to an increase of permeation/retention of drugs.

Keeping constant the amount of the oil (10%), the design an experimental study to optimize the amount of the formulation components seeking a combination that would result in a stable NE with a smallest droplet size and highest zeta potential and BZP association efficiency was performed. A Box-Behnken Design allowed evaluating simultaneously the effect of egg-lecithin, DOTAP and BZP concentrations on the physicochemical properties formulations. The Box-Behken Design matrix with independent variables in coded form and observed responses are presented in the Table 3.

Table 3. Box-Behken Design and results obtained for each dependent variable.

Run	Lecithin	DOTAP	BZP	Size (nm)	Zeta (mV)	AE (%)
1	-1	-1	0	183.2	40.0	87.5
2	0	0	0	160.3	57.0	92.7
3	-1	0	-1	191.8	46.0	100.0
4	0	1	1	144.5	58.9	78.3
5	1	-1	0	182.8	28.0	100.0
6	-1	1	0	180.4	75.7	88.6
7	-1	0	1	182.0	60.1	59.1
8	1	1	0	139.5	54.3	100.0
9	0	-1	-1	170.3	36.0	100.0
10	0	1	-1	133.3	74.3	100.0
11	1	0	-1	163.9	52.8	100.0
12	0	-1	1	183.5	25.1	61.3
13	1	0	1	170.8	39.9	81.0
14	0	0	0	167.3	42.6	100.0
15	0	0	0	163.4	49.3	99.9

Figure 2 shown the contour plots, which exhibit the response variation tendency within the specified range of the independent variables and also show the influence of each of these on the other.

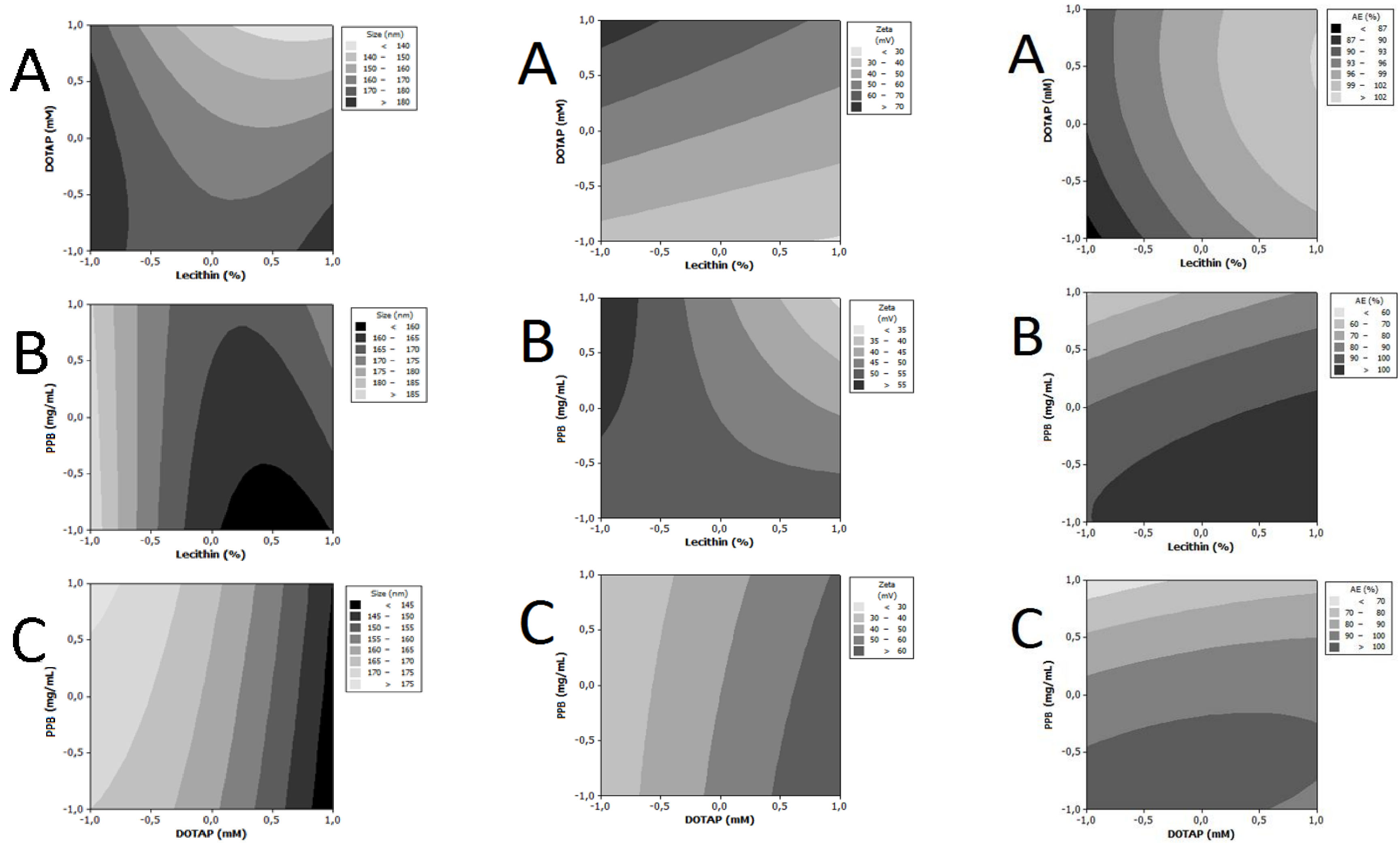

Figure 2. Contour plot for the droplet average size (2a) zeta potential (2b) BZP association efficiency (2c) obtained by plotting : (A) concentration of lecithin versus versus concentration of DOTAP; (B) concentration of lecithin versus concentration of BZP, and (C) concentration of DOTAP versus concentration of BZP.

From these results it was possible to determine mathematically, using the Mini Tab® software, the formulation with smaller particle size and higher zeta potential and AE. The coefficients for each independent variable (linear, square and interaction) showed significance as follows: lecithin have an influence on the size and AE, DOTAP have an influence on the size and zeta potential and BZP only in the AE (Table 4).

Table 4. Coefficients for each independent variable in the Box-Benhken Design (linear, square and interaction)

	Size	**Zeta**	**AE**
Constant	163.667 (0.000)*	49.6556 (0.000)*	97.5333 (0.000)*
Lecithin	-10.050 (0.009)*	-5.8500 (0.071)	5.7250 (0.007)*
DOTAP	-15.262 (0.001)*	16.7625 (0.001)*	2.2625 (0.141)
BZP	2.687 (0.313)	-3.1375 (0.274)	-15.0375 (0.000)*
Lecithin:lecithin	13.517 (0.012)*	0.4847 (0.903)	-1.6917 (0.416)
DOTAP:DOTAP	-5.708 (0.167)	-0.603 (0.872)	-1.8167 (0.385)
BZP:BZP	-0.058 (0.987)	-0.4403 (0.911)	-10.8167 (0.002)*
Lecithin:DOTAP	-10.125 (0.031)*	-2.3500 (0.545)	-0.2750 (0.887)
Lecithin:BZP	4.175 (0.273)	-6.7500 (0.121)	5.4750 (0.031)*
DOTAP:BZP	-0.500 (0.889)	-1.1250 (0.768)	4.2500 (0.068)

*Significant for $p < 0.05$.

Based on these results, the optimal formulation (NE_{BZP}) was defined containing lecithin 182 mg, DOTAP 20.96 mg and BZP 6.7 mg, with a final PPBs concentration of 0.67mg/mL. This formulation was prepared five times to verify whether the response variables had consistent values, i. e., prove that these samples showed reproducibility of the results. These analyses were carried out (in triplicate) just after simple emulsification procedure and over 30 days. The results showed a

mean average size of 140.56±5.22 nm, zeta potential of +60.72±3.07 and AE of 99.55±1.09%. No significant differences ($p<0.05$) were detected after 30 days of storage at 4 °C, demonstrating a short-term stability of the optimized formulation. In addition, significant variation was not observed concerning pH (5.6±0.3) and PI (≤ 0.21±0.04) values.

3.2 Skin and mucosa retention of BZP from nanoemulsions

BZP permeation/retention from NE_{BZP} through porcine ear skin and esophageal mucosa was evaluated by using Franz-type diffusion cells. BZP content in different layers (expressed as garcinol) was determined by a validated bioanalytical HPLC method (FASOLO *et al.*, 2016). The human skin has three distinct layers (epidermis, dermis and hypodermis) and its main function is to protect against water loss and penetration of external agents, also exerting important role in thermoregulation and excretion of various substances (WALTERS and ROBERT, 2002). As shown in table 5, BZP were found in all layers of intact skin (up to 2.58 $\mu g/cm^2$) from NE_{BZP} after 8 h of permeation, mainly in the dermis (~2 $\mu g/cm^2$). To evaluate the permeation of substances through mucous membranes, among many models, oral and esophageal mucosa, from the porcine origin, are used to mimic human tissue (HOOGSTRAATE *et al.*, 1996). Oral and esophageal mucosa have a stratified squamous epithelium, squamous and non-keratinized supported by connective tissue fibers and the lipid composition is qualitatively and quantitatively similar (SQUIER and KREMER, 2001; CONSUELO *et al.*, 2005a; CONSUELO *et al.*, 2005b). As shown in Table 5, approximately ~13 $\mu g/cm^2$ of BZP were detected into intact esophageal mucosa after 8 h of kinetics showing a higher permeability of BZP through mucosa when compared to the skin. No BZP was detected in the receptor compartment of Franz diffusion cells after permeation studies using intact skin and mucosa.

Table 5. BZP permeation and retention through intact and impaired porcine skin and mucosa.

Sample			BZP (µg/cm^2)	BZP (µg/g)
Skin	Intact	Stratum corneum	0.20 (0.02)	-
		Epidermis	0.32 (0.03)	11.33 (0.92)
		Dermis	2.08 (0.06)	7.36 (0.44)
		Receptor fluid	ND	-
	Impaired	Epidermis	0.79 (0.05)	27.97 (3.85)
		Dermis	5.74 (0.36)	20.32 (2.26)
		Receptor fluid	ND	-
Mucosa	Intact	Tissue	13.2 (1.44)	93.45 (10.02)
		Receptor	ND	-
	Impaired	Tissue	25.1 (1.83)	193.16 (21.22)
		Receptor fluid	0.62 (0.04)[b]	

ND: not detected; [a]Mean of six replicates (RSD); [b]µg/mL

The mucocutaneous fungal infections may cause the skin to become cracked and sore and blisters and pustules may also occur. Once the treatment of fungal infections occurs under these conditions, BZP permeation/retention experiments were also carried out on previously impaired tissue to gather information on the tissue condition on BZP permeation/retention. The literature reported the use of a tape stripping technique to impair the skin (JENSEN *et al.*, 2011; SPAGNUL, BOUVIER-CAPELY and PHANETAL, 2011; BIDONE *et al.*, 2015). A significant higher amount of BZP was detected ($p < 0.05$) in the dermis when tissue was previously impaired in comparison with intact skin (about 3-fold) demonstrating the effect of a lesion in the cutaneous tissue on BZP permeation. Such a result may be related to the partial removal of the outermost layer of the epidermis (as demonstrated in Figure 3b), the stratum corneum, that consists of corneocytes (rich dead cells keratin) located in an

intercellular matrix (SCHAEFER, REDELMEIER and BENECH-KIEFFER, 1999; BOUWSTRA and HONEYWELL-NGUYEN, 2002). However, even in this condition, no BZP was detected in the acceptor compartment, suggesting that incorporation of BZP into NE might modulate the transepidermal diffusion of bioactive compounds (CEVC, 2004). Similar results were observed for impaired tissue when the upper layers of the esophageal mucosa were partially removed (Figure 5d). Approximately twice BZP (25 $\mu g/cm^2$) was detected in the injured porcine esophageal mucosa. On the opposite that was observed for skin, the previous removal of upper layer of esophageal mucosa allowed the permeation of BPZ towards the acceptor compartment of Franz-diffusion cells (0.62 $\mu g/cm^2$ of BZP).

To have a better insight on the permeation/retention of BZP, confocal fluorescence microscopy studies were performed in order to visualize the distribution of a fluorescent dye (Nile red) incorporated into NE_{BZP} through the porcine skin/mucosa layers after 8 h of treatment. Figure 3 shows the distribution of fluorescent dye (Nile red) on intact and injured skin and mucosa after permeation from NE_{BZP}. Regardless tissue condition, the fluorescence of Nile red is visibly distributed in tissues after treatment. The fluorescence intensity seems to be higher in both impaired skin and mucosa when compared with intact tissues, in line with BZP distribution from nanoemulsions. Taken together, the injury could remove parts of the surface layer of mucosa and stratum corneum, decreasing tissue barrier, and leading to an increase of Nile red permeation/retention in tissues (BIDONE *et al.*, 2015; MATTOS *et al.*, 2015).

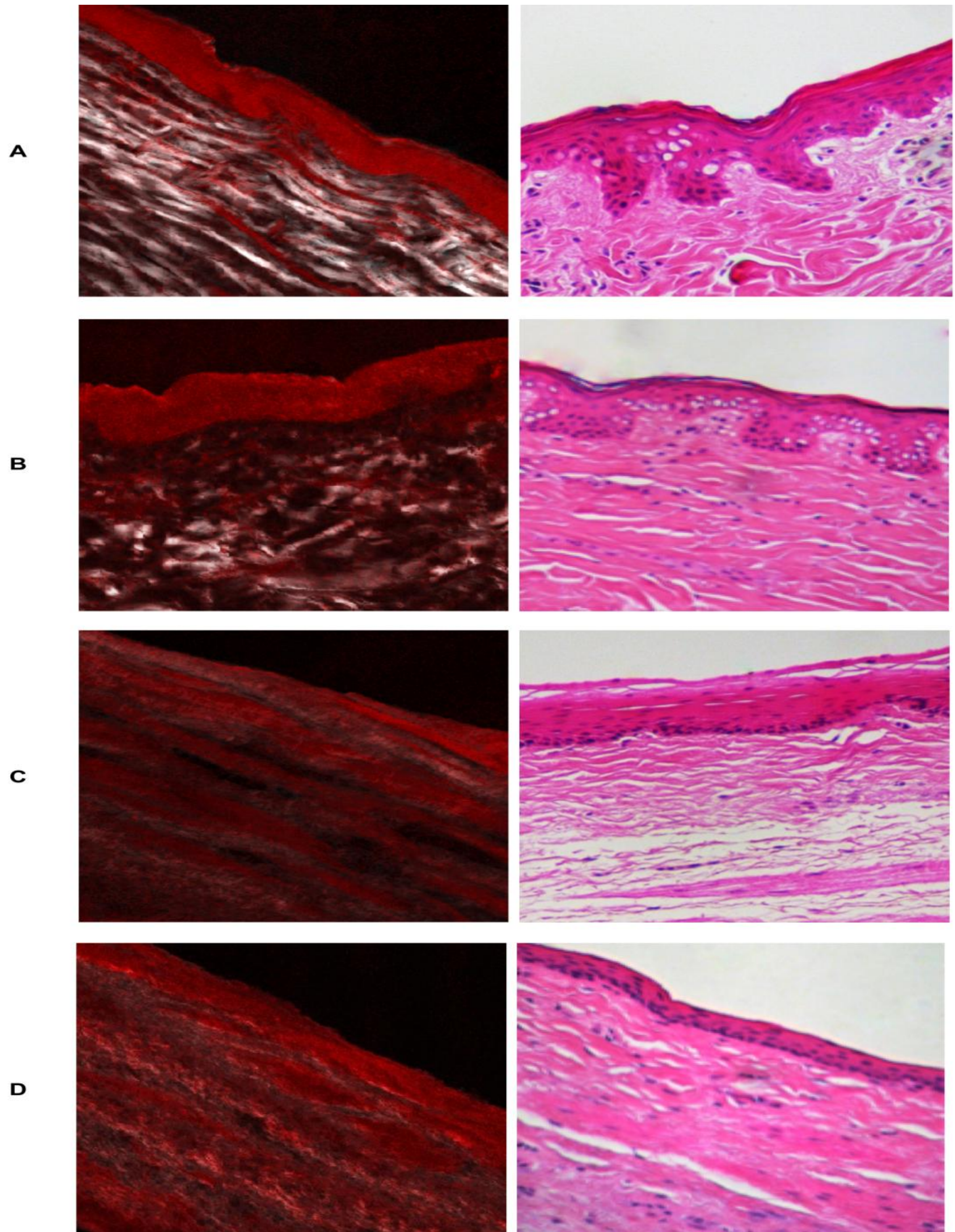

Figure 3. Fluorescent (left line) and histological (right line) images of intact skin (A), impaired skin (B), intact mucosa (C) and impaired mucosa (D).

3.3. Antifungal activity of BZP-loaded nanoemulsions

Once we got to know the permeation/retention profile of BZP in the porcine skin and mucosa, the next step was to investigate the antifungal activity of these compounds against NAC species. It is known that *Candida spp.* are the major human fungal pathogens that cause both deep (invasive candidiasis) or superficial (including cutaneous and mucosal) infections (SARDI *et al.*, 2013) and act binding to skin and mucosal surfaces degrading epidermal keratin which allows the invasion (LEHMAN, BRUCE and ROGERS, 2009). These yeasts are responsible for 35-65% of all candidaemias and occur more frequently in patients with hematologic malignancies, preterm neonates and recipients of a marrow transplant. *Candida albicans* is the most prevalent species involved in invasive fungal infections but the frequency of infections caused by NAC is increasing due to the development of antimicrobial resistance and the restricted number of antifungal drugs (SARDI *et al.*, 2013).

In this context, a variety of natural products can be used as a prototype for the development of new drugs. An important class of these compounds, BZP, has several activities reported in the literature including the antimicrobial activity (LOKVAM *et al.*, 2000; MATSUHISA *et al.*, 2002). Previous literature has shown the antimicrobial activity of BZP against different microorganisms as is the case of kolanone – isolated from the fruit pulp of *Garcinia kola* and active against *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Streptococcus pneumoniae* and *Candida albicans* (HUSSAIN *et al.*, 1982) – and 3-geranyl-2,4,6-trihydroxybenzophenone, active against *C. albicans, C. neoformans, S. aureus* and methicillin-resistant *Straphylococcus aureus* with MICs of 25.0, 3.13, 3.13 and 3.13 μg/mL, respectively (ZHANG *et al.*, 2002). In this study, the antifungal susceptibility test was performed with BZP and NE_{BZP} against NAC clinical isolates and ATCC strains to determine MIC values and, additionally, these results were confirmed by MTT cell damage assay (Table 6).

Table 6. MIC and cell damage of BZP and NE_{BZP} against NAC.

Strain	BZP		NE_{BZP}	
	MIC[a]	Cell Damage[b]	MIC[a]	Cell damage[b]
CG RL34	0.654	77,60 (2.06)	1.308	78,84 (1.68)
CG RL34m	0.654	79.46 (2.93)	1.308	86,30 (2.96)
CP RL27	1.308	93.19 (1.84)	1.308	98.54 (2.11)
CP RL27m	1.308	88.48 (1.91)	1.308	97.87 (1.88)
CK ATCC 6258	0.654	90.99 (2.21)	2.617	85.76 (1.89)
CK 03	2.617	85.97 (1.79)	2.617	96.02 (1.84)
CT ATCC 750	2.617	95.07 (1.93)	2.617	97.22 (1.99)
CT 72A	2.617	93.63 (2.01)	2.617	97.03 (1.86)

[a] µg/mL; [b] % (RSD)

The results showed a marked activity against NAC tested for BZP and NE_{BZP}, exhibiting MIC values from 0.654 to 2.617 µg/mL, with respective cell damage in the range of 78 - 97% (approximately) to these concentrations. The control formulation prepared without BZP (blank NE), did not show antifungal activity and the positive control (amphotericin B) showed a MIC value of 0.327 to all strains. In a recent study of our research group, PIPPI and coworkers (2015) evaluated the ability of *C. parapsilosis* and *C. glabrata* to develop phenotypic resistance to a benzophenone enriched fraction of BRP as compared to fluconazole (FLC), and a pharmacological synergism between them. They observed an increase in the MIC values of fluconazole for all strains with the development of resistance to most of them, whereas no isolate became less susceptible to BRP. A synergism (BRP+FLC) was observed, suggesting that BRP could be a possible therapeutic strategy for the treatment of infections related to FLC-resistant *Candida* spp.

4 CONCLUSIONS

Our results revealed a rational optimization of the NE composed by BZP (a benzophenone-rich *n*-hexane extract), egg-lecithin, isopropyl myristate, DOTAP and purified water, obtained by spontaneous emulsification, which showed small droplet size and high zeta potential and AE. The permeation/retention behavior of these compounds showed appropriate, since it seeks an application in human skin and mucosa. Also, this study point to the strong antifungal capacity of this formulation containing natural products extracted from BRP and can be considered a promising alternative for the topical treatment of infections caused by NAC strains.

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7. DISCUSSÃO GERAL E PERSPECTIVAS

As candidíases, infecções causadas por espécies do gênero *Candida*, são classificadas em superficiais, com ocorrência cutânea e em mucosas, até infecções profundas, sistêmicas, de alta gravidade. Esta última acomete, principalmente, pacientes imunocomprometidos (JAYATILAKE e SAMARANAYAKE, 2010). Anualmente, a candidíase vulvovaginal está entre os principais problemas ginecológicos que afetam mulheres no mundo inteiro, sendo causada, principalmente, por *C. albicans.* Apesar disso, a identificação de espécies de CNA, como *C. tropicalis*, *C. parapsilosis* e, especialmente, *C. glabrata* como causadoras dessa infecção tem sido relatada e sua incidência vem aumentando nos últimos anos (RAGUNATHAN *et al.,* 2014; GONÇALVES *et al.,* 2015).

Um grande desafio para a clínica diz respeito à resistência aos antifúngicos usuais empregados, como o fluconazol, resultando na maioria das vezes, em ineficácia do tratamento das candidíases (ZOMORODIAN *et al.,* 2011). Assim, diante das limitações relacionadas aos antifúngicos existentes, a descoberta de novos compostos, incluindo produtos naturais, com potencial atividade antifúngica torna-se essencial. TAKAISI-KIKUNI e SCHILCHER (1994) demonstraram possíveis mecanismos de ação para a atividade antifúngica exibida pela própolis. Foi observado efeito na membrana dos patógenos, bem como na parede celular e na replicação do DNA dos micro-organismos. Assim, a evolução da resistência torna-se dificultada, uma vez que diversos mecanismos de adaptação necessitariam ser desenvolvidos pelos fungos, requerendo várias mutações concomitantes.

Nesse sentido, considerando as propriedades atribuídas à própolis já relatadas na revisão da literatura deste trabalho, a primeira etapa do presente estudo consisitiu em investigar a atividade da própolis vermelha brasileira (PVB) contra cepas de CNA. Frações da PVB foram obtidas com solventes de polaridade crescente através de maceração à temperatura ambiente. O ensaio antifúngico foi conduzido utilizando um total de nove cepas de CNA. A CIM das frações foi determinada pelo método de microdiluição em caldo, com concentrações variando entre 1,9 a 500 µg/mL. Ensaio colorimétrico (MTT) foi utilizado para confirmar o dano celular da fração *n*-hexano (HEX), utilizando também outras cepas resistentes.

Entre as frações testadas, HEX apresentou maior potencial antifúngico, alcançando valores de CIM de 1,95 µg/mL contra *C. parapsilosis*. Este valor é semelhante aos relatados para fármacos de referência como equinocandinas, azóis ou polienos (ANDES *et al.*, 2010; BRAUTASET *et al.*, 2011; MELETIADIS *et al.*, 2012). Para *C. glabrata, C. krusei* e *C. tropicalis* os valores de CIM obtidos foram variáveis (1,95-250 µg/mL), sendo esta variabilidade descrita para fungos emergentes tais como CNA (YASHWANT *et al.*, 2013). O dano celular (MTT) variou entre 80,66-94,44%, com extensa morte celular causada pela HEX. Destacam-se ainda os efeitos contra cepas resistentes de *Candida*, principalmente *C. glabrata* e *C. parapsilosis*. A potencial capacidade antifúngica evidenciada pela HEX está relacionada com os compostos lipofílicos, provalmente BPPs, anteriormente detectadas na PVB.

Resultados similares foram encontrados em estudo de QUEIROZ (2010), no qual própolis coletadas em três estados brasileiros (Paraná, Alagoas e Bahia) foram utilizadas para a preparação de extrato etanólico e frações hexano, diclorometano e acetato de etila. O extrato e as frações foram submetidos à avaliação do potencial antifúngico, frente a seis espécies de *Candida*. Os resultados de CIM variaram de 1,0 a 62,5 µg/mL para os extratos brutos ativos, sendo que o extrato obtido da própolis da Bahia apresentou baixa atividade. A fração diclorometano da própolis coletada em Alagoas foi a mais ativa, sendo caracterizados compostos isoflavonoídicos, como medicarpina, vestitol e formononetina.

A atividade antifúngica da própolis brasileira obtida no estado de Minas Gerais também foi avaliada em pacientes com candidíase oral, sendo utilizado como controle positivo a nistatina (tratamento padrão). Neste estudo, os pacientes seguiram o seguinte esquema posológico: aplicação tópica na lesão e na superfície da prótese dentária quatro vezes ao dia, durante sete dias. Todos os pacientes tratados com o extrato etanólico de própolis demonstraram uma regressão da lesão, similar àquela observada nos pacientes tratados com nistatina. A eficácia desse extrato no tratamento da candidíase oral é de grande interesse para a saúde pública no Brasil, uma vez que a própolis é um produto barato e acessível à população (SANTOS *et al.,* 2005).

Valores de CIM para o extrato etanólico da própolis coletada no Paraná (Brasil) frente a biofilmes formados por cepas de *C. albicans* foram determinados por CAPOCI e colaboradores (2015). Esses valores variaram de 68,35 a 546,87 µg equivalente de ácido gálico/mL (conteúdo total de fenóis). A concentração de 546,87 µg/mL foi capaz de causar morte a 75,8% das cepas isoladas. Assim, esse extrato de própolis foi ativo na inibição da formação de biofilme de *C. albicans* isoladas de pacientes com candidíase vulvovaginal.

Estudos com amostras de própolis de fora do país também são relatados na literatura. O extrato etanólico de própolis oriunda da Índia (MURUGAN *et al.,* 2008) apresentou valores de CIM (700 µg/mL) superiores aos encontrados no presente estudo frente a cepas de *Candida*, sendo menos ativo que o produto brasileiro. Da mesma maneira, nos estudos desenvolvidos por YALFANI, KHOSRAVI e PIROUZ (2013), os autores avaliaram o extrato etanólico da própolis, coletada no Irã, e demonstraram valores de CIM entre 3,83 e 9,33 mg/mL, frente a isolados clínicos de *C. albicans.*

De modo geral, a maioria dos estudos realizados no país presentes na literatura se refere à própolis verde; em contrapartida, nos últimos anos, observa-se um aumento no número de trabalhos com a PVB. De acordo com ALENCAR e colaboradores (2007), a PVB apresenta compostos bioativos inéditos e incomuns, os quais não são encontrados nos outros tipos. Essa composição diversa caracteriza-a como uma importante fonte de substâncias com potenciais atividades biológicas (TRUSHEVA *et al.,* 2006; OLDONI *et al.,* 2011).

Estudo recente de BEZERRA *et al.* (2015a) demonstrou o efeito do extrato hidroetanólico da PVB em amostras de secreção vaginal humana contendo espécies de *Candida*. Destas, 81,25% foram sensíveis formando halos de inibição de crescimento superiores a 10 mm. Os autores sugerem que a menor concentração do extrato da própolis (25%) apresentou o maior efeito antifúngico. Ainda, essa atividade da PVB não foi aumentada quando foi variada a concentração do extrato testada. Resultados semelhantes foram observados pelo mesmo grupo de pesquisa, quando avaliado o extrato frente a isolados clínicos de *Candida* da cavidade oral. Dentre as cepas ensaiadas, 61% foram sensíveis ao extrato da PVB, com atividade antifúngica pronunciada na menor concentração (25%) (BEZERRA *et al.,* 2015b).

No que se refere à composiação química, mais de 300 fitocompostos já foram identificados em várias amostras de própolis, como: flavonoides, derivados de ácidos benzoicos, benzaldeído e derivados, ácido cinâmico e derivados, outros alcoóis, cetonas, fenóis e compostos heteroaromáticos, terpenos, incluindo sesqui e triterpenos, hidrocarbonetos alifáticos, minerais, esteróis, açúcares e aminoácidos, além de novas substâncias que ainda estão sendo descobertas durante a caracterização química de diferentes tipos de própolis (WALKER e CRANE, 1987; MARICA *et al.,* 2004; CROCI *et al.,* 2009; TRUSHEVA *et al.,* 2010; ALMUTAIRI *et al.,* 2014; BANKOVA *et al.,* 2014). Isso demonstra a complexidade química dessas amostras, podendo variar de acordo com a escolha do extrato e do método de obtenção, que por sua vez, pode refletir nos efeitos biológicos encontrados. PARK e colaboradores (1998a) verificaram que, dependendo da concentração etanólica utilizada para a preparação dos extratos, ocorre grande variação na concentração de flavonoides, um dos principais grupos responsáveis pela ação farmacológica, causando diferenças nas respostas biológicas encontradas.

A utilização de métodos analíticos para a quantificação de fármacos e seus metabólitos precisa, necessariamente, da prévia validação destes, seja para fins de estudos analíticos e/ou bioanalíticos. Sabe-se que um método é considerado validado no momento em que cumprir todos os requisitos estabelecidos pelas agências reguladoras presentes em diferentes países (ICH, 2005; EMEA, 2011; FDA, 2014). Na revisão da literatura realizada durante este estudo foi observada a ausência da métodos analíticos validados para quantificar os compostos majoritários (BPPs) encontrados no extrato hexano da PVB. Compostos lipofílicos de PVB têm recebido atenção crescente devido a alguns relatos interessantes a respeito de suas atividades biológicas (TRUSHEVA *et al*., 2006; ALENCAR *et al.*, 2007) e, neste contexto, este estudo teve como objetivo avaliar a composição química do extrato hexânico da PVB (HEXred) por CLUE-DAD-EM.

A investigação química resultou na identificação de BPPs neste extrato, sendo elas oblongifolina, gutiferona E e/ou xantochimol. Após, um método isocrático de CLAE-UV foi validado para a determinação do teor total de BPPs (a 260 nm) expresso

como garcinol, um diasteroisômero da gutiferona E disponível comercialmente. O método desenvolvido provou ser capaz de quantificar adequadamente o garcinol em solução ou na presença das matrizes avaliadas, evidenciando um baixo efeito da matriz. Além disso, revelou ser específico, linear, preciso, exato e robusto, características fundamentais para que um método possa ser considerado válido e apto para a finalidade a que se destina. Neste contexto, o desenvolvimento e a validação do método para a quantificação de um marcador da fração lipofílica da PVB torna-se um ponto de referência para mais estudos de preparações padronizadas deste produto natural.

Com base nestes resultados, cabe destacar a importância de fontes naturais brasileiras, como a própolis, na triagem de extratos bioativos, propiciando um futuro desenvolvimento de formulações capazes de melhorar a atividade do produto, seja através da vetorização da ação ou pela melhoria na forma de liberação do ativo. A incorporação de extratos, frações ou compostos bioativos isolados a partir de extratos em nanoemulsões têm sido objeto de estudo do nosso grupo de pesquisa (FASOLO *et al.,* 2009; BIDONE *et al.,* 2014;. NEMITZ *et al.,* 2015.). No presente trabalho, devido à reduzida hidrossolubilidade das benzofenonas, HEXred foi incorporado em NE, compostas de um núcleo oleoso de miristato de isopropila estabilizado por lecitina de gema de ovo e DOTAP e água. Essa forma farmacêutica foi selecionada sabendo-se que as propriedades da nanoestrutura obtida podem modular a penetração/permeação dos compostos ativos, no que se refere ao uso tópico, otimizando a eficácia do tratamento (CEVC, 2004).

Estudos incorporando extratos de própolis em formulações, visando uso tópico, já foram descritos na literatura. No estudo realizado por BITTENCOURT e colaboradores (2014), os autores obtiveram extrato etanólico seco da própolis vermelha, padronizado em 2% de flavonoides, que foi adicionado aos excipientes para formular duas emulsões (creme Polawax® e creme Lanette®), um creme-gel (Hostacerin SAF®) e dois géis (Natrosol® e Aristoflex®), contendo 5 mg de própolis/g de base. A concentração fungicida mínima (CFM) foi igual a 647,5 µg/mL, para o extrato da própolis vermelha, frente a *C. albicans*. Dentre as bases estudadas, o creme

Lanette® foi a que apresentou melhor característica físico-química para a incorporação e veiculação do ativo, sendo mais efetivo na inibição do crescimento da levedura.

Quanto ao uso da própolis em formulações com base nanotecnológica, BUTNARIU e GIUCHICI (2011) apontaram a mesma como um dos componentes ativos de NEs com proteção contra raios UV-A. Sua associação com licopeno resultou em um potente modulador do estresse oxidativo, reduzindo processos pró-oxidantes e aumentando o sequestro de radicais livres. Ainda, os autores relataram que as NEs conferem melhores efeitos terapêuticos que as formulações convencionais, com base no controle da liberação local por um longo período de tempo, provavelmente aumentando a sua eficiência e aceitação na pele. A aplicação local em roedores da NE experimental (própolis e licopeno) conferiu analgesia e apresentou baixa toxicidade.

Sabendo que a composição destes nanosistemas pode desempenhar um papel importante nas propriedades físico-químicas das NEs, na eficiência de associação, bem como sobre a permeação/retenção de compostos bioativos através da pele, um desenho experimental, o Box-Behnken, foi utilizado como ferramenta na otimização da formulação. A grande vantagem deste é que o analista pode controlar os fatores que ele acredita ser relevante nas variáveis de resposta do experimento, conduzindo à previsão de valores de resposta para todas as combinações possíveis de variáveis independentes e também para a identificação do melhor ponto experimental. (ERIKSSON, 2008; HONARY, EBRAHIMI e HADIANAMREI, 2014). Como etapa prévia, a definição do núcleo oleoso foi realizada através de estudo de solubilidade do garcinol em três óleos diferentes, sendo que o miristato de isopropila apresentou os melhores resultados, em comparação aos TCM e ao ODD. O miristato de isopropila é um núcleo oleoso correntemente utilizado na composição de nanoemulsões (ROWE *et al.*, 2012).

As três variáveis independentes escolhidas foram quantidades (em mg) de lecitina, DOTAP e benzofenonas e como variáveis dependentes optou-se pelo tamanho médio das gotículas (nm), potencial zeta (mV) e eficiência de associação (%), visando obter uma formulação ótima (com menor tamanho de gotícula e maiores potencial zeta e eficiência de associação). Os coeficientes para cada variável independente (linear,

quadrático e interação) evidenciaram que a lecitina apresentou influência no tamanho e na eficiência de associação; o DOTAP apresentou influência no tamanho e no potencial zeta e as BPPs apenas na eficiência de associação. Com base nestes resultados, a formulação ótima foi definida como aquela contendo 182 mg de lecitina, 20,96 mg de DOTAP e 6,7 mg BPPs.

Uma vez otimizada a NE, a permeação/retenção das BPPs através da pele da orelha e mucosa esofágica, ambas de origem suína, foi avaliada usando células de difusão do tipo Franz. BPPs foram encontradas em todas as camadas de pele intacta (até 2,58 $\mu g/cm^2$) após 8 h de permeação, principalmente na derme (~ 2 $\mu g/cm^2$). Aproximadamente 13 $\mu g/cm^2$ foi detectado na mucosa esofágica intacta após 8 horas de cinética o que mostra uma permeabilidade mais elevada das BPPs através da mucosa quando comparado com a pele. As BPP não foram detectadas no fluido receptor das células de difusão após o estudo de permeação através da pele e mucosa intactas.

Sabendo que as infecções fúngicas mucocutâneas podem causar fissuras e bolhas nos tecidos, os experimentos de permeação/retenção das BPPs também foram realizados nos tecidos previamente lesados. Este procedimento é realizado, através da utilização de uma fita adesiva (*tape-stripping*) para promover lesão na pele e através de raspagem com bisturi para promover lesão na mucosa (JENSEN *et al.*, 2011; SPAGNUL-BOUVIER *et al.*, 2011; BIDONE *et al.*, 2015). Uma maior quantidade de BPPs foi detectada na derme quando o tecido foi previamente lesado, em comparação com a pele intacta (cerca de 3 vezes), demonstrando o efeito de uma lesão no tecido cutâneo sobre a permeação destes compostos. Tal resultado pode estar relacionado com a remoção parcial da camada mais externa da epiderme, o estrato córneo, que é constituído por corneócitos (células mortas ricas em queratina) localizado em uma matriz intercelular (SCHAEFER, RELDEMEIER e BENECH-KIEFFER, 1999; BOUWSTRA e HONEYWELL-NGUYEN, 2002). No entanto, nesta mesma condição, não foram detectadas BPPs no fluido receptor, sugerindo que a incorporação destes compostos em NE pode modular a difusão transepidérmica de compostos bioativos (CEVC, 2004). Resultados semelhantes foram observados para a mucosa esofágica lesada em relação à mucosa íntegra. Entretanto, ao contrário do que foi observado para

a pele, a prévia remoção da camada superior da mucosa esofágica permitiu a permeação das BPPs para o fluido receptor de células de Franz.

Para ter um melhor conhecimento sobre os resultados de permeação/retenção, estudo de microscopia confocal de fluorescência foi realizado a fim de visualizar a distribuição de um corante fluorescente (vermelho do Nilo) incorporado na formulação. Independentemente da condição do tecido, a fluorescência do vermelho do Nilo é visivelmente distribuída nos tecidos após o tratamento, sugerindo uma maior intensidade em ambos os tecidos lesados quando comparado com os tecidos intactos. A lesão promovida pode remover partes da camada superficial da mucosa e camada córnea, reduzindo a função barreira, o que leva a um aumento do corante nos tecidos (BIDONE *et al.*, 2015; MATTOS *et al.*, 2015).

A atividade antifúngica do extrato e PVB e das formulações desenvolvidas foi, em uma última etapa, avaliada frente a especies de CNA. Segundo SARDI e colaboradores (2013) espécies de *Cândida* são os principais fungos patogênicos humanos que causam tanto infecções sistêmicas (candidíase invasiva) quanto superficiais (incluindo cutânea e mucosa). Estes agem através da ligação com a superfície da pele e mucosa degradando a queratina epidérmica, o que permite a invasão (LEHMAN, BRUCE e ROGERS, 2009). *Candida albicans* é a espécie mais prevalente envolvida em infecções fúngicas invasivas, mas a frequência de infecções causadas por espécies de CNA vem aumentando devido ao desenvolvimento de resistência antimicrobiana e ao número restrito de fármacos antifúngicos (SARDI *et al.*, 2013). Assim, os produtos naturais possuem uma grande variedade de compostos que podem servir como um protótipo para o desenvolvimento de novos fármacos, onde as benzofenonas constituem uma classe importante com diversas atividades já descritas na literatura, tais como anti-HIV-1 *in vitro* (FULLER *et al.*, 1999) e citotóxicas (SEO *et al.*, 2000; HOU *et al.*, 2001; WILLIAMS *et al.*, 2003), especialmente a atividade antimicrobiana (LOKVAM *et al.*, 2000; MATSUHISA, *et al.*, 2002).

Neste trabalho, o teste de susceptibilidade antifúngica foi realizado com as BPPs e com a NE contendo BPPs contra isolados clínicos de CNA e cepas ATCC para

determinar os valores de CIM e, complementarmente, estes resultados foram confirmados por ensaio de dano celular (MTT). Os resultados mostraram uma atividade acentuada contra CNA testado para as BPPs e NE contendo BPPs, exibindo valores de CIM entre 0,654-2,617 µg/mL, com respectivos danos celulares no intervalo de 78-97% (aproximadamente) nestas concentrações. A formulação controle preparado sem BPPs (NE branco), não apresentou atividade antifúngica.

Recentemente, PIPPI e colaboradores (2015) avaliaram a capacidade de *C. parapsilosis* e *C. glabrata* em desenvolver resistência fenotípica de uma fração enriquecida de BPPs em comparação ao fluconazol, e um sinergismo farmacológico entre eles. Os autores observaram um aumento nos valores de CIM de fluconazol para todas as cepas, com o desenvolvimento de resistência da maioria delas, ao passo que nenhum isolado se tornou menos suscetível a fração. Além disso, foi observada uma sinergia (fração+fluconazol), sugerindo que a fração pode ser uma estratégia terapêutica para o tratamento de infecções relacionadas com cepas de *Candida* resistentes ao fluconazol. Outros autores demonstraram esta atividade antimicrobiana de BPPs contra diferentes micro-organismos como é o caso da kolanona – isolada a partir da polpa do fruto de *Garcinia kola* e ativa contra *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Streptococcus pneumoniae* e *C. albicans* (HUSSAIN *et al.*, 1982), benzofenona-3-geranilo-2,4,6-tri-hidroxi ativa contra *C. albicans*, *C. neoformans*, *S. aureus* resistente a meticilina e *Staphylococcus aureus* com CIM de 25,0, 3,13, 3,13 e 3,13 µg/mL, respectivamente (ZHANG *et al.*, 2002).

Algumas perspectivas decorrem dos resultados obtidos ao longo da presente tese de doutorado. A primeira delas é a utilização de tecnologia verde na extração dos compostos ativos da PVB. A utilização de fluídos supercríticos, tais como o CO_2 supercrítico, na extração de produtos naturais tem mostrado um considerável potencial para futuros processos tecnológicos. Apresenta diversas vantagens em relação aos solventes utilizados comumente como baixa viscosidade e reduzida tensão superficial (sendo uma escolha interessante para a extração de substâncias a partir de uma matriz sólida) e, principalmente, porque é um processo limpo (TAYLOR, 1996). Esta linha de trabalho iniciou a ser explorada recentemente em um estudo em colaboração com

pesquisadores do Laboratório de Operações Unitárias da Faculdade de Engenharia da PUC/RS, cujos resultados iniciais estão apresentados em uma publicação apresentada em anexo.

A segunda perspectiva consiste na incorporação da nanoemulsão contendo as BPPs da PVB em um hidrogel. Esta matriz polimérica tridimensional caracteriza-se como um sistema mucoadesivo que pode proporcionar um aumento do tempo de contato *in situ* dos compostos ativos e promover uma melhor absorção dos mesmos (EDSMAN e HAGERSTROM, 2005).

A terceira e última perspectiva consiste na avaliação da atividade anti-fúngica *in vivo* desta formulação. Sabe-se que a ligação de espécies de *Candida* a superfícies mucosas é um passo importante no processo infeccioso e que modelos experimentais *in vivo* (como, por exemplo, modelos de infecção vaginal com estes micro-organismos) têm sido extremamente úteis na determinação da susceptibilidade e do tratamento da infecção (CARRARA *et al.*, 2010).

8. CONCLUSÕES

- Dentre os extratos obtidos da PBV com solventes de polaridade crescente, a maior atividade frente a cepas de *Candida* não albicans foi observada para o extrato *n*-hexano;
- O extrato *n*-hexano de PBV é constituído majoritariamente por BPPs (aproximadamente 70%);
- A validação de um método analítico por CLAE-UV permitiu a determinação do teor de BPPs totais em extratos de PVB expresso em garcinol;
- O método desenvolvido provou ser específico, linear, preciso, exato e robusto e capaz de quantificar adequadamente o garcinol em solução ou na presença das diferentes matrizes avaliadas;
- A utilização de um desenho experimental, o Box-Behnken, permitiu otimizar uma nanoemulsão contendo BPPs na concentração de 0,67mg/mL e as propriedades físico-químicas da formulação otimizada (diâmetro nanométrico das gotículas e carga positiva) são consideradas adequadas para o uso tópico proposto;
- As BPPs foram encontradas tanto na mucosa quanto nas diferentes camadas da pele (principalmente na derme), ambas de origem suína, sendo que em condições de lesão nestes tecidos a quantidade encontrada destes compostos foi duas a três vezes superior;
- A atividade antifúngica da formulação otimizada comprovou o efeito frente às cepas de CNA – *C. krusei, C. glabrata, C. tropicalis* e *C. parapsilosis* – com valores de CIM <2,617 µg/mL e com o dano celular superior a 78%;
- O conjunto dos resultados obtidos sugerem que a NE otimizada apresenta um potencial promissor para ser utilizada topicamente no tratamento de infecções fúngicas mucocutâneas causadas por espécies de CNA.

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ANEXO

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Polyprenylated Benzophenones Enriched Extracts obtained by Supercritical Carbon Dioxide from the Dry Ethanolic Extract of Brazilian Red Propolis

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Keywords:	Brazilian red propolis, benzophenones, antifungal activity, UPLC/MS, CO2 supercritical

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Polyprenylated Benzophenones Enriched Extracts obtained by Supercritical Carbon Dioxide from the Dry Ethanolic Extract of Brazilian Red Propolis

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Abstract

Natural products are usually seen as a valuable source of new drugs and propolis, a resinous substance obtained by bees by collecting secondary metabolites from plants of the region, is well known as a potent antimicrobial source due to its variety of bioactive compounds. The aim of this work was to submit a sample of Brazilian red propolis to supercritical CO_2 extraction with subsequent chemical characterization by HPLC and UPLC/MS, and evaluation of antifungal activity against four strains of *Candida glabrata*. It was also carried out the experimental extraction curve, yield *versus* extraction time, and the data were modeled by dynamic model based on a one-dimensional mass balance for the extract. The method proved to be selective for the extraction of benzophenones (BZP) which were characterized by UPLC/MS. The results of analyses by HPLC demonstrated a strong correlation between the BZP and the antifungal activity. The supercritical extract that presented the highest amounts of BZP was the one subjected a 300 bar, and was the most active against *Candida glabrata* strains. Thus, Brazilian red propolis BZP extracts shows potential as source of new antifungal drug.

Keywords: Brazilian red propolis, supercritical extraction, benzophenones, antifungal activity.

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Abreviations: SFE, Supercritical Fluid Extraction; **MIC,** Minimal Inhibitory Concentration; **SC**, Supercritical; **BZP**, benzophenones.

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1 Introduction

The study of natural products as therapeutic agents have been carried out for many years and today a wide variety of chemical structures are used for the development of new drugs by the pharmaceutical industry. Used as an alternative to treat some infections, propolis is a complex mixture of resinous, gummy and balsamic materials obtained from buds, flowers and plant exudates collected by bees mixed with salivary secretions, pollen and wax. It contains approximately 50% of resin and balsam, 30% of wax, 10% of essential and aromatic oils, 5% of pollen, and 5% of organic residues. The chemical composition is highly variable and depends on the local flora at the site of collection, from which the bees collect the exudates [1]. Factors of chemical variability include seasonality, climate, illumination and altitude, among others [2].

Different types of propolis have been widely used in folk medicine since ancient times for their therapeutic properties such as antibacterial, anti-inflammatory, and antifungal among other beneficial activities [3, 4, 5]. Brazilian propolis was classified by Park and coworkers [6] in twelve major groups. Recent attention has been given to a new group, classified as Group 13, named *red propolis* due to its intense red color [7]. For this propolis sample several biological and pharmacological properties have been described, including antibacterial, antifungal, citotoxic, among others [7, 8, 9, 10].

Recently, aiming to identify the active compounds, Fasolo et al. [11] partitioned Brazilian red propolis within increasing polarity solvent and verified that polyprenylated benzophenones were the main compounds present in the *n*-hexane extract. Further, this extract demonstrated important activity against non-*albicans Candida* strains [12].

Propolis cannot be used as a raw material and must be submitted to extraction to remove insoluble material such as waxes and detritus from the hive. Most propolis formulations are prepared with ethanol, resulting in an ethanolic extract of propolis (EEP) or diluted in

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aqueous ethanol and, less frequently, with water extracts, which present a wide array of compounds [6, 13, 14].

CO_2 is a nonpolar solvent and has great affinity with nonpolar compounds, like waxes. The high amount of these compounds in raw propolis may lead to low yield values in supercritical extraction. Due to this reason, a pretreatment of the samples is recommended in order to reduce the wax content like the addition of acetone, a solvent well known as a good wax remover. Maceration of raw propolis with ethanol is commonly used to reduce the wax content by generating a soluble portion, with most of medicinal compounds, and an insoluble portion, where waxes predominate. Paviani et al. [14] conducted supercritical extraction from raw propolis and its dry ethanol extract using ethanol as co-solvent in increasing concentrations and observed that the yields from extraction of raw propolis without addition of co-solvent afforded much smaller amounts (3-7%). When using supercritical solvent with increasing concentrations of ethanol, the yield increased significantly, reaching values up to 51%. In this study, several works employing the method to extract the compounds from different types of propolis were cited. Nevertheless, as far as we know, there are no studies with red Brazilian propolis obtained by supercritical carbon dioxide concerning the antifungal activity of these extracts against *Candida glabrata*.

The aim of this study was to investigate the influence of pressure on the global yield and chemical composition of the supercritical extracts obtained from filter papers impregnated with EEP of Brazilian red propolis as well as to determine the antifungal activity of the extracts against *Candida glabrata*.

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2. Materials and Methods

2.1 Material

Red propolis samples (Natucentro® (Minas Gerais, Brazil) were frozen, crushed, weighed and thoroughly extracted with ethanol at room temperature to obtain the crude ethanol extract (EEP). This procedure was performed by diluting ethanol 70% (v/v) in 100 g of red propolis fragments. The extracts were concentrated under vacuum distillation and deposited homogeneously on filter paper sheets. Subsequently, after dry, the sheets were weighed and the amount of impregnated EEP was calculated by decreasing the weight of the sheets before and after the impregnation. The sheets with EEP were then cut into small pieces (6 x 4 cm) that were submitted to supercritical carbon dioxide extraction. This process was performed in triplicate, resulting in an amount of 180 g of EEP impregnated on the filter sheets.

2.2 Supercritical fluid extraction (SFE)

The impregnated sheets were separated in three amounts of 60 g and subjected to supercritical extraction with a 99.9% supercritical carbon dioxide (Air Products) at 40 °C in a Pilot Equipment [15]. For each amount of 60 g, a different pressure condition was used: 90 bar, 150 bar and 300 bar. The rate of solvent was 0.1667 g/s.

The equipment includes an air driven liquid pump (Maximator G35-CO_2) for solvent delivery, a 500 mL high-pressure extraction vessel, and a separator flask. The extraction vessel was supplied with a heating jacket and an automated temperature controller. Heating tapes were used throughout the apparatus to maintain a constant temperature in the extraction section. To ensure a constant and steady solvent delivery the pump head was cooled by a circulating fluid, which passed through a chiller. Flow rates and accumulated gas volumes passing through the apparatus were measured using a flowmeter assay

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(Sitraus F C Massflo 2100 – Siemens, with accuracy of < 0.1%). Hoke (USA) micrometering valves were used for flow control throughout the apparatus. Heating tapes with automated temperature control were also used around this valve to prevent both freezing of the solvents and solid solute precipitation following depressurization. Pressure in the extractor was monitored with a digital transducer system, Novus 8800021600, acquired from Novus Produtos Eletrônicos (Brazil) with a precision of ±1.0 bar. The temperature controller was connected to thermocouples (PT-100, with an accuracy of 0.5K).

The extract yield curves *versus* time were constructed by determining, in every 5 minutes, the mass of extract obtained. Extractions had an average duration of approximately two hours for each pressure condition.

2.3 HPLC analysis

HPLC analyses (Agilent, 1200 with UV) were performed based on the method described by Chang et al. [16]. The system consisted of a G1322 vacuum degasser, G1311, a quaternary pump, manual injector, G1314B column oven and a UV/VIS detector. The chromatographic data were recorded and processed by LC Solution software version 1.24 SP2. For separations a Waters Nova Pack C18 column (5 μm, 4.6 x 250 mm) and a Waters Nova-Pack C18, 60Å, guard column (3.9 mm × 20 mm) were used.

An amount of 30 mg of the supercritical extracts were dissolved in 1.0 mL of methanol (Merck) and filtered on a Millipore filter (0.22 μm, Whatman). A volume of 20 μL of each sample was injected into the device. The mobile phase used was methanol HPLC grade and water/formic acid (0.5%), starting with 20% methanol and after 40 minutes, increasing to 100%, with a flow rate of 1 mL/min. The wavelength used for detection was 345 nm and the run lasted 60 minutes.

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2.4 UPLC/MS analysis

The 150 bar fraction was analyzed by Ultra High Performance Liquid Chromatography-Mass Spectrometry (UPLC/MS) using a UPLC Waters Acquity® with Photodiode array detector. A C18 reversed-phase column (Acquity UPLC BEH 50 mm x 2.1 mm, 1.7 µm, Waters) was used operating at 45 °C. Mass Spectrometer Q-TOF micro Micromass was used to identify the major compounds present in the samples. The analysis was performed using direct infusion positive ion mode electrospray ionization mass spectrometry (ESI(+)-MS).

The following parameters were set to spray chamber: capillary voltage 3.0 kV, sample cone voltage 30 V, extraction cone voltage 3.3 V, desolvation temperature 300 °C, source temperature 120 °C, Collision energy 4.0 V was used for MS spectra and variable energy from 20 to 40V for MS/MS spectra. Nitrogen and Argon were used as nebulizer and collision gas, respectively. Data analyses were processed using Masslynx V 4.1 software. The sample was dissolved in methanol (HPLC grade) to obtain final concentration of 1 mg/mL and filtered through a 0.45 µm pore size membrane (Millipore, Bedford, USA) before injection into the UPLC/MS system. Elution of sample was performed using a linear gradient system and the mobile phases consisted of a mixture of water:formic acid (100:0.1, *v/v*) (A) and methanol (100, *v*) (B), as described by Fasolo et al. [11]. The gradient profile was: 0.0–1.54 min from 40 to 65% of B, 1.54-4.38 min from 65 to 70% of B, 4.38-7.21 min from 70 to 75% of B, 7.21-8.00 min from 75 to 80% B, 8.00-11.00 min from 80 to 85% of B, 11.00-11.46 min 85% of B, 11.46-12.88 min from 85 to 90% of B, 12.88-14.29 min 90% of B, 14.29-15.71 min from 90 to 40% of B and finally, to restore the initial conditions, 15.71-16.00 min 40% of B. The flow-rate was 0.4 mL/min and the injection volume was 5 µL. The detection was at 260 nm in the photodiode array detector. The UV spectra were recorded with a 200-400 nm range.

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2.5 Antifungal activity

The antifungal activity of the supercritical EEP was investigated against three strains of *Candida glabrata* (RL 02, RL 02 and CG 03). Minimal inhibitory concentration (MIC) of the fraction was determined by the broth microdilution method according to M27- A3 documents determined by the Clinical Laboratory and Standards Institute [17], with RPMI-MOPS (RPMI 1640 medium containing l-glutamine, without sodium bicarbonate – Sigma–Aldrich Co., St. Louis, USA – buffered to pH 7.0 with 0.165 mol/L MOPS buffer – Sigma). The concentrations of the extracts ranged from 1.9 to 500 µg/mL and 100 µL-aliquots were inoculated of a flat-bottom 96-well microtiter. MICs values were defined as the lowest concentration of the samples at which the microorganism tested did not demonstrate visible growth in 48 h. Amphotericin B kindly supplied by Cristália® (Brazil) was used as positive control. The experiments were carried out in triplicate.

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2.6 Mathematic Modelling

A dynamic model was used for the mathematical representation of the supercritical extraction in this study [18]. It is based on a one-dimensional mass balance for the extract, considering the hypothesis of a linear behavior for the solid *vs.* liquid-phase equilibrium relationship.

Supercritical Fluid:

$$\frac{\partial C}{\partial t} = -v\frac{\partial C}{\partial z} - \frac{(1-\varepsilon)}{\varepsilon}\rho\frac{\partial q}{\partial t} \quad (1)$$

Solid phase:

$$\frac{\partial q}{\partial t} = -k_{TM}(q - q') \quad (2)$$

where C is the concentration of extract in the fluid phase and q is the extract mass fraction in the solid matrix, both measured throughout the bed height (z) and time (t), v is the

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interstitial fluid velocity through the bed, ε is the bed porosity, k_{TM} is the overall mass transfer coefficient, ρ is the density of the solid matrix. The phase equilibrium relationship is described by the linear correlation $q'=K.C$, where K is the equilibrium constant.

The model was developed in the Simulator EMSO [19] to solve the system of equations proposed, wherein the system is solved by a multi-stage integrator. The global coefficient of mass transfer (k_{TM}) and equilibrium constant (K) were estimated by weighted least squares method, using a flexible polyhedra optimizer.

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3. Results and discussion

As already described, propolis is not very soluble in supercritical CO_2, but becomes more soluble in CO_2 + ethanol [20]. The impregnation of the EEP in filter papers was performed to remove waxes and resins due to the great difficulty of extraction from the raw propolis, which caused equipment pipe obstruction in previous tests, for its high wax and resins content. The supercritical extracts from EEP impregnated in filter papers showed increased returns in relation to the increase of the pressure. The yields of the tree different SC-CO_2 extracts, submitted at 90, 150 and 300 bar and 40 °C were 8.6%, 11.8% and 39.9%, respectively. When raising the pressure on the process, the solvating power of CO_2 rises due to its density elevation. Consequently, there is an increased ability to solubilize compounds of high molecular mass [21]. The yields were obtained by calculating the ratio of the mass extracts and mass of EEP impregnated on filter sheets. A gain of almost four times in yields of red propolis extract can be observed to 300 bar in relation to other pressure conditions.

3.1 HPLC and UPLC/MS analysis

Brazilian red propolis presents various classes of substances in its composition. The main compounds are isoflavonoids, pterocarpans, chalcones, flavonoids and polyprenylated

benzophenones [22, 23]. Polyprenylated benzophenones have been reported as the most abundant structures present in Brazilian red propolis [22, 23, 24]. Considering the similarity of the HPLC chromatograms obtained by Cabral et al. [25] and Fasolo et al. [11], we postulated that the most lipophilic compounds verified in our chromatograms (150 and 300 bar, retention time of approximately 44 min) were benzophenones (Figure 1). The peak attributed to benzophenones represented 8% and 16% of the extract obtained at 150 and 300 bar, respectively, being present as traces in the 90 bar extract. Thus, in order to identify the compounds, UPLC/MS analyses were performed, by using the methodology previously reported by Fasolo et al. [11]. The peak attributed to benzophenones in the HPLC, as expected, was related to the polyprenylated benzophenones xanthochymol and/or guttiferone E. These compounds are isomers having a difference only in a double bond position (Figure 2), and are usually obtained as an inseparable mixture [11, 22, 23]. The mass spectrum of the peak attributed to xanthochymol and/or guttiferone E is shown in Figure 3.

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Figure 1

Figure 2

Figure 3

3.2 Antifungal activity

In a previous work, Pippi et al. [12] have demonstrated the activity of the *n*-hexane extract of Brazilian red propolis against fluconazole resistant strains of *C. glabrata* and *C. parapsilosis*. As the main compounds of the extract were benzophenones [11], the authors attributed the activity to these compounds. In order to further confirm this assumption, we decide to investigate the activity of the supercritical EEP against *C. glabrata* strains.

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The antifungal activity of supercritical EEP showed results consistent with the chemical analysis (Table 1). The strains of *Candida glabrata* were susceptible to SC-CO_2 extracts of propolis obtained in higher pressures. As noted by the HPLC analysis, the concentration of benzophenone is directly proportional to the pressure of the extraction system. Table 1 shows a significant increase in antifungal activity in accordance with the increase in pressure and concentration of benzophenones.

Table 1

3.3 Mathematical Modelling

Figure 4 shows the experimental and modeling yield *versus* time curves obtained at 90, 150, 300 bar and 40 °C. It is possible to observe that simulation of the extraction process was conducted with high accuracy, which is confirmed by the high values of the determination coefficients (R^2). The determination coefficient values are shown Table 2, as well as the mathematical model parameters.

Figure 4

Table 2

Traditionally, the supercritical fluid extraction behavior *versus* time presents has two stages: the first period is controlled by the phase equilibrium between the extract and the supercritical fluid employed. In the second period the resistance to the solute mass transfer from raw material internal structure is predominant [26, 27]. In this study it was observed that the effect of phase equilibrium is very important during the extraction, as evidenced by the extensive region where the extraction curve shows behavior close to linear. The use of EPP impregnated on filter paper sheets as raw material can justify this behavior, because

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traditionally the raw materials used in supercritical fluid extraction process are vegetable structures and the resistance to the solute mass transfer is related to solute diffusion of within of the plant cells to solvent phase [27, 28].

4 Concluding remarks

It can be observed in the extraction process that the pressure directly influences the chemical composition of the EEP, justifying the highest concentration of benzophenones when using higher pressures. The results of HPLC analyzes demonstrated a strong correlation between the benzophenones and the antifungal activity, whereas the most active fraction was that showed high levels of these compounds.

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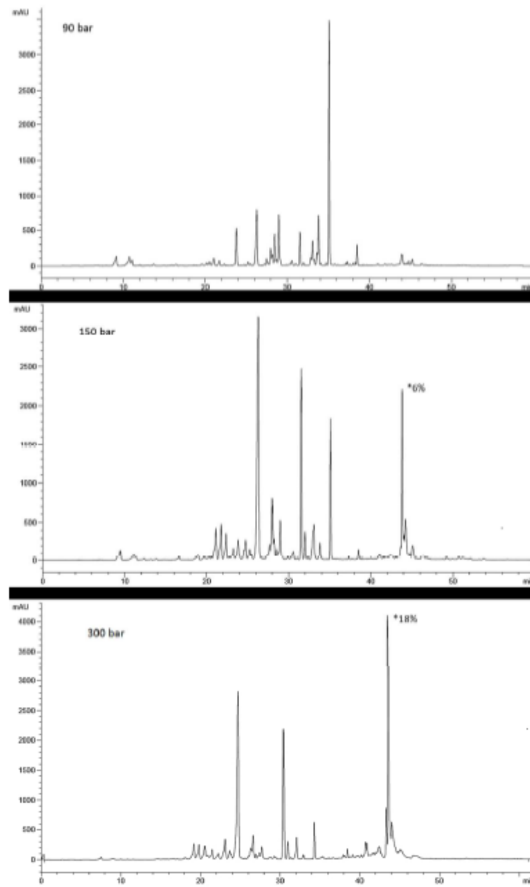

Figure 1. HPLC of SC-CO2 extracts of EEP (*) guttiferone E and/or xanthochymol.
172x290mm (150 x 150 DPI)

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Figure 2. Chemical structures of polyprenylated benzophenones (1) guttiferone E and xanthochymol (2).
712x264mm (96 x 96 DPI)

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Figure 3. Mass spectrum of the peak attributed to guttiferone E and/or xanthochymol.

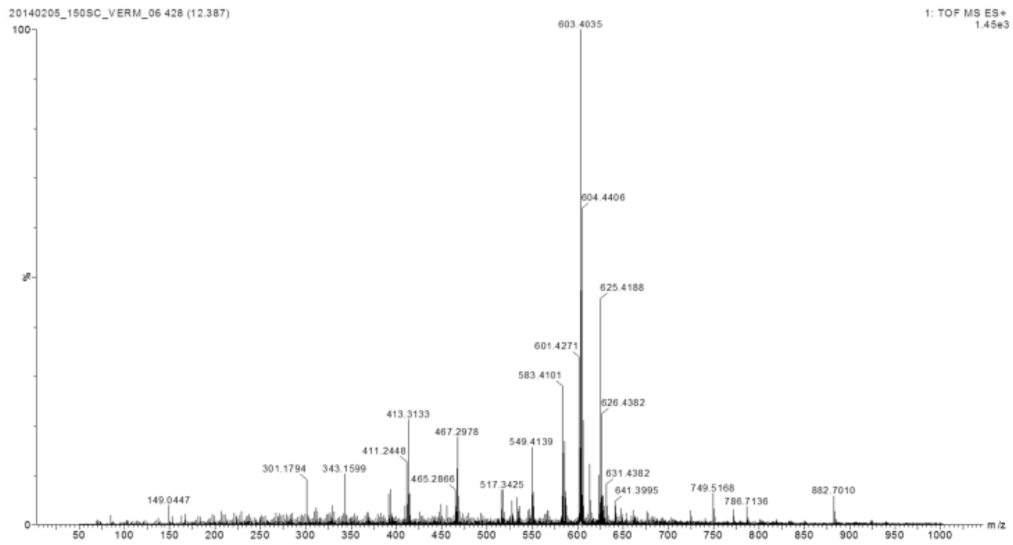

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Figure 4. Supercritical extraction *yields* curves and experimental data *versus* time for EEP obtained at 313.15 K and 300 bar (▲), 150 bar (■), 90 bar (◊) and mathematical model (—).

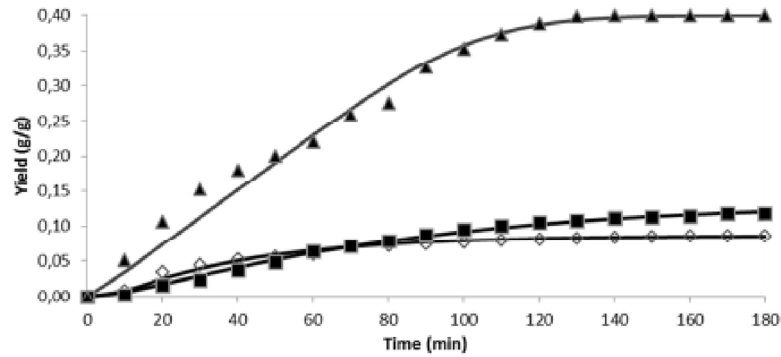

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Table 1. Minimum inhibitory concentration (MIC) of the EEP obtained by supercritical CO_2 against three strains of *Candida glabrata*: RL 02, RL 02, and CG 03.

P(bar)	Minimum Inhibitory Concentration		
	RL02	RL03	CG03
90	Partial inhibition of growth in 500 µg/mL*	Partial inhibition of growth in 500, 250 and 125 µg/mL*	Partial inhibition of growth in 500, 250 µg/mL*
150	62.5 µg/mL	250.0 µg/mL	15.6 µg/mL
300	7.8 µg/mL	125.0 µg/mL	3.9 µg/mL

* Not observed total inhibition of yeast growth, but there was a reduction in growth compared to the positive control.

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Table 2. Parameters of extraction curves fitted to experimental data for the EEP at 40 °C and 90, 150 and 300 bar using supercritical CO_2 as solvent.

P(bar)	Parameters		R^2
	K (m^3kg^{-1})	K_{TM} (s^{-1})	
90	1.174E-04	2.531E-04	0.9865
150	1.010E-03	4.383E-04	0.9978
300	1.020E-03	5.337E-04	0.9934

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