UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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Potenciação da ação de produtos lipofílicos provenientes de espécies de *Hypericum* nativas do sul do Brasil

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Potenciação da ação de produtos lipofílicos provenientes de espécies de *Hypericum* nativas do sul do Brasil

Tese apresentada por Gabriela de Carvalho Meirelles
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RESUMO

Plantas do gênero *Hypericum* (Hypericaceae) são reconhecidas fontes de moléculas com fins terapêuticos. Para espécies nativas do sul do Brasil, atividades como antifúngica e antinociceptiva já foram relatadas, atribuídas principalmente a compostos extraídos em suas frações lipofílicas como derivados de floroglucinol, benzopiranos e benzofenonas. Neste estudo, o potencial sinérgico entre frações lipofílicas de *H. carinatum* e o fármaco fluconazol, frente a fungos leveduriformes emergentes, foi avaliado por duas metodologias distintas: *checkerboard* e isobolograma. Para isolados de *Candida krusei* e *C. famata* o efeito da associação foi superior ao do fármaco isolado. Dessa forma, o perfil de suscetibilidade observado sugere que a fração esteja auxiliando a ação do fármaco. Ainda abordando o potencial terapêutico de espécies de *Hypericum,* a investigação da atividade antinociceptiva (via oral) do benzopirano HP1 de *H. polyanthemum*, quando incorporado em nanoemulsões, foi avaliada. Os resultados demonstraram que HP1 pode ser adequadamente incorporado em nanoemulsões, dada sua solubilidade no núcleo oleoso. Em relação ao efeito antinociceptivo, nanoemulsões contendo HP1 demonstraram o mesmo efeito do composto livre, em magnitude, porém em dose inferior. A redução da dose ativa sugere que uma melhor solubilização do composto possa ter ocorrido quando o mesmo está inserido em nanoemulsões. Nesse contexto, estudos de permeabilidade intestinal *ex vivo* (Ussing chambers) de HP1, na sua forma livre e incorporado em nanoemulsões, foram realizados. Os resultados demonstraram que a permeabilidade intestinal do benzopirano HP1, quando incorporado em nanoemulsões, foi cerca de 4 vezes maior em relação a forma livre. Além disso, experimentos de lipólise *in vitro* mostraram que enzimas presentes no trato gastrointestinal são hábeis em hidrolisar nanoemulsões a espécies coloidais, mais solúveis e facilmente absorvíveis pelas células intestinais. Ainda, a permeabilidade intestinal do benzopirano HP1, na sua forma livre, no sentido absortivo foi maior que no sentido secretório indicando que transportadores ativos estão, ao menos em parte, auxiliando a absorção deste composto pelas células intestinais. Dessa forma, com vistas a elucidar o provável transportador ativo de HP1, dada a semelhança estrutural deste benzopirano com moléculas canabinoides e a relação existente entre os sistemas opioide e canabinoide, a influência deste último na absorção de HP1 foi

investigada. Os resultados demonstraram que o benzopirano HP1 pode estar relacionado ao sistema canabinoide, mas a natureza dessa ligação, seja de transporte, agonismo/antagonismo ou físico-química, não foi possível de ser elucidada. Os resultados obtidos nesta tese são relevantes à medida que espécies de fungos leveduriformes emergentes se mostram cada vez mais resistentes aos fármacos comumente utilizados. Além disso, a importância destes resultados se dá pela viabilidade de incorporação do benzopirano HP1 em nanoemulsões e a capacidade desses sistemas em reduzir a dose ativa no benzopirano HP1 por uma maior solubilização do composto e assim, melhor absorção. Dessa maneira, os resultados deste trabalho representam o alto potencial biológico de espécies de *Hypericum* e abrem possibilidade para mais estudos utilizando estas plantas.

Palavras-chave: absorção, antifúngico, antinociceptivo, HP1, *Hypericum*, nanoemulsão, permeabilidade intestinal, sinergismo.

ABSTRACT

Potentiation of action of lipophilic products from *Hypericum* species native to south Brazil. Plants from genus *Hypericum* (Hypericaceae) are recognized as a source of therapeutical agents. To south Brazil species, acitivities like antifungal and antinociceptive had already been demonstrated, attributed mainly to compounds from lipophilic fractions as phloroglucinol derivatives, benzophenones and benzopyrans. In this study, antifungal potential of lipophilic fractions of *H. carinatum* and fluconazole against emerging yeasts was evaluated by two methodologies for multiple dose-response analyzes: checkerboard and isobologram. To *Candida krusei* and *C. famata* isolates the effect of association was higher than the effect of fluconazole alone. Thus, the susceptibility profile observed for these species suggests that, somehow, the fractions are facilitating the action of drug. Still on therapeutical potential of *Hypericum* species, the antinociceptive study of a benzopyran (HP1) isolated from *H. polyanthemum*, incorporate in nanoemulsions, was evaluated. The results demonstrated that HP1 could be incorporated in a nanoemulsion system, given the high solubility in the oil core. Regarding the antinociceptive effect, HP1 loaded in nanoemulsions showed the same effect of free form, in magnitude, at lower doses. These results suggest a better solubilization of HP1 when loaded in nanoemulsions, and, thus, better absorption by organism. In this context, *ex vivo* intestinal permeability studies (Ussing chambers) of HP1 free form and loaded in nanoemulsions were performed. The results showed that the intestinal permeability of HP1 loaded in nanoemulsions were about 4 times higher than HP1 free form. Besides, the intestinal permeability of HP1 free form in absorptive direction was higher than secretory direction indicating that active transporters are, at least in part, involved in HP1 intestinal absorption. Thus, in order to elucidate the probable active transporter of HP1 and since its structure looks like a cannabinoid molecule and there is a relation between the opioid and cannabinoid pathways, the influence of intestinal cannabinoid system in HP1 absorption was investigated. The results indicated that the benzopyran HP1 may be related to cannabinoid system, but the nature of this interaction: transport, agonism/antagonism or physico-chemical is still unknown. The outcomes obtained are relevant since the resistance of emerging yeast species to available drugs, used for a variety of fungal infections, is increasing. The importance of these findings lies also in the feasibility of incorporating HP1 into nanoemulsions, and the capacity of these systems in reduce the antinociceptive active doses, by higher solubilization, and thus,

absorption. Then, together the results represent the high biological potential of *Hypericum* species and open new possibilities to further studies with these plants.

Keywords: absorption, antifungal, antinociceptive, HP1, *Hypericum*, nanoemulsion, intestinal permeability, sinergism.

SUMÁRIO

1 INTRODUÇÃO

Produtos naturais são uma importante fonte de novos protótipos para o desenvolvimento de fármacos. Alguns estudos indicam que de 1981 a 2014, parte significativa dos medicamentos aprovados pela agência regulatória norte americana (Food and Drug Administration - FDA) eram derivados de produtos naturais (BUTLER, 2008; CRAGG e NEWMANN, 2016), demonstrando que, apesar dos processos atuais serem direcionados a fármacos sintéticos, aqueles de origem natural ainda são fundamentais dada sua elevada diversidade química.

Hypericaceae é uma extensa família botânica, na qual 80% de sua diversidade se deve ao gênero *Hypericum*, com cerca de 500 espécies apresentando constituintes químicos com atividades biológicas diferenciadas (CROCKETT e ROBSON, 2011). A espécie mais estudada, *H. perforatum* nativa da Europa, é comercializada na forma de extrato padronizado para o tratamento de depressão leve a moderada (MÜLLER, 2005).

Evidências científicas apontam para resultados biológicos relevantes envolvendo extratos e substâncias isoladas de espécies de *Hypericum* nativas do Rio Grande do Sul. Dentre estas podemos citar atividades antifúngica (BARROS et al., 2013) e antinociceptiva (VIANA et al., 2003; HAAS et al., 2010; STOLZ et al., 2012) que foram observadas para espécies com forte tendência em acumular compostos fenólicos como flavonoides (DALL´AGNOL et al., 2003), derivados de floroglucinóis (ROCHA et al.,1995; FERRAZ et al., 2002; NÖR et al., 2004), xantonas (ROCHA et al., 1994), entre outros.

BARROS e colaboradores (2013) avaliaram o efeito antifúngico de frações lipofílicas, ricas em derivados de floroglucinol diméricos, de cinco espécies de *Hypericum* nativas do Rio Grande do Sul: *Hypericum carinatum*, *H. caprifoliatum*, *H. linoides*, *H. polyanthemum* e *H. myrianthum*. Os resultados deste trabalho foram bastante promissores principalmente para *H. carinatum*, com indícios de que os derivados diméricos de floroglucinóis e benzofenonas sejam os responsáveis pela atividade antifúngica apresentada. Dessa forma, o presente estudo se direciona a estudos de associação desta fração com o fármaco fluconazol.

Esses estudos são justificáveis a medida que uma gama de fungos, antes não patogênicos, são responsáveis por causar uma série de doenças em humanos, as chamadas infecções oportunistas. Esses fungos denominados emergentes acometem

em maior proporção, pacientes imunossuprimidos (GULLO, 2009). Visto que a patogenicidade dos mesmos ainda é desconhecida, a suscetibilidade a agentes antifúngicos é muito variada, fazendo com que novas alternativas de combate a esses micro-organismos sejam necessárias (CHEN et al., 2010).

Ainda em relação a espécies de *Hypericum* nativas do Rio Grande do Sul, há um crescente interesse no estudo de frações lipofílicas e metabólitos provenientes das mesmas, em especial *H. polyanthemum*, acerca de efeitos sobre o sistema nervoso central. Os primeiros estudos sobre atividade antinociceptiva (VIANA et al., 2003) das espécies nativas começaram há cerca de duas décadas. Os resultados bastante relevantes deram origem a uma série de trabalhos, tanto com frações quanto com produtos isolados (HAAS et al., 2010; STOLZ et al., 2012; 2014).

Estima-se que aproximadamente 20% da população europeia e 31% da norte-americana, em especial mulheres e idosos, sofram de dor (van HECKE et al., 2013). No Brasil, não existem dados concretos sobre a epidemiologia da dor, porém eles se direcionam a seguir a tendência mundial fazendo com que o estudo de novas alternativas terapêuticas contra esse sintoma, que muitas vezes pode ser caracterizado como doença propriamente dita, seja de suma importância. Dessa forma, visto que a dor possui uma natureza pluridimensional, o uso de medicamentos analgésicos com mecanismos de ação distintos, se faz cada vez mais necessário (SBED, 2012).

Em 2010, HAAS e colaboradores evidenciaram ação antinociceptiva pronunciada de um dos principais metabólitos presentes em *H. polyathemum*, o benzopirano HP1. Apesar desta atividade ser bastante importante, a baixa solubilidade do metabólito em água impede que estudos mais aprofundados sobre essa ação sejam realizados. Além disso, a absorção oral de compostos lipofílicos é extremamente influenciada pela solubilidade em água e taxa intrínseca de dissolução (massa do composto dissolvida por unidade de tempo e volume) que irão determinar baixa permeabilidade intestinal e, em última análise, baixa biodisponibilidade (DESAI et al, 2012). Assim, a incorporação em sistemas nanoestruturados surge como uma importante ferramenta para contornar a problemática da solubilidade e avaliar a ação deste benzopirano em modelos de antinocicepção.

Entre os sistemas nanoestruturados destacam-se sistemas de liberação lipídicos que englobam combinações de lipídeos naturais com surfactantes, co-surfactantes e co-solventes (PORTER et al., 2007). Compostos lipofílicos apresentam

taxa de dissolução limitada, o que determina baixa permeação através da membrana do epitélio intestinal. Sendo assim, a incorporação dos mesmos em sistemas de liberação lipídicos faz com que a fase de dissolução não seja necessária visto que os compostos estão apresentados na forma de uma fase coloidal dispersa (CHAKRABORTY et al., 2009). Além disso, a digestão lipídica da formulação aumenta a dispersão da molécula aumentando assim a absorção intestinal (NARANG et al., 2007).

Dessa forma, a incorporação de compostos lipofílicos em sistemas de liberação lipídicos, como nanoemulsões triglicerídicas, é benéfica para o aumento da absorção intestinal de moléculas lipofílicas como o benzopirano HP1.

2 OBJETIVOS

2.1 Objetivo Geral

Avaliar o potencial sinérgico entre a fração lipofílica de *H. carinatum* com o fármaco fluconazol contra fungos leveduriformes emergentes. Além disso, incorporar o benzopirano HP1, de *H. polyanthemum*, em nanoemulsões a fim de verificar a influência da formulação na sua ação antinociceptiva por via oral. Por fim, avaliar a permeabilidade intestinal do benzopirano HP1 na sua forma livre e incorporado em nanoemulsões para elucidar os possíveis mecanismos de absorção intestinal.

2.2 Objetivos Específicos

Capítulo 1

- ✓ Avaliar a capacidade de sinergismo da fração lipofílica em estudo associada ao fármaco mais utilizado no combate de fungos leveduriformes, fluconazol, por duas metodologias:
  - *Checkerboard*;
  - Análise Isobolar.

Capítulo 2

- ✓ Isolar o benzopirano HP1 de *Hypericum polyanthemum*;
- ✓ Desenvolver e caracterizar nanoemulsões contendo HP1 pelo método da emulsificação espontânea;
- ✓ Avaliar o efeito antinociceptivo de HP1 livre e contido em nanoemulsões pelo teste da placa aquecida;
- ✓ Verificar possível dano motor, nas doses ativas, de HP1 livre e contido em nanoemulsões pelo teste do rota-rod;

Capítulo 3

- ✓ Avaliar a permeabilidade intestinal de HP1 livre e incorporado em nanoemulsões pelo modelo de câmaras de Ussing (*ex vivo*);
- ✓ Verificar o possível envolvimento de transportadores ativos na permeabilidade intestinal de HP1 pelo modelo de câmaras de Ussing (*ex vivo*);
- ✓ Verificar o comportamento de nanoemulsões contendo HP1 frente a enzimas pelo método da lipólise *in vitro*;
- ✓ Verificar a influência da camada mucosa que envolve as células intestinais na absorção de HP1, livre e incorporado em nanoemulsões, pelo método da bio-mucoadesão *ex vivo*.

3 Capítulo 1- Avaliação da atividade antifúngica da fração lipofílica de *Hypericum carinatum* e estudos de associação desta fração com fluconazol

Os resultados apresentados neste capítulo foram obtidos nos Laboratórios de Farmacognosia e de Pesquisa em Microbiologia Aplicada sob orientação dos professores **Dr. Gilsane von Poser** e **Dr. Alexandre Meneghello Fuentefria**, respectivamente.

Desde o início dos anos 80, infecções fúngicas têm chamado atenção devido ao alto número de pacientes afetados, principalmente aqueles com o sistema imunológico comprometido (GULLO, 2009; SILVA et al., 2012). Além disso, muitos fungos que antes não causavam morbidade e mortalidade passaram a ser extremamente patogênicos e as infecções causadas pelos mesmos foram denominadas de oportunistas. Nessa gama de micro-organismos oportunistas destacam-se espécies de *Candida*, *Aspergillus* e *Cryptococcus* (NUCCI e MARR, 2005; CDC, 2010; BYRNES et al., 2011; PFALLER et al., 2012).

A terapêutica contra as infecções oportunistas é baseada em fármacos derivados de azóis (ex.: fluconazol, voriconazol) e no derivado poliênico anfotericina B (BARKER et al., 2004; PFALLER et al., 2010; 2014). Quando introduzidos no mercado, estes compostos mostraram alta eficácia como agentes antifúngicos (LEWIS, 2011), porém a constante evolução dos micro-organismos, o uso indiscriminado dos agentes antifúngicos e os efeitos adversos severos levam a necessidade constante de introduzir novos fármacos no mercado e/ou promover a associação de novas substâncias com fármacos já existentes a fim de obter maior eficácia e menores efeitos adversos.

O gênero *Hypericum* engloba várias espécies utilizadas na medicina tradicional (BUSSMANN et al, 2010; van VURREN e NAIDO, 2010) e reconhecidas por apresentarem uma série de atividades biológicas. Dentre estas, podemos citar atividades antifúngica (BARROS et al., 2013; DULGER e DULGER, 2014), antibacteriana (DALL´AGNOL et al., 2003) e antitumoral (JAYASURIYA et al., 1989).

Dessa forma, com base em relatos de atividade antifúngica do gênero *Hypericum* e na necessidade de introduzir alternativas para o tratamento de infecções oportunistas, o presente estudo objetivou avaliar o sinergismo da fração lipofílica de *H. carinatum* com o fármaco fluconazol frente a fungos leveduriformes emergentes.

3.1 O gênero *Hypericum*

Hypericaceae é uma extensa família botânica que compreende nove gêneros*:* *Cratoxylum* Blume, *Eliea* Cambess., *Harungana* Lamarck, *Hypericum* L., *Lianthu*s N. Robson, *Santomasia* N.Robson, *Thornea* Breedlove & McClintock, *Triadenum*

Rafinesque, e *Vismia* Vand. Dentre estes, o que apresenta maior diversidade é o gênero *Hypericum* (CROCKETT e ROBSON, 2011) que compreende várias espécies utilizadas na medicina tradicional, incluindo a espécie mais estudada atualmente, *H. perforatum* L., proveniente da Europa.

Estudos demonstram que extratos de espécies brasileiras de *Hypericum* apresentam atividade antifúngica principalmente em suas frações lipofílicas (FENNER et al., 2005; BARROS et al., 2013). Essas espécies são ricas em floroglucinóis diméricos (uliginosina B, hiperbrasilol B e japonicina A), considerados marcadores taxonômicos das que são encontradas no do sul do Brasil (FERRAZ et al., 2002; NÖR et al., 2004), bem como apresentam outros metabólitos com padrão estrutural de floroglucinóis como as benzofenonas (von POSER et al., 2006). Assim, neste estudo foi escolhida a fração lipofílica de *H. carinatum* (Figura 3.1) em continuação ao trabalho desenvolvido por BARROS e colaboradores (2013).

Figura 3.1 *Hypericum carinatum* Griseb.

3.2 Fungos leveduriformes emergentes

Os fungos leveduriformes emergentes se tornaram relevantes há aproximadamente duas décadas com o aumento do número de casos da Síndrome da Imunodeficiência Adquirida (SIDA), utilização indiscriminada de antimicrobianos de amplo espectro, quimioterapia citotóxica e elevado número de transplantes (SILVA et al., 2010; PFALLER et al., 2012). Outros fatores que contribuíram para o aumento das infecções causadas por fungos emergentes foram enfermidades que debilitam o

sistema imune de pacientes levando a ruptura das barreiras mucosas e cutâneas, defeitos no número e na função dos neutrófilos e na imunidade celular ocasionando assim, disfunções metabólicas (SEGAL et al., 2006).

Entre as espécies emergentes, destacam-se as do gênero *Candida*. *Candida albicans* é a espécie predominante em infecções invasivas (RUHNKE, 2006; HORN et al., 2009; PFALLER et al., 2012). No entanto, dados epidemiológicos têm demonstrado um aumento em infecções por espécies de *Candida* não-*albicans* e outras espécies de fungos leveduriformes menos comuns. As espécies mais comumente encontradas em infecções, após *C. albicans,* são *C. glabrata, C. parapsilosis*, *C. tropicalis* e *C. krusei* (MICELI et al., 2010; LOCKHART et al., 2012; GUINEA, 2006), chamando atenção o alto número de infecções por *C. glabrata* nos Estados Unidos da América e em países da Europa. Porém, dados de outros países como Brasil, demonstram que infecções por *C. glabrata* não são tão comuns, como aquelas provocadas por *C. tropicalis* e *C. parapsilosis* (COLOMBO et al., 2006).

As espécies do gênero *Candida* são geralmente encontradas como comensais nos tratos gastrointestinal e genital de pacientes sadios, todavia se tornam potenciais patógenos em pacientes debilitados causando infecções superficiais e sistêmicas (MICELI et al., 2011). As principais razões da virulência desses micro-organismos são a versatilidade na adaptação a vários habitats diferentes e a formação de biofilmes, os quais aumentam a capacidade de aderência a superfícies facilitando as infecções (RAMAGE et al., 2005).

3.3 Terapêutica contra fungos leveduriformes emergentes

A resistência de espécies de *Candida* não-*albicans* é um dos principais desafios na terapêutica, uma vez que a maioria das espécies oportunistas se mostra menos suscetível aos fármacos comumente utilizados (Quadro 3.1).

Quadro 3.1 Suscetibilidade de algumas espécies de *Candida* não-*albicans* a fármacos azólicos utilizados na terapêutica*

Espécie	Azóis	
	Fluconazol	**Voriconazol**
C. glabrata	Suscetível (dose dependente) a resistente	Suscetível (dose dependente) a resistente
C. tropicalis	Suscetível	Suscetível
C. parapsilosis	Suscetível	Suscetível
C. krusei	Resistente	Suscetível (dose dependente) a resistente

*Adaptada de Miceli e colaboradores, 2010.

O conceito de suscetível ou resistente a certos fármacos é relativo, devido a constante modificação que esses micro-organismos sofrem a fim de se adaptar no combate a esses fármacos. Neste trabalho foram utilizados os *breakpoints* para testes *in vitro* descritos no documento M27-S4 da CLSI (Clinical and Laboratory Standard Institute, EUA), 2012. Esses valores estão apresentados no quadro 3.2.

Quadro 3.2 *Breakpoints* para espécies de *Candida* frente ao fluconazol de acordo com a CLSI, 2012.

Espécie	Fluconazol (CIM)[£]		
	S	**SDD**	**R**
C. albicans	≤2	4	≥8
C. tropicalis	≤2	4	≥8
C. glabrata	-	≤32	≥64
C. parapsilosis	≤2	4	≥2
C. krusei	R	R	R
*C. famata**	0,5	-	≥8

[£]S: Sensível, SDD: Sensibilidade Dose Dependente, R: Resistente. Concentração Inibitória Mínima (CIM) em µg/mL. *Não consta na CLSI, porém há relatos na EUCAST (European Comittee on Antimicrobial Susceptibility Test), pelo método preconizado na CLSI, que esses valores de CIM procedem para 16 isolados avaliados.

A terapêutica utilizada contra espécies de *Candida* segue a cronologia de acordo com a evolução das pesquisas e dos próprios micro-organismos.

Desde sua introdução, em 1958, a anfotericina B tem sido utilizada como tratamento padrão para doenças fúngicas sistêmicas, porém seus efeitos adversos acentuados e a nefrotoxicidade relacionada à dose fizeram com que novas alternativas fossem necessárias objetivando os mesmos efeitos com menores relatos de toxicidade (LEWIS, 2011).

Os primeiros fármacos azólicos introduzidos no mercado (miconazol e cetoconazol) não se mostraram ideais por sua baixa biodisponibilidade e alta toxicidade oral. No entanto com a introdução do fluconazol esses problemas foram, em parte, resolvidos, pois o mesmo apresentou excelente biodisponibilidade oral e farmacocinética linear com ampla distribuição entre tecidos como câmara vítrea ocular e o fluido cerebral espinhal, além da baixa toxicidade (REX et al., 1994). Ainda, esse fármaco também se mostrou efetivo contra candidíase orofaríngea em pacientes com

SIDA (Síndrome da Imunodeficiência Adquirida), embora ainda fosse problemático em tratamentos prolongados (KONTOYIANNIS e LEWIS, 2002). Embora o fluconazol fosse a melhor escolha, ainda apresentava problemas como a inefetividade contra algumas espécies de *Candida* e de *Aspergillus*. Dessa forma, a introdução dos triazóis de amplo espectro, voriconazol (2002) e posaconazol (2006), levou a melhora no tratamento de pacientes imunocomprometidos, sendo mais efetivo do que a anfotericina B, até então utilizada nesses casos (HERBRECHT et al., 2002). Porém, apesar do amplo espectro, esses fármacos possuem alta variabilidade farmacocinética e podem interagir com outros medicamentos.

Por fim, a introdução das equinocandinas, as quais apresentam mecanismo de ação específico para a parede celular fúngica, demonstrou um avanço no quesito efeitos adversos, visto que não danifica a célula humana. Todavia, o baixo espectro de ação ainda constitui um empecilho a sua utilização (LEWIS, 2011). No entanto, o último guia publicado pela IDSA (Infectious Disease Society of America), em 2009, recomenda a associação de equinocandinas com azóis de amplo espectro para o tratamento de candidemias (PAPPAS et al., 2009).

Além de espécies de *Candida*, um micro-organismo emergente que tem atingido vários pacientes é *Cryptococcus neoformans.* A infecção tem ampla distribuição mundial e acomete principalmente pacientes que possuem SIDA, com mais de 80% dos casos associados aos mesmos (COX e PERFECT, 2013). O modo de infecção ocorre, geralmente, por inalação de propágulos do meio ambiente (PERFECT e CASADEVAL, 2002), sendo que as lesões se assemelham ao câncer o que pode dificultar o diagnóstico. Os fármacos de escolha no tratamento dessa infecção são, na maioria dos casos, a combinação de anfotericina B com 5-flucitosina (SAAG et al., 2000). Porém em pacientes não responsivos, já há relatos do uso dos novos fármacos azólicos (FLORES et al., 2012; LOCKHART et al., 2012). Não existem *breakpoints* estabelecidos para a suscetibilidade dessa espécie aos antifúngicos utilizados na terapêutica, portanto este trabalho seguiu valores encontrados em um estudo multicêntrico para as espécies de *C. neoformans* e *C. gattii*, 16 µg/mL (ESPINEL-INGROFF et al., 2012).

Apesar de existirem fármacos novos para o tratamento de infecções fúngicas oportunistas, o fluconazol continua sendo amplamente utilizado na terapêutica por ser

um tratamento acessível e de baixo custo para a maior parte dos países, acarretando um aumento no número de espécies resistentes a este fármaco, e até mesmo, aos fármacos mais novos (RENAME, 2014).

3.4 Terapias combinadas

Em virtude de falhas terapêuticas no combate a infecções causadas por micro-organismos emergentes, o uso de terapias combinadas tem sido uma estratégia para o tratamento de infecções fúngicas sistêmicas (ZARAGOZA e PEMAN, 2012). A possibilidade de ação em múltiplos alvos da célula fúngica significa um grande avanço no estudo de infecções invasivas, visto que a associação de fármacos já existentes com outros não utilizados para este fim ou com produtos naturais pode levar a uma reversão da resistência de determinadas espécies aos compostos já utilizados na terapia antifúngica.

A principal vantagem das terapias combinadas é alcançar o efeito sinérgico utilizando doses mais baixas de cada componente da associação, objetivando não só melhor ação antifúngica, mas também redução da toxicidade (KONTOYIANNIS e LEWIS, 2002). No entanto, essas associações também podem causar efeitos como antagonismo, aditivismo ou indiferença, não justificando o uso da associação (BERENBAUM, 1989; BOUCHER, 2006).

Há diversas técnicas *in vitro* para analisar o tipo de interação entre compostos visando atividade antifúngica. Entretanto, é importante mencionar que essas técnicas podem não representar o comportamento da associação *in vivo* (CUENCA-ESTRELLA, 2004). As técnicas mais utilizadas para analisar interações entre dois compostosa antifúngicos são: método da difusão em disco, *checkerboard*, isobolograma e *time kill*. A maioria dessas técnicas utiliza índices que permitem a classificar o tipo de associação (JOHNSON et al., 2004).

O *checkerboard* consiste na análise da interação entre dois compostos em concentrações múltiplas da sua concentração inibitória mínima (CIM), tanto para mais quanto para menos (JOHNSON et al., 2004). Em contrapartida, o isobolograma leva em consideração curvas concentração-resposta dos compostos em separado a fim de

prever qual a proporção da associação dos mesmos será testada visando melhor ação antifúngica (TALLARIDA et al., 2006) .

Assim, baseado no uso das terapias combinadas, os resultados apresentados nesse capítulo têm por objetivo demonstrar a influência da fração lipofílica de *Hypericum carinatum* na ação antifúngica do fluconazol através de duas metodologias, *checkerboard* e isolobolograma. Os resultados serão apresentados na forma de um manuscrito científico.

MANUSCRITO 1 – Synergistic antifungal activity of the lipophilic fraction of *Hypericum carinatum* Griseb. and fluconazole.

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Original article

Synergistic antifungal activity of the lipophilic fraction of *Hypericum carinatum* and fluconazole

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Abstract

Hypericum species, Hypericaceae, are recognized as a source of therapeutical agents. Purified fractions and isolated compounds have been shown antimicrobial activity. As the indiscriminate use of antifungals and the increase of infections caused by emerging species are leading to the search of new alternative treatments, the aim of this study was to continue the study with *Hypericum carinatum* Griseb. lipophilic fraction, rich in phloroglucinol derivatives, investigating the effect of its association with fluconazole against emerging yeasts (*Candida krusei*, *C. famata*, *C. parapsilosis* and *Cryptococcus neoformans*). The synergistic activity between *H. carinatum* lipophilic fraction and fluconazole was assessed by two methodologies for multiple dose-response analysis: checkerboard and isobologram. Regarding synergistic experiments, the effect of the association was higher than the effect of fluconazole alone against *Candida krusei* and *C. famata* isolates (MIC fluconazole decreased about eight and four folds, respectively), suggesting that, somehow, *H. carinatum* lipophilic fraction compounds

are facilitating the action of this drug. On the other hand, when tested against *Cryptococcus neoformans* and *C. parapsilosis*, fluconazole showed better results than the association. Thus, against *Candida krusei* and *C. famata*, the lipophilic fraction of *H. carinatum* was able to reduce the MIC values of fluconazole and could be considered as a potential alternative to be used against emerging yeast species.

Keywords: Candida, checkerboard, fluconazole, *Hypericum carinatum*, isobologram, synergistic activity

Introduction

Fungal infections are associated with high morbidity and mortality rates. In the last decades, emerging fungal infections, also called opportunistic infections, have drawn attention due to the high number of immunocompromised patients affected (Silva et al., 2012). Some species of *Candida* and *Cryptococcus*, previously considered nonpathogenic, are now recognized as opportunistic pathogens responsible for deep-seated mycoses (Vandeputte et al., 2012; Alcazar-Fuoli and Mellado, 2014).

The high incidence of infection by *Candida* species is due to many factors such as imunossupressive therapies, invasive surgical procedures and use of broad-spectrum antibiotics (Pfaller et al., 2012). *Candida albicans* is still the most prevalent species but infections caused by non-*Candida albicans* (NCA) have significantly increased, bringing even more worrying scenario due to high resistance to antifungal exhibited by these microorganisms (Pfaller et al., 2010; 2012). Since the epidemiology of these fungal infections is currently changing, new alternatives are needed in case of antifungal therapy failure (Alcazar-Fuoli and Mellado, 2014).

Because of yeasts inconstant susceptibility profiles and lack of different molecular targets, drug combinations appear as a strategy for therapy due to the multiplicity of targets (Musiol et al., 2014). The main advantage of these combinations is the synergistic interaction, in which the antifungal activity is better than the individual effects of each compound.

Plants from genus *Hypericum*, Hypericaceae, are an important source of therapeutic agents. Purified fractions and isolated compounds have shown antibacterial and antifungal activities (Barros et al., 2013; Dulger and Dulger, 2014). Barros et al. (2013) have reported the antifungal activity of lipophilic extracts of five *Hypericum* species (*H. carinatum*, *H. caprifoliatum*, *H. linoides*, *H. myriathum* and *H.*

polyanthemum) against several emerging fungal strains, with better results for *H. carinatum*. According to these authors, dimeric phloroglucinol derivatives (uliginosin B, hyperbrasilol B and japonicin A), present in lipophilic fractions could be responsible for the antifungal activity showed by *Hypericum* species. Other compounds with phloroglucinol pattern such as benzopyrans and benzophenones also showed antifungal activity.

Due to the indiscriminate use of antifungals and the increase of infections caused by emerging species new alternative treatments are necessary. Thus, the aim of this work was to continue the study with *Hypericum carinatum* Griseb. lipophilic fraction (LF), investigating the effect of its association with fluconazole against the emerging yeasts *Candida krusei*, *C. famata*, *C. parapsilosis* and *Cryptococcus neoformans*. The synergistic activity between LF and fluconazole was assessed by two methodologies for multiple dose-response analysis: checkerboard and isobologram.

Materials and methods

Plant material

Aerial parts of *Hypericum carinatum* Griseb., Hypericaceae, were collected in Rio Grande do Sul, Brazil, in December of 2009. Voucher specimens are deposited in the herbarium of Federal University Rio Grande do Sul (ICN). Plants collection was authorized by IBAMA (Brazilian Institute of Ambient Media and Renewable Natural Resources) (nº 003/2008, protocol: 02000.001717/1008-60).

Lipophilic fraction preparation

The dried and powdered plant material (*ca*. 500 g) was extracted with hexane at room temperature. The extract was pooled, evaporated to dryness under reduced pressure, and the epicuticular waxes were removed by acetone treatment. The lipophilic fraction (LF) was stored at −20 °C until biological and chemical evaluation.

LF was analyzed by HPLC using a Shimadzu 600 pump (LC-6AD) and a Shimadzu SPD-10A dual absorbance detector. The separations were carried out with an isocratic solvent system (60% acetonitrile: 40% water) to benzophenones determination and (95% acetonitrile, 5% water, 0.01% trifluoroacetic acid) to phloroglucinol derivatives using a Waters Nova-Pack C_{18} column (4 µm, 3.9 mm x 150 mm) adapted to a Waters Nova-Pack C_{18} 60 Å (3.9 mm x 20 mm) guard column. The

flow rate was 1 ml/min, the detector sensitivity was 1.0 Aufs, and the detection was performed at 270/220 nm at room temperature.

Constituents were identified by comparison with the retention times of the authentic samples and co-injection of isolated compounds. The yields were expressed in % (weight compound per weight dry extract) as mean of two injections.

LF toxicity

The experimental protocol was approved by Local Ethical Committee (Protocol 23081, UNIPAMPA). The toxicity of LF was evaluated by cell viability test and comet assay, according to Güez et al. (2012), analyzing three different fraction concentrations: 500, 250 and 100 µg/ml.

Fungal strains

Four resistant strains to fluconazole were used in this study. Interpretative criteria of resistance were used according to breakpoints from M27-S4 document (CLSI, 2012) to *Candida* and according to Espinel-Ingroff et al. (2012) to *Cryptococcus neoformans*. All strains are deposited in the Mycology Collection of Federal University of Rio Grande do Sul, Brazil: *Candida famata* (RL23) originates from hemoculture, *C. krusei* (CK03) from National Program of Quality Control, *C. parapsilosis* (RL11) from urine and *Cryptococcus neoformans* (HCCRY 01) from environment (environmental pathogenic). *C. krusei* ATCC 6258 was included as control in the susceptibility testing.

Antifungal activity

The screening for antifungal activity was carried out with a concentration of 500 µg/ml. In order to achieve the test concentration, samples were solubilized with dimethyl sulfoxide 2% (DMSO) and sabouraud dextrose broth (SDB). Further, the minimal inhibitory concentration (MIC) was determined by the broth microdilution method according to M27-A3 protocol (CLSI, 2008). The MIC was defined as the lowest concentration of LF in which the microorganism tested did not demonstrate visible growth. In microdilution experiments, samples were solubilized with DMSO 2% and RPMI-MOPS medium (RPMI 1640 medium containing L-glutamine, without sodium bicarbonate buffered to pH 7.0 with 0.165 mol/l of MOPS buffer. The

concentrations of LF ranged from 1.9 to 500 µg/ml and all experiments were carried out in duplicate. Control with DMSO 2% was previously performed.

Association studies

Checkerboard assay

The effect of fluconazole combined with LF was evaluated in quadruplicate using the checkerboard method (Johnson et al., 2004) with slightly modifications. The fluconazole final concentrations ranged from 0.5 to 32 µg/ml for *C. famata* and *C. neoformans*, and 4 to 64 µg/ml for *C. krusei* and *C. parapsilosis.* On the other hand, the concentration of LF ranged from 31.25 to 250 µg/ml for *C. famata* and *C. neoformans* and 4 to 250 µg/ml for *C. krusei* and *C. parapsilosis*. Plates were incubated at 37 ºC for 48 h and then, the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used to assess the fungal cell viability. Interaction was evaluate algebraically by determining the fractional inhibitory concentration index (FICI) defined as the sum of the MIC of each drug in combination, divided by the MIC of the drug used alone. An FICI ≤ 0.5 is considered synergistic; > 0.5 and ≤ 1 additive; >1 and ≤ 4 indifferent, and > 4 antagonistic (Kontoyiannis and Lewis, 2003).

Isobologram

The isobologram was performed with the association of LF and fluconazole against *C. krusei* (CK03) and *C. parapsilosis* (RL11).

A curve concentration-effect of LF or fluconazole was determined with logarithmic concentrations, in order to obtain the IC_{50} (Inhibitory Concentration 50%) by non-linear regression. Then, with these results, curves concentration-effect of association were also performed by non-linear regression (Tallarida, 2006; 2007). The proportion of combinations is demonstrated in Table 1.

Table 1. Proportion of combinations used in isobologram studies

Yeasts Strains	**Fluconazole concentration (%IC_{50})**	**LF concentration (%IC_{50})**
C. krusei CK03	50	50
	25	25
	12.5	12.5
	6.25	6.25
	3.125	3.125
	70	30
	35	15
	17.5	7.5
	8.75	3.75
	4.38	1.875
	2.19	0.938
	1.095	0.496
C. parapsilosis RL11	50	50
	25	25
	12.5	12.5
	6.25	6.25
	3.125	3.125
	70	30
	35	15
	17.5	7.5
	8.75	3.75
	4.38	1.875
	2.19	0.938
	1.095	0.496

C. parapsilosis RL11: IC_{50} Fluconazole: 26.55 µg/ml and IC_{50} LF: 174.7 µg/ml; *C. krusei* CK03: IC_{50} Fluconazole: 35.58 µg/ml and IC_{50} LF: 35.76 µg/ml.

Theoretical additive curves (IC_{50} add) were calculated to each combination according the equation:

Conc. add = *f* x Conc. fluconazole + (1-*f*) x Conc. Fraction

where, Conc. Fluconazole and Conc. Fraction represent the equi-effective concentration of each treatment alone and *f* is the fraction of each sample that composes the active concentration of association (in this study two *f* values 0.5 (50:50) and 0.7 (70:30) were used). Conc. add is the total concentration and it variance add was calculated by this equation:

$$\text{Var } IC_{50} \text{ add} = f^{*}2 \times \text{Var } IC_{50} \text{ fluconazole} + (1-f)^{*}2 \times \text{Var } IC_{50} \text{ fraction}$$

From these variances, confidence intervals were calculated according the proportion of each sample in the association. Besides, the interaction magnitude was calculated through interaction index (γ), following the formula:

$$\gamma b = \text{dose fluconazole } IC_{50 \text{ mixture}} + \text{dose fraction } IC_{50 \text{ mixture}}$$

The interaction index is an indicator of the potency of the association. Values next to 1 indicate additive interaction; values higher than 1, antagonistic interaction, and values lower than 1, synergistic interaction (Grabovsky and Tallarida, 2004).

Statistical analysis

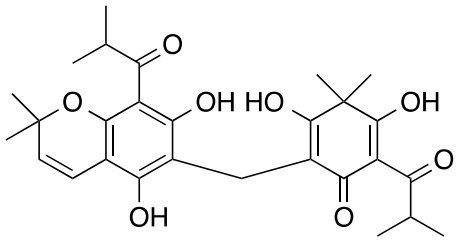
Checkerboard and toxicity data were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's test (Sigma Stat 3.2 software, Jandel ScientificCorporation®). In checkerboard, the difference between antifungal activity of fluconazole alone and in combination with LF was evaluated. Differences were considered statistically significant at $p < 0.05$. The isobologram data were performed with Student t test, where IC_{50} mixture is significantly shorter than IC_{50} calculated (IC_{50} add) to a determined combination, there is a synergistic interaction (Codd et al., 2008). The non-linear regression analysis was performed using GraphPad Prism® version 4.02.

Results and Discussion

Chemical analysis

HPLC analysis were carried out to quantify the major constituents of LF. As demonstrated by Barros et al. (2013), the main constituents of *H. carinatum* lipophilic fraction are the phloroglucinol derivative uliginosin B (**1**) (1.65 ± 0.08%) and the

benzophenones cariphenone A (**2**) (0.08 ± 0.001%) and cariphenone B (**3**) (0.58 ± 0.009%), confirming the previous results.



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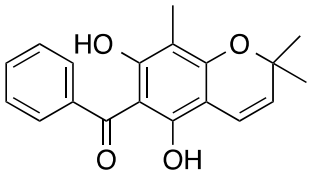

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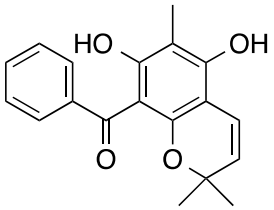

3

LF toxicity

The investigated fraction (LF) did not show toxic effects at the concentration used (250 µg/ ml) in association studies as demonstrated in the Fig.1. According to these results, the concentration of 500 µg/ml showed DNA damage (Fig.1A) as well as reduced cellular viability (Fig.1B). Therefore, the higher LF concentration used at this study (250 µg/ml) is considered safe by these two toxicity methodologies.

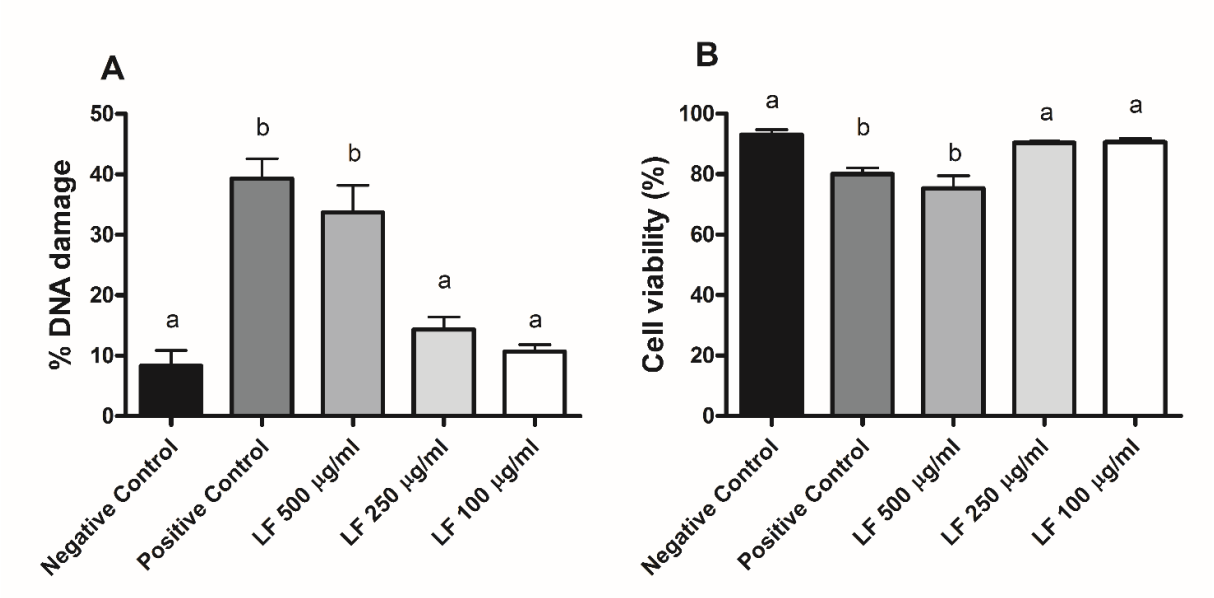

Fig. 1. (A) DNA damage index determined by comet assay and (B) Cell viability in leucocytes for *H. carinatum* lipophilic fraction (LF) in three different concentrations. Phosphate buffered saline (PBS) was used as negative control and hydrogen peroxide (10 µM) (H_2O_2) as positive control in both experiments. DMSO 2% was used as diluent control in these assays. Vertical bars are mean ± SD of three different replicates. Different letters represents significant differences at $p<0.05$ (Tukey test).

Antifungal activity

Concerning the antifungal capacity, LF was capable of inhibit the fungal growth in a moderate way (Table 2). This capacity may be attributed to the presence of dimeric phloroglucinol derivatives as uliginosin B (**1**) and the benzophenones cariphenone A (**2**) and cariphenone B (**3**). These results are in accordance with those described by Barros et al. (2013).

Table 2. Minimal Inhibitory Concentration (MIC) of *Hypericum carinatum* lipophilic fraction (LF) against emerging yeasts strains

Species	Strains	MIC[a]LF
Candida famata	*RL23*	250
Candida krusei	*CK03*	>1000
Candida parapsilosis	*RL11*	250
Cryptococcus neoformans	*HCCRY01*	125

[a]MIC (µg/ml): Minimal inhibitory concentration

Association studies

The results obtained in the checkerboard analysis (Fig. 2) are interesting, since LF was capable of reduce the fluconazole MIC values for all species tested. For *C. neoformans, C. krusei* and *C. parapsilosis* the fluconazole MIC decreased about eight fold (% Cell damage = 75.6%, ICIF = 0.375; % Cell damage = 91.2%, ICIF = 0.25 and % Cell damage = 71.3%, ICIF = 0.5, respectively), while for *C. famata* this value was about four fold (% Cell damage = 94.4%, ICIF = 0.5). Nevertheless, for *C. neoformans* and *C. parapsilosis*, the fluconazole MIC was capable of achieve a higher cell damage in comparision with association. Therefore, the use of the combinations is only justified when decrease of drug dose is needed, especially in cases where the microorganisms are resistant to this azole.

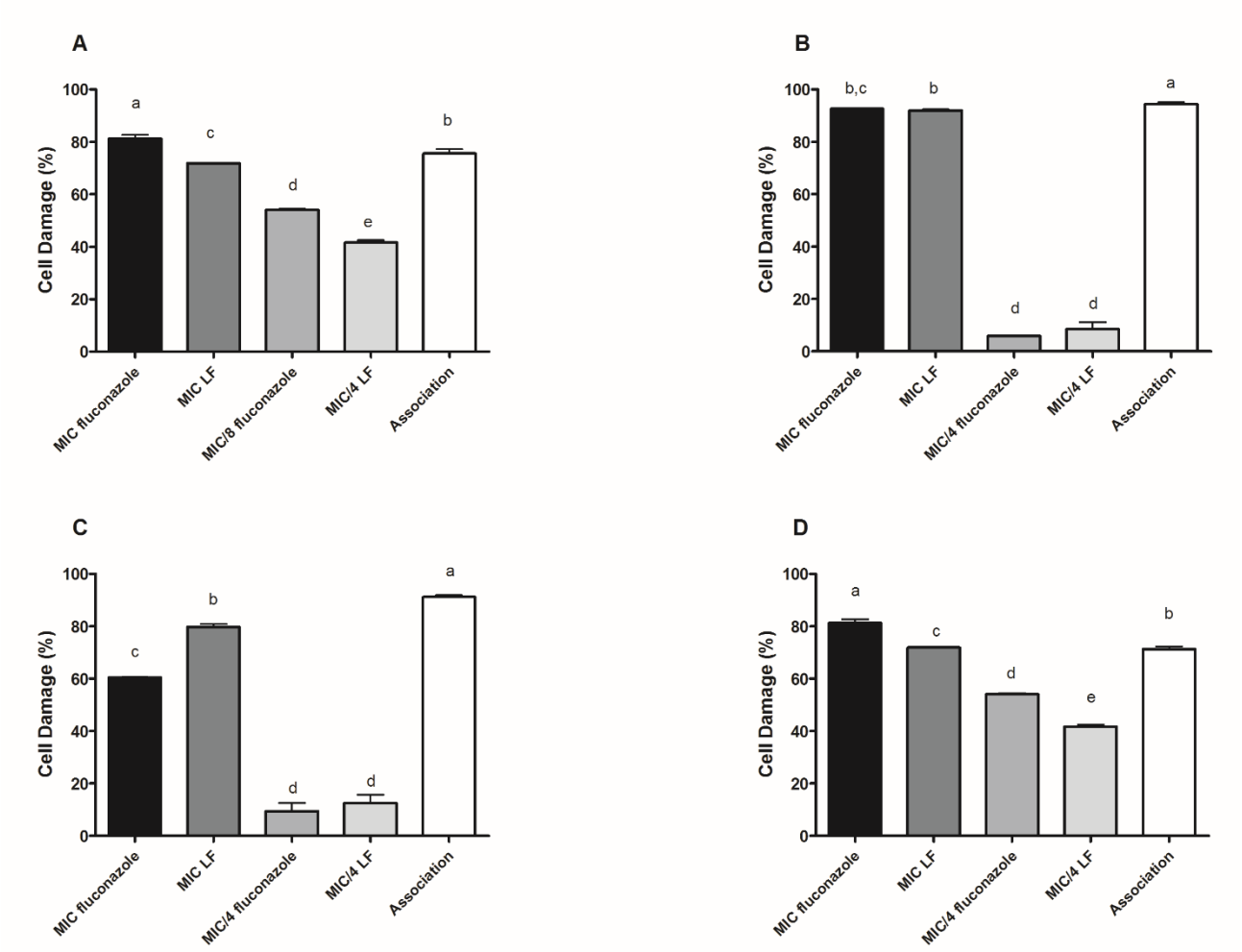

Fig. 2 Interaction between the lipophilic fraction of *H. carinatum* (LF) and fluconazole against *C. neoformans* HCCRY01 (MIC_{Fluco}=32 µg/ml, MIC_{LF}=250 µg/ml, Association=$MIC/8_{Fluco}$:$MIC/4_{LF}$) (A), *C. famata* RL 23 (MIC_{Fluco}=8 µg/ml, MIC_{LF} =250 µg/ml, Association=$MIC/4_{Fluco}$:$MIC/4_{LF}$) (B), *C. krusei* CK03 (MIC_{Fluco}=32 µg/ml, MIC_{LF}>250 µg/ml, Association=$MIC/4_{Fluco}$:$MIC/4_{LF}$) (C) and *C. parapsilosis* RL 11 (MIC_{Fluco}=32 µg/ml, MIC_{LF} =250 µg/ml, Association=$MIC/8_{Fluco}$:$MIC/4_{LF}$) (D) by checkerboard method, stained with tetrazolium salt MTT. Vertical bars are mean ± SD of four different replicates. Different letters represents significant differences at p<0.05 (Tukey test).

Concerning the isobologram analysis the curves concentration effect of each compound tested (fluconazole and LF) showed IC_{50} values of 35.58 µg/ml and 35.76 µg/ml for *C. krusei* and 26.55 µg/ml and 174.7 µg/ml for *C. parapsilosis*, respectively. It is important to note that this methodology was not applied to *C. famata* and

Cryptococcus neoformans due to the impossibility of to construct dose response curves with fluconazole alone.

The results obtained in the isobologram (Fig. 3), are in agreement with those obtained the checkerboard analysis, where synergistic effect was found to both species tested (*C. krusei* CK 03 and *C. parapsilosis* RL11). The interaction index (γ) was less than 1 for all proportions tested for *C. krusei* ($\gamma_{50:50}$=0.36; $\gamma_{70:30}$= 0.57) and for *C. parapsilosis* ($\gamma_{50:50}$=0.79; $\gamma_{70:30}$= 0.75). However, this index against *C. parapsilosis* was closer to 1, indicating a probable presence of additive effect instead of synergistic, corroborating with the ICIF (0.5) found for this association in the checkerboard analysis.

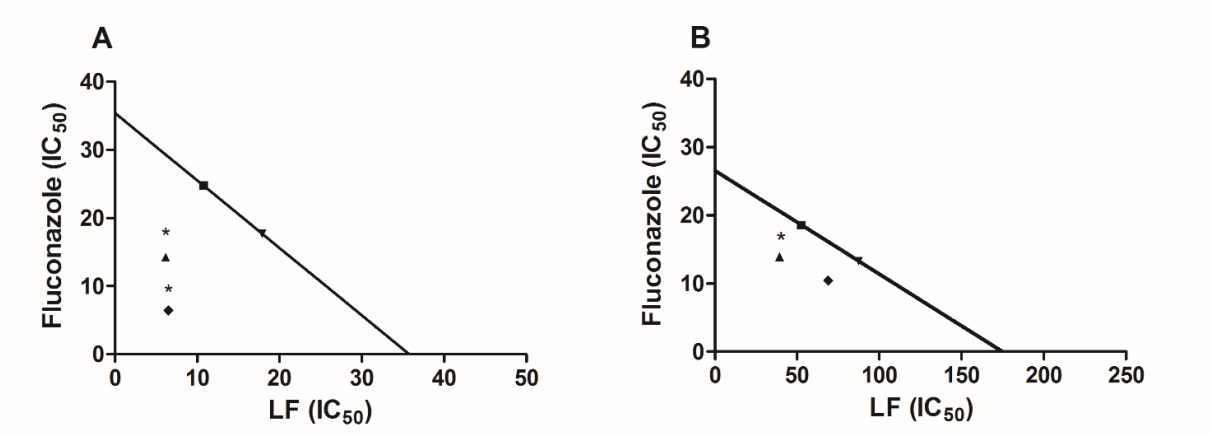

Fig. 3 Interaction analysis of fluconazole with the lipophilic fraction of *H. carinatum* (LF) (IC_{50}) against *C. krusei* (CK03) (A) and *C. parapsilosis* (RL11) (B). The continuous line represents the additivity line and the points the experimental combinations at different levels. * represents significant differences between DeqADD (calculated) and Deqmix (experimental) with p<0.05. (■) Additive Equieffective Concentration (70:30), (▲) Concentration equi-effective of the association (70:30), (▼) Additive Equi-effective Concentration (50:50) and (◆) concentration equi-effective of the association.

The increased incidence of systemic infections caused by NCA species and the high mortality rates due to acquired resistance against drugs current utilized is worrisome, as well as the high incidence of polymicrobial fungal infections (Ruhnke, 2014; Trifilio et al., 2015). Therefore, the association between different compounds could be an excellent strategy to reduce the drug doses, and thus, achieve the resistance reversion.

There are two hypotheses to lipophilic fraction of *H. carinatum* decreases the MIC of fluconazole. The first could be related to the general action mechanism of phenolic compounds, change the fungal dimorphism (Zhang et al., 2011) and/or opening of membrane ionic channels (Rao et al., 2010) both found for *C. albicans*. The second hypothesis lies in the fact that some benzophenones are able to block the cytochrome P-450 (Podust et al., 2007). Nevertheless, since it is a fraction, the synergistic effects of the bioactive compounds mixture could be responsible by increase the effectivity of itself, and then, the antifungal effect is achieved by a sum of mechanisms (Wagner, 2011)

Some studies report association between extracts and antifungal drugs such as essential oils in association with ketoconazole against several fungal species (Giordani et al., 2004) and benzophenone enriched fraction from Brazilian red propolis with fluconazole and anidulafungin against *C. parapsilosis* and *C. glabrata* (Pippi et al., 2015). On the other hand, many studies have demonstrated the association between plant metabolites and antifungal drugs against *Candida* species. For example, the association of the tannin punicalagin and fluconazole against *C. albicans* and *C. parapsilosis* (Endo et al., 2010) and flavonoids (catechin, quercetin and epigallocatechin gallate) associate with fluconazole against *C. tropicalis* (Da Silva et al., 2014).

There are no doubts that combined therapy between LF and fluconazole is benefic, but further studies must be performed in order to determine the nature of this interaction. The analysis of isolated compounds of this fractions alone and/or combined with fluconazole is needed aiming to standardize this association in cases where the monotherapy with fluconazole is ineffective.

Conclusion

The results of this study reinforce the use of *Hypericum* species as source of products with biological importance. Association studies are very significant, especially in emerging fungi, which are worldwide distributed and frequent causes of infections in immunocompromised patients. The lipophilic fraction of *H. carinatum* was able to reduce the MIC of fluconazole, probably by facilitating the access of the drug within the fungal cell. These results are important due to the increasing resistance of emerging yeast species to available drugs used for a variety of fungal infections and the exploration of potential alternative therapeutic sources for multidrug therapy.

Authors' contributions

GCM (PhD student) contributed in fraction preparation, chemical characterization, biological studies (antifungal activity and association studies), analysis of data and drafted the paper. BP contributed to biological studies (antifungal activity and association studies – checkerboard) and critical reading of manuscript, CH contributed to biological studies (antifungal activity), FMCB contributed to chemical characterization, LFSO contributed to toxicity studies, GLVP and AMF supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

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Conflict of interest

The authors have no conflict of interest to declare.

References

Alcazar-Fuoli, L., Mellado, E., 2014. Current status of antifungal resistance and its impact on clinical practice. Br. J. Haematol. 166, 471-484.

Barros, F.M.C., Pippi, B., Dresch, R.R., Dauber, B., Luciano, S.C., Apel, M.A., Fuentefria, A.M., von Poser, G.L., 2013. Antifungal and antichemotactic activities and quantification of phenolic compounds in lipophilic extracts of *Hypericum* spp. native to South Brazil. Ind. Crops Prod. 44, 294-299.

CLSI M27 – A3, 2008. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts: Third Informational Supplement. Wayne, PA, USA: Clinical and Laboratory Standards Institute.

CLSI M27-S4, 2012. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts: Fourth Informational Supplement. Wayne, PA, USA: Clinical and Laboratory Standards Institute.

Codd, E.E., Martinez, R.P., Molino, L., Rogers, K.E., Stone, D.J., Tallarida, R.J., 2008. Tramadol and several anticonvulsants synergize in attenuating nerve injury-induced allodynia. Pain 134, 254-262.

Da Silva, C.R., De Andrade Neto, J.B., De Sousa Campos, R., Figueiredo, N.S., Sampaio, L.S., Magalhães, H.I.F., Cavalcanti, B.C., Gaspar, D.M., De Andrade,

G.M., Lima, I.S.P., De Barros Viana, G.S., De Moraes, M.O., Lobo, M.D.P., Grangeiro, T.B., Nobre, H.V., 2014. Synergistic effect of the flavonoid catechin, quercetin, or epigallocatechin gallate with fluconazole induces apoptosis in *Candida tropicalis* resistant to fluconazole. Antimicrob. Agents Chemother. 58, 1468-1478.

Dulger, G., Dulger, B., 2014. Antifungal activity of *Hypericum havvae* against some medical *Candida* yeast and *Cryptococcus* species. Trop. J. Pharm. Res. 13, 405-408.

Endo, E.H., Garcia Cortez, D.A., Ueda-Nakamura, T., Nakamura, C.V., Dias Filho, B.P., 2010. Potent antifungal activity of extracts and pure compound isolated from pomegranate peels and synergism with fluconazole against *Candida albicans*. Res. Microbiol. 161, 534-540.

Giordani, R., Regli, P., Kaloustian, J., Mikaïl, C., Abou, L., Portugal, H., 2004. Antifungal effect of various essential oils against *Candida albicans*. Potentiation of antifungal action of amphotericin B by essential oil from *Thymus vulgaris*. Phyther. Res. 18, 990-995.

Grabovsky, Y., Tallarida, R.J., 2004. Isobolographic analysis for combinations of a full and partial agonist: curved isoboles. J. Pharmacol. Exp. Ther. 310, 981-986.

Güez, C.M., Waczuk, E.P., Pereira, K.B., Querol, M.V.M., da Rocha, J.B.T., de Oliveira, L.F.S., 2012. *In vivo* and *in vitro* genotoxicity studies of aqueous extract of *Xanthium spinosum*. Braz. J. Pharm. Sci. 48, 461-467.

Johnson, M.D., Macdougall, C., Ostrosky-zeichner, L., Perfect, J.R., Rex, J.H., 2004. Minireview Combination antifungal therapy. Antimicrob. Agents Chemother. 48, 693-715.

Kontoyiannis, D.P., Lewis, R.E., 2003. Combination chemotherapy for invasive fungal infections: What laboratory and clinical studies tell us so far. Drug Resist. Updat. 6, 257-269.

Musiol, R., Mrozek-Wilczkiewicz, a, Polanski, J., 2014. Synergy against fungal pathogens: working together is better than working alone. Curr. Med. Chem. 21, 870-893.

Pfaller, M. A., Andes, D., Diekema, D.J., Espinel-Ingroff, A., Sheehan, D., 2010. Wild-type MIC distributions, epidemiological cutoff values and species-specific clinical breakpoints for fluconazole and *Candida*: Time for harmonization of CLSI and EUCAST broth microdilution methods. Drug Resist. Updat. 13, 180-195.

Pfaller, M., Neofytos, D., Diekema, D., Azie, N., Meier-Kriesche, H.U., Quan, S.P., Horn, D., 2012. Epidemiology and outcomes of candidemia in 3648 patients: Data from the prospective antifungal therapy (PATH Alliance®) registry, 2004-2008. Diagn. Microbiol. Infect. Dis. 74, 323-331.

Pippi, B., Lana, A.J.D., Moraes, R.C., Güez, C.M., Machado, M., de Oliveira, L.F.S., Lino von Poser, G., Fuentefria, A.M., 2015. *In vitro* evaluation of the acquisition of resistance, antifungal activity and synergism of Brazilian red propolis with antifungal drugs on *Candida* spp. J. Appl. Microbiol. 118, 839-850.

Podust, L.M., von Kries, J.P., Eddine, A.N., Kim, Y., Yermalitskaya, L. V., Kuehne, R., Ouellet, H., Warrier, T., Altekös ter, M., Lee, J.S., Rademann, J., Oschkinat, H.,

Kaufmann, S.H.E., Waterman, M.R., 2007. Small-molecule scaffolds for CYP51 inhibitors identified by high-throughput screening and defined by X-ray crystallography. Antimicrob. Agents Chemother. 51, 3915-3923.

Rao, A., Zhang, Y., Muend, S., Rao, R., 2010. Mechanism of antifungal activity of terpenoid phenols resembles calcium stress and inhibition of the TOR pathway. Antimicrob. Agents Chemother. 54, 5062-5069.

Ruhnke, M., 2014. Antifungal stewardship in invasive *Candida* infections. Clin. Microbiol. Infect. 20 Suppl 6, 11-18.

Silva, S., Negri, M., Henriques, M., Oliveira, R., Williams, D.W., Azeredo, J., 2012. *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: Biology, epidemiology, pathogenicity and antifungal resistance. FEMS Microbiol. Rev. 36, 288-305.

Tallarida, R.J., 2007. Interactions between drugs and occupied receptors. Pharmacol. Ther. 113, 197-209.

Tallarida, R.J., 2006. An overview of drug combination analysis with isobolograms. J. Pharmacol. Exp. Ther. 319, 1-7.

Trifilio, S., Zhou, Z., Fong, J.L., Zomas, A., Liu, D., Zhao, C., Zhang, J., Mehta, J., 2015. Polymicrobial bacterial or fungal infections: incidence, spectrum of infection, risk factors, and clinical outcomes from a large hematopoietic stem cell transplant center. Transpl. Infect. Dis. 17, 267-274.

Vandeputte, P., Ferrari, S., Coste, A.T., 2012. Antifungal resistance and new strategies to control fungal infections. Int. J. Microbiol. 2012, 1-27.

Wagner, H., 2011. Synergy research: Approaching a new generation of phytopharmaceuticals. Fitoterapia 82, 34-37.

Zhang, L., Chang, W., Sun, B., Groh, M., Speicher, A., Lou, H., 2011. Bisbibenzyls, a new type of antifungal agent, inhibit morphogenesis switch and biofilm formation through upregulation of DPP3 in *Candida albicans*. PLoS One 6, 1-8. doi:10.1371/journal.pone.0028953

4 Capítulo 2- Atividade antinociceptiva de nanoemulsões contendo o benzopirano HP1 de *Hypericum polyanthemum*

Os resultados apresentados neste capítulo foram obtidos nos Laboratórios de Farmacognosia, Desenvolvimento Galênico, Psicofarmacologia Experimental, sob orientação dos professores **Dra. Gilsane Lino von Poser**, **Dr. Helder Ferreira Teixeira** e **Dra. Stela Maris Kuze Rates**, respectivamente.

A Associação Internacional de Estudos da Dor define dor como “uma experiência sensorial e emocional desagradável associada a um dano real ou potencial dos tecidos ou descrita em termos de tais lesões” (IASP, 2014). A dor possui dois principais componentes: a sensação dolorosa e a reatividade emocional a dor (LOESER e TREEDE, 2008). Em modelos animais apenas a sensação dolorosa é analisada.

Estima-se que 1 em cada 5 adultos sofra de dor e que 1 em cada 10 seja diagnosticado com dor crônica anualmente (IASP, 2014). Essa enfermidade afeta todas as populações sem discriminar idade, sexo, raça ou geografia. Além disso, pode ser caracterizada como aguda, crônica, intermitente ou uma combinação das três. A dor pode ter sérias consequências como depressão, inabilidade de manter relações sociais e profissionais e possíveis pensamentos suicidas. Dessa forma, é imperativo que o tratamento da dor seja feito de uma forma rápida e eficaz (GOLDBERG e McGEE, 2011).

Há vários medicamentos utilizados para o tratamento da dor e a escolha se dá com base no tipo e intensidade da mesma. Apesar disso, a introdução de novas alternativas terapêuticas se faz necessária em virtude do grande número de pacientes afetados e dos efeitos adversos que os medicamentos, hoje utilizados, podem causar. Dessa forma, espécies vegetais podem constituir uma excelente fonte de compostos para este fim (CALIXTO et al., 2000; ALMEIDA et al., 2001).

Hypericum polyanthemum Klotzsch ex Reichardt é uma planta bianual nativa do sul do Brasil reconhecida por apresentar ação antinociceptiva (VIANA et al., 2003). Essa planta apresenta como principais metabólitos ativos o derivado de floroglucinol uliginosina B (NÖR et al., 2004) e os benzopiranos HP1 (6-isobutiril-5,7-dimetóxi-2,2-dimetil-benzopirano), HP2 (7-hidróxi-6-isobutiril-5-metóxi-2,2-dimetil-benzopirano) e HP3 (5-hidróxi-6-isobutiril-7-metóxi-2,2-dimetil-benzopirano) (FERRAZ et al., 2001) (Figura 4.1).

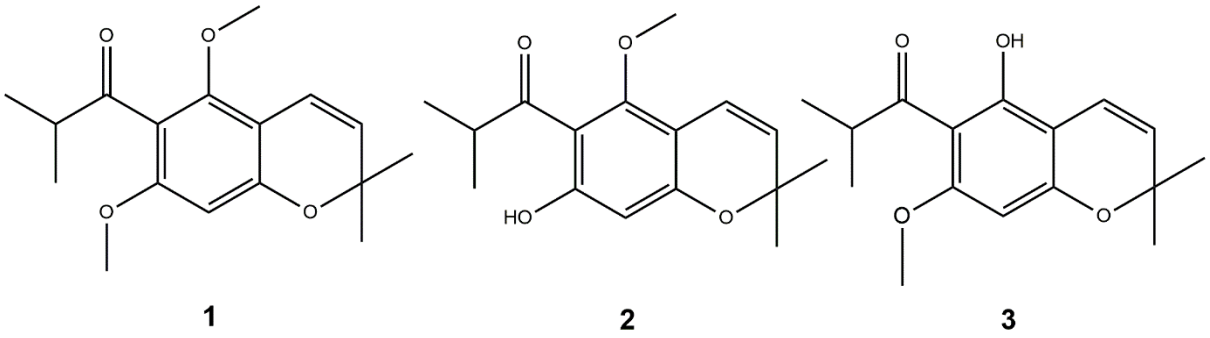

Figura 4.1 Benzopiranos de *H. polyanthemum*: HP1 (**1**), HP2 (**2**), HP3 (**3**).

O benzopirano HP1 apresenta ação antinociceptiva mediada pelo sistema opioide como já evidenciado por estudos deste grupo (HAAS et al., 2010). Ainda, estudos na literatura científica destacam a importância do núcleo benzopirano como responsável por atividades farmacológicas ligadas ao sistema nervoso central como por exemplo tonabersat e carabersat (anticonvulsivantes) (UPTON et al., 1997; PARSONS et al., 2011), espinosina (ansiolítico) (LIU et al., 2015) e o canabinoide Δ-^{9}-tetrahidrocanabinol, o THC (antinociceptivo e psicotrópico) (WELCH et al., 1999).

Evidências apontam para uma relação entre os sistemas opioide e canabinoide, inclusive compartilhando efeitos farmacológicos como antinocicepção, hipotermia, inibição de atividade locomotora, hipotensão e sedação (MANZANERES et al., 1999; MASSI et al., 2001). Especificamente para ação antinociceptiva, essa relação pode ser bidirecional ou sinérgica. Além disso, esses receptores são expressos em áreas anatômicas relevantes para a analgesia como medula espinhal e corno dorsal, onde podem ser co-localizados no mesmo neurônio (WELCH e STEVENS, 1992; HOHMANN et al., 1999; SALIO et al., 2001).

A via oral é considerada a mais conveniente e econômica devido ao conforto e fácil aceitação pelo paciente (FUCHS et al., 2004; BARROS, 2010; BRUNTON et al., 2011). Para alcançar efeito sistêmico, os fármacos devem atingir o sistema vascular, o que requer prévia dissolução no suco gástrico. No entanto, devido à baixa solubilidade em água, a absorção de compostos lipofílicos, como o benzopirano HP1, pela via oral é frequentemente deficiente. Em 2010, estimou-se que cerca de 70% das novas entidades candidatas a fármacos apresentavam baixa solubilidade em água (KU e DULIN, 2010).

Para contornar estes obstáculos, fármacos lipofílicos têm sido incorporados em sistemas de liberação lipídicos, como nanoemulsões óleo em água (McCLEMENTS, 2013) com o objetivo de aumentar sua solubilidade e melhorar seus aspectos biofarmacêuticos, visando melhor absorção pela via oral (KOTTA et al., 2012). As nanoemulsões possuem várias características peculiares devido ao tamanho de partícula reduzido, que as habilitam como excelentes sistemas transportadores para compostos lipofílicos como (McCLEMENTS, 2011):

(a) Permitem que enzimas digestivas ajam com mais rapidez e liberem o conteúdo da nanoemulsão que será então absorvido;

(b) Facilitam a permeação na mucosa que recobre células epiteliais no intestino delgado, facilitando a absorção do composto;

(c) Possuem a capacidade de serem transportadas diretamente para as células epiteliais pelo mecanismo transcelular ou paracelular;

(d) A solubilidade em água de um composto lipofílico aumenta em função da diminuição do tamanho das partículas, o que pode promover uma melhor absorção do mesmo.

Assim, devido à baixa solubilidade do benzopirano HP1 (5µg/mL), este estudo objetiva a incorporação do mesmo em nanoemulsões e a influência na sua ação antinociceptiva quando incorporado nessas formulações

4.1 *Hypericum polyanthemum*

Hypericum polyanthemum (Figura 4.2) possui como principais metabólitos secundários o derivado de floroglucinol uliginosina B e os benzopiranos HP1, HP2 e HP3, sendo eles responsáveis pelas atividades biológicas atribuídas a essa espécie.

Figura 4.2 *Hypericum polyanthemum* (Foto G. von Poser)

Dentre as atividades biológicas atribuídas a uliginosina B, destacam-se potencial antinociceptivo não mediado pelo sistema opioide (STOLZ et al., 2012). Para os benzopiranos, estudos demonstram efeito inibidor da monoaminoxidase (GNERRE et al., 2001), antinociceptivo mediado pelo sistema opioide (HP1) (HAAS et al., 2010), entre outros.

4.2 Nocicepção

A dor é composta por dois fatores interligados, a nocicepção e a reatividade emocional à dor.

A nocicepção se refere à atividade do sistema nervoso aferente, induzida por estímulos nocivos, sejam eles exógenos (mecânicos, químicos, físicos e biológicos), ou endógenos (inflamação, aumento do peristaltismo, isquemia tecidual). Sua percepção em nível periférico se dá em estruturas situadas nas terminações nervosas livres, denominadas nociceptores (FUCHS et al., 2004).

A reatividade emocional à dor corresponde a interpretação afetiva dessa sensação, influenciada por estados psicológicos, experiências anteriores e fatores culturais, sociais e ambientais (FUCHS et al., 2004).

O manejo farmacológico da dor tem sido realizado através de terapia com multifármacos, ou seja, diferentes fármacos com mecanismos de ação distintos, minimizando os efeitos adversos (EKSTEROWICZ, 2010). A escolha dos fármacos se dá se acordo com a origem da dor. Os medicamentos utilizados podem ser divididos em três categorias: analgésicos não-opioides, analgésicos opioides e co-analgésicos (adjuvantes) (AMERICAN PAIN SOCIETY, 2008).

Os analgésicos não opioides incluem paracetamol, aspirina e analgésicos não esteroides e são utilizados para o tratamento da dor leve a moderada. Os analgésicos opioides como morfina, oxicodona, tramadol e codeína, são utilizados para o tratamento da dor mais severa e são, quando possível, evitados em virtude de seus efeitos adversos pronunciados. Por fim, os co-analgésicos incluem anestésicos e relaxantes musculares que podem auxiliar no alívio da dor (WUHRMAN e COONEY, 2011).

Apesar de existirem diversas opções terapêuticas para o combate à dor, os efeitos adversos que estes medicamentos podem causar são prejudiciais ao paciente, quando em usos prolongados. Analgésicos não opioides podem induzir úlcera gastrointestinal, sangramentos e complicações cardiovasculares em virtude da inibição da enzima cicloxigenase (COX) (WOODCOCK, 2009; TRELLE et al., 2011) e opiodes podem provocar dano cognitivo, depressão respiratória e dependência por ativação das vias opioides cerebrais (BENYAMIN et al.,2008; von KROFF et al., 2011; AMERICAN PAIN SOCIETY, 2008). Dessa forma, o tratamento da dor deve ser realizado de forma rápida e precisa a fim de evitar a dor crônica, situação onde há necessidade de tratamentos farmacológicos prolongados, acarretando possíveis riscos ao paciente.

Nesse contexto, novas alternativas terapêuticas como o uso de compostos oriundos de plantas são uma importante estratégia para o tratamento e controle da dor, com o objetivo de diminuir os efeitos adversos e assim melhorar a qualidade de vida de pacientes que sofrem dessa injúria.

4.3 Sistemas opioide e canabinoide

Entre as opções existentes para o tratamento da dor estão os fármacos da classe do opioides como morfina, codeína, tramadol, entre outros (ROSENBLUM et al., 2008). O sistema opioide inclui quatro principais subtipos de receptores: mi (μ), kapa (ƙ), delta (∆) (KIEFFER, 1995) e o receptor do tipo opioide (ORL-1) (MEUNIER et al., 1995; 1997). Evidências sugerem que os fármacos considerados analgésicos fortes estão relacionados com a ativação do receptor mi (μ) (KIEFFER e GAVERIAUX-RUFF, 2002).

Estudos prévios deste grupo comprovaram que a atividade antinociceptiva do benzopirano HP1 é relacionada com o sistema opioide, visto que a ação foi bloqueada pelo antagonista naloxona (HAAS et al., 2010). Este mesmo estudo indicou que a curva dose resposta deste composto segue o padrão de forma de sino ou "U" invertido, característica de alguns fármacos opioides como a buprenorfina (DUM et al., 1981; LISAZOIN et al., 1991). A buprenorfina apresenta esse comportamento devido ao agonismo parcial do receptor mi (μ) (HEEL et al., 1979) e, em altas doses, a ativação do receptor do tipo opioide (ORL-1) (WNENDT et al., 1999; BLOMS-FUNKE et al., 2000; HUANG et al., 2001). A ativação deste último pode desencadear resposta pró-nociceptiva ou, ao menos, anular respostas antinociceptivas (MOGIL e PASTERNAK. 2001). Ainda, essa molécula pode funcionar como agonista (TYERS, 1980) ou antagonista (LEANDER, 1988) do receptor kapa (ƙ) opioide.

Evidências demonstram similaridades comportamentais, anatômicas e bioquímicas entre os sistemas opioide e canabinoide (subtipos CB_1 e CB_2) (KIEFFER, 1995; HOWLETT et al., 2002). Ativação de um desses sistemas pode produzir efeitos comportamentais semelhantes incluindo antinocicepção, hipotermia, sedação, inibição da motilidade intestinal e depressão motora (MANZANARES et al., 1999). Ainda, os dois tipos de receptores são encontrados em regiões cerebrais reconhecidas por participar da antinocicepção (CICHEWICZ, 2004), sugerindo que possam agir sozinhos ou em conjunto. Além disso, o receptor mi (μ) opioide e o CB_1 canabinoide podem ser co-localizados no mesmo neurônio, inclusive compartilhando o mesmo RNA mensageiro, evidenciando a forte relação entre esses dois sistemas (SHAPIRA et al., 2000; 2003).

Estudos clínicos e pré-clinicos demonstraram que a interação entre os sistemas opioide e canabinoide pode levar a aplicações terapêuticas promissoras no tratamento da dor. Por exemplo, ligantes simultâneos dos receptores mi (μ) opioide e o CB_1 canabinoide se mostram analgésicos em potencial sem causar tolerância (LE NAOUR et al., 2013; FERNÁNDEZ-FERNÁNDEZ et al., 2014)

4.4 Sistemas nanoestruturados como carreadores de compostos lipofílicos pela via oral

A administração de compostos pela via oral é um dos métodos mais tradicionais em virtude da conveniência e concordância do paciente (PLAPIED et al., 2011). Para alcançar a circulação sistêmica e atingir o sistema alvo, os compostos necessitam atravessar o trato gastrointestinal.

Muitos compostos com fins terapêuticos possuem baixa solubilidade, baixa permeabilidade e/ou reduzida estabilidade no trato gastrointestinal e assim, apresentam baixa biodisponibilidade oral (ENSIGN et al., 2012). Por exemplo, paclitaxel, aprovado para o tratamento de câncer de ovário e mama, tem biodisponibilidade menor que 6% quando administrado pela via oral (MALINGRE et al., 2001) e as principais razões desta baixa absorção residem na baixa solubilidade em água, afinidade por bombas de efluxo e rápida metabolização pelas enzimas do citocromo *P*-450 no trato gastrointestinal (ZHANG e BENET, 2001).

Com vistas a contornar os problemas de absorção de compostos lipofílicos, sistemas de liberação lipídicos têm sido descritos como alternativas para aumentar a solubilidade e permeabilidade, prevenir a degradação enzimática na parede intestinal e superar as barreiras de bombas de efluxo para facilitar a disponibilidade no tecido ou célula de interesse (GANTA et al., 2010). Em especial, destacam-se formulações compostas por combinações de lipídeos naturais, com surfactantes, co-surfactantes e co-solventes (PORTER et al., 2007) como por exemplo, as nanoemulsões.

Nanoemulsões são sistemas heterogêneos óleo em água com tamanho de partícula usualmente <500 nm. A versatilidade destes sistemas está baseada nos diferentes tipos de óleos e modificadores de superfície que podem ser utilizados (SARKER, 2005). O núcleo oleoso desse tipo de formulação é capaz de incorporar adequadamente compostos muito pouco solúveis em água (TIWARI e AMIJI, 2006). Esses sistemas são usualmente estabilizados com surfactantes anfifílicos como lecitina de gema de ovo, um dos componentes das membranas celulares, portanto, compatíveis com sistemas biológicos.

Há diversos estudos que demonstram que nanoemulsões podem aumentar a absorção oral de compostos (YANG e BENITA, 2000; TIWARI e AMIJI, 2006; VYAS et al., 2008; BALI et al. 2010). Os mecanismos já descritos são:

(1) Composto lipofílico presente na sua forma solúvel na nanoemulsão, não necessitando a fase de dissolução no trato gastrointestinal (WICKLINE e LANZA, 2003);

(2) Transferência direta dos compostos pela fusão das gotículas de óleo com as membranas biológicas (WICKLINE e LANZA, 2003);

(3) Gotículas oleosas da nanoemulsão podem ser absorvidas pelo sistema linfático (NISHIOKA e YOSHINO, 2001).

A carga da superfície das nanoemulsões também pode influenciar na disposição *in vivo* e *clearence*, e assim aumentar as interações com as membranas. Essas cargas são expressas em termos de potencial zeta e são resultantes da ionização de compostos formando uma monocamada na interface óleo/água (TIWARI, 2007).

Dessa forma, os resultados apresentados neste capítulo têm por objetivo demonstrar a incorporação do benzopirano HP1 em nanoemulsões e a influência na sua ação antinociceptiva quando incorporado nesses sistemas de liberação.

MANUSCRITO 2- Nanoemulsion improves the antinociceptive activity of HP1, a benzopyran from *Hypericum polyanthemum*.

Manuscrito em preparação

Nanoemulsion improves the antinociceptive activity of HP1, a benzopyran from *Hypericum polyanthemum*.

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Abstract

The genus *Hypericum* (Hypericaceae) represents a source of compounds with antinociceptive activity. Among them, our group has been studying the benzopyran HP1 from *Hypericum polyanthemum* Klotzsch ex Reichardt. Despite its antinociceptive effect already demonstrated in mice, the low solubility of HP1 could represent an obstacle to further studies aiming at developing new analgesic drugs. The nanotechnology has been considered a fruitful approach to improve the solubility and bioavailability of drugs. In this context, the aim of this study was to investigate the impact of nanoencapsulation on the antinociceptive effect of HP1 in mice hot-plate test. The results revealed that the benzopyran HP1 was successful incorporated into a nanoemulsion system, given its high solubility in the oil phase. Regarding the pharmacological effect, HP1 (15, 30, 45 and 60 mg/kg, p.o.) in both forms, free and loaded in a nanoemulsion, displayed the pattern of bell-shapped dose response curve. HP1 loaded nanoemulsion displayed the maximal antinociceptive effect at lower dose than HP1 free-form. The highest effect of free compound was found at 45 mg/kg, while the HP1 loaded in a nanoemulsion reached the same effect at 30 mg/kg. These results suggests that the effect found might be attributed to an increase of solubility and, thus, better absorption by the organism. The results obtained in this study corroborate with literature data, where several studies demonstrate absorption enhancement and thus, improvement of biopharmaceutical aspects, such as bioavailability when a compound is loaded in nanoemulsions.

Keywords: antinociceptive, *Hypericum polyanthemum*, HP1, nanoemulsion

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Introduction

The International Association for the Study of Pain (IASP) and the World Health Organizaton (WHO) define pain as a “unpleasant sensory and emotional experience associated with actual or potential tissue damage” (WHO, 2007; IASP, 2014). Pain is considered a major clinical, social, and economic problem in populations around the world (HENSCHKE et al., 2015). Besides, is the defining feature for several diseases diagnoses and could serve as an index of severity and activity of an underlying condition, a prognostic indicator, and a determinant of health service use (MCBETH and JONES, 2007).

There are many drug options to pain treatment and the decision is taken based on the type and intensity of symptoms. On the other hand, there are a high number of patients affected by this injury and the adverse effects of drugs clinically used are pronounced (WOODCOCK, 2009; TRELLE et al., 2011). The mainly adverse effects are constipation, depression, tolerance, dependence, gastrits and ulcers (BRUNTON et al., 2011). Therefore, new therapeutic alternatives are needed and the vegetal species could constitute an excellent source of products to this purpose (CALIXTO et al., 2000).

Hypericum polyanthemum Klotzsch ex Reichardt is a biannual plant native to South Brazil that presents as main active metabolites the phloroglucinol derivative uliginosin B (NÖR et al., 2004) and the benzopyrans HP1 (6-isobutyryl-5, 7-dimethoxy-2, 2-dimethyl-benzopyran), HP2 (7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethyl-benzopyran), and HP3 (5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl-benzopyran) (FERRAZ et al., 2001). To benzopyran HP1 (Figure 1), the antinociceptive activity has already been demonstrated in rodents (HAAS et al., 2010). In spite of this activity, the low water solubility (5 μg mL^{-1}) of this compound constitutes an obstacle to *in vivo* further studies.

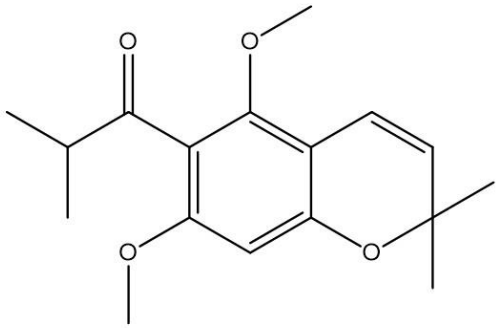

Figure 1. Chemical structure of benzopyran HP1.

Regarding administration route, the oral delivery is the dominant pathway of drug therapy and will continue to be due to safety, efficacy, and easily access with minimal discomfort for patients when compared with other routes (LENNERNÄS, 2007). To reach the systemic effects, drugs must achieve the vascular system, which need previous dissolution in the gastric juice of stomach. However, given the low water solubility, the absorption of lipophilic compounds, as HP1, by this route is frequently deficient. Besides low solubility, there are many barriers that could hamper the absorption of this type of compound, like limited absorption site, metabolizing intestinal enzymes and hepatic first pass metabolism (DAHAN and HOFFMAN, 2007)

To overcome these hindrances, lipophilic compounds have been incorporated in lipidic release systems (POUTON et al., 2006) aiming to increase solubility and improve biopharmaceutical aspects, such as permeation and bioavailability (KOTTA et al., 2012). Among these systems, nanoemulsions have drawn attention by peculiar characteristics due to the low size of particles. These systems present more stability and ease penetration in the intestinal mucosa, which enable them as excellent carrier systems to lipophilic compounds (RAJPOOT et al., 2011; McCLEMENTS, 2011; 2013).

Thus, given the low water solubility of benzopyran HP1, this study aims the incorporation of this compound in a triglyceride nanoemulsion and to verify the impact of nanoemulsification on HP1 antinociceptive effect in the mice hot-plate test.

Material and Methods

Plant material

Flowering aerial parts of *Hypericum polyanthemum* were collected in Caçapava do Sul, Rio Grande do Sul state, Brazil, in September of 2011. Plants were identified by Dr. Sérgio Bordignon (UNILASALLE, RS). Voucher specimens were deposited in the herbarium of Federal University Rio Grande do Sul (ICN). Plant collection was authorized by IBAMA (Brazilian Institute of Ambient Media and Renewable Natural Resources) (nº 003/2008, protocol: 02000.001717/1008-60).

Extraction and isolation

The dried and powdered plant material (ca. 500 g) was successively extracted with *n*-hexane at room temperature (solvent renewal each 24 hours). The extracts were pooled, evaporated to dryness under reduced pressure, and the epicuticular waxes were removed by acetone treatment. This fraction was stored at −20 °C until isolation process.

The acetone-soluble fraction was subjected to dry column vacuum chromatography (DCVC) on silica gel 60 GF_{254} as stationary phase and *n*-hexane–dichlorometane (100:0→50:50) gradient as mobile phase in order to obtain enriched fractions of HP1. Then, these fractions were submitted to centrifugal planar chromatography (CPC) (Chromatotron®, model 7924 T, Harrison Research, San Bruno, CA, USA) on silica gel 60 GF_{254} as stationary phase and *n*-hexane–ethyl acetate (100:0→50:50) as mobile phase to obtain the compound HP1.

The identity of HP1 was confirmed by Nuclear Magnetic Resonance (^{1}H and ^{13}C NMR) and mass spectrometry (Supplementary Data). These results were compared

with previous data of the compound (FERRAZ et al., 2001). Besides, the purity was confirmed by High Performance Liquid Chromatography (HPLC) using a system previously validated composed by acetonitrile (60%) and water (40%) as mobile phase and using a Waters Nova-Pack C18 column (4 µm, 3,9 mm x 150 mm) adapted to a guard column Waters Nova-Pack C18 60Å (3,9 mm x 20 mm). The flow rate was 1mL/min at λ=230 nm in 25 ºC.

Characteristics of benzopyran HP1

To evaluate the characteristics of water solubility, the partition coefficient (log $P_{o/w}$) was performed in computational software (ACD Labs Release 2012 – File version 14.1, Build 65894, 17 Sep 2013). After that, the compound solubility in the nanoemulsion oil core was assessed. For this purpose, an excess of HP1 was added in 500 µL of medium chain triglycerides (MCT), under agitation, for 24 hours. Then, the solutions were centrifuged and the supernatant was diluted with methanol HPLC grade (1:400). The samples were evaluated by HPLC using the same method described previously.

Nanoemulsion preparation

The nanoemulsions were prepared by means of spontaneous emulsification method (YU et al., 1993; BOUCHEMAL et al., 2004). The oil core (8% of MCT, 2% egg lecithin and HP1) and aqueous components (1% of polyssorbate 80) were dissolved in ethanol:acetone (50:50) mixture and ultrapure water, respectively. Increasing amounts of HP1 (1.5, 3.0, 4.5 and 6.0 mg/mL) were added to organic phase. This phase was poured, slowly, under moderate magnetic stirring, on the aqueous phase. The solvent excess was eliminated under reduced pressure in 50 ºC until the final volume required (5 mL).

Nanoemulsion characterization

Particule size

The nanoemulsions were characterized according the particle size and polydispersion index through dynamic light scattering, in Malvern Zetasizer ZS (3000HS Zetasizer, Malvern Instruments, Worcestershire, UK) equipment. The results were expressed as mean of three determinations of three different nanoemulsions.

Zeta potential

The zeta potential was determined according the particles eletroforetic mobility in Malvern Zetasizer ZS (3000HS Zetasizer, Malvern Instruments, Worcestershire, UK) equipment. The results were expressed as mean of three determinations of three different nanoemulsions.

Morphological evaluation

To evaluate the oil droplets morphology, the nanoemulsions were visualized in transmission electronic microscopy transmission electronic microscopy (JEOL JEM 1200 Exll), in 120kV voltage and 300.000x magnification. The samples were prepared 24 hours before analysis and maintained in desiccator. After preparation, samples were deposited in cuprum metallic supports (200 mesh) and submitted to negative contrast with uranile acetate solution (2%).

Pharmacological study

Animals

Male CF1 mice (25–30 g) from the breeding colony of Federal University of Santa Maria (UFSM) were used. Before the experiments, the animals were housed in plastic cages of 17x28x13 cm, 6 per cage, under a 12 h light/dark cycle (lights from 7:00 to 19:00h) at constant temperature (23 ºC ± 2). The animals were kept in an

exhaustion system (ventilated shelves Alesco®) and monitored humidity, with free access to standard certified rodent diet and tap water. The experiments were performed between 10:00 to 16:00 hours, with 1 hour of adaptation in the experimental room. Before the administration by oral route, the animals were fasted by 2 hours. All protocols were approved by local research ethical committee (UFRGS) (nº 26390) and comply with the Brazilian law and the CIOMS (Council for International Organization of Medical Sciences International) (INTERNATIONAL GUIDING PRINCIPLES FOR BIOMEDICAL RESEARCH INVOLVING ANIMALS, 2012)

Drugs and treatments

The following drugs were used: morphine (Cristalia®) and haloperidol (Galena®). HP1 free form was dissolved in a mixture of polyssorbate 80 (2%) and saline solution (0.9% NaCl) and HP1 loaded in nanoemulsions was administered directly. The control animals received vehicle (saline solution with 2% of polyssorbate 80 or blank nanoemulsions). The doses were chosen according to previous studies from our group (HAAS et al., 2010). Besides, the sample size was nine (9) animals per group considering an alfa value of 0.01, test power 0.9 and the statistic test for each experiment.

Hot plate test

For hot plate test, the mice were habitude to the nonfunctioning apparatus for 1 minute. After that, the animals were placed on the functioning hot plate (Ugo Basile®) at 53 ± 2 ºC (AMRESH et al., 2007) to determine baseline responsiveness. The latency time was counted to the mice lick one of its hind paws or jump. After that, the animals were withdrawn of hot plate. The mice that presented baseline reaction of more than 20 s were eliminated from experiment.

The selected animals received the respectively treatments by oral route (1 mL/100 g), in the following doses: HP1 free form (15, 30, 45 and 60 mg/kg), HP1 loaded nanoemulsions (15, 30, 45 and 60 mg/kg), vehicle control, blank nanoemulsion and morphine as positive control (10 mg/kg). After 60 minutes, the mice were placed again in the hot plate and the latency time was measured. In the second session, a

maximum latency time of 40 s was imposed in order to avoid tissue damage. The percentage of maximum possible antinociceptive effect was calculated according the follow equation:

$$\% \text{ MPE} = \frac{\text{(Post drug latency- pre-drug latency)}}{\text{(Cut-off latency- pre-drug latency)}} \times 100$$

Motor Coordination test (Rota Rod)

For the motor coordination test, the rota rod apparatus was used (DUNHAM and MIYA, 1956). The apparatus consist of a cylinder of 3 cm in diameter rotating at 5 rpm. One day before testing, the animals were trained once during five minutes. On the test day, the mice were trained again for five minutes and those that were able to stay 90 seconds balanced on the rota rod were selected to analysis. The selected mice received HP1 free form and HP1 loaded nanoemulsions at active doses, vehicle control, blank nanoemulsion, morphine (10 mg/kg) and haloperidol as positive control (4 mg/kg).

Statistical analysis

Data were analyzed by one way analysis of variance (ANOVA) and two-way repeated measure analysis of variance (ANOVA), both followed by Student-Newmann-Keuls test. All results were expressed as mean ± S.E.M. The analyses were performed using Sigma Stat 2.03 software (Jandel Scientific Corporation, San Rafael, CA, USA). Differences were considered statistically significant at $p < 0.05$.

Results and Discussion

Physicochemical properties of nanoemulsions

The nanoemulsions were characterized in terms of droplet size, polydispersity index and zeta potential. The results are presented in Table 1.

Table 1. Nanoemulsion characteristics

	Φ (nm)[1]	**PDI[2]**	**Zeta potential (mV)**	**Content (mg/mL)**
HP1 (1.5 mg/mL)	169.65 ± 1.317	0.118 ± 0.038	-27.917 ± 0.383***	1.46 ± 0.001
HP1 (3.0 mg/mL)	174.00 ± 6.986	0.114 ± 0.003	-22.544 ± 1.887*	2.94 ± 0.014
HP1 (4.5 mg/mL)	164.43 ± 0.833	0.134 ± 0.0001	-15,933 ± 0.513	4.28 ± 0.001
HP1 (6.0 mg/mL)	159.3 ± 4.30	0.218 ± 0.017***	-26.65 ± 3.65**	5.99 ± 0.124
Blank	168.06 ± 10.98	0.118 ± 0.005	-16.578 ± 1.412	-

[1]Droplet size; [2] Polydispersion index

***Statistical difference by One Way ANOVA followed by Tukey´s test related to blank nanoemulsion at $p<0.001$

**Statistical difference by One Way ANOVA followed by Tukey´s test related to blank nanoemulsion at $p<0.01$

*Statistical difference by One Way ANOVA followed by Tukey´s test related to blank nanoemulsion at $p<0.05$

The results indicated that the spontaneous emulsification generated monodisperse nanoemulsions (polydispersion index < 0.25) with average of particle size around 170 nm. The benzopyran HP1 seems to be incorporated in the internal phase of formulations. These results could be explained by the low HP1 water solubility, since HP1 partition coefficient octanol/water ($logP_{o/w}$) was 4.3 ± 0.39, characteristic of lipophilic compounds.

The solubility in the nanoemulsion oil core (MCT) was also evaluated, finding the value of 41.25 ± 0.97 mg/mL, which is very high and promotes the compound solubilization inside the oil core. The zeta potential ranged from -15 mV to -28 mV. The negative charge, attributed to egg lechitin and MCT presence, contributes to nanoemulsion stability by repulsion between droplets (LAWRENCE, 1996). Significative differences were found between blank and HP1 loaded nanoemulsions with higher values, in modulus, when the compound is encapsulated, probably due to HP1 adsorption in the interface o/w. The higher HP1 solubility in the oil core and the zeta potential more negative indicate that part of the molecule is inside of the oil core and part is in the interface o/w associated with surfactants.

The nanoemulsions droplet morphology was evaluated by Transmission Eletronic Microscopy (TEM) as represented in figure 2. The photomicrographs revealed spherical structures with average diameter between 100 and 200 nm, defined interface without deposit of compound.

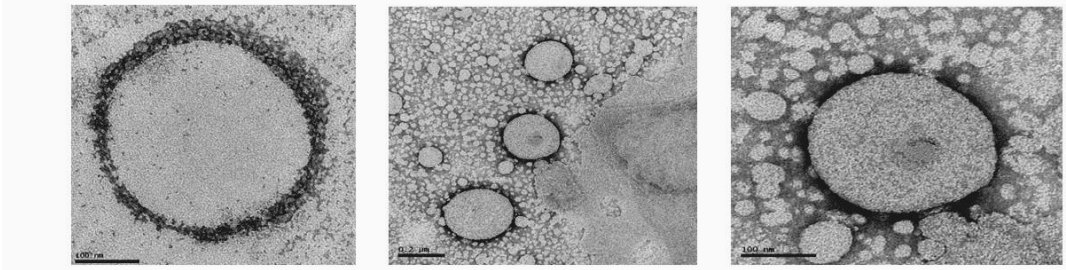

Figure 2. Nanoemulsion micrographs after HP1 incorporation (magnification 300.000x).

Antinociceptive activity

Regarding pharmacological results, HP1 loaded in nanoemulsions had it active dose reduced as shown in figure 3. The maximum effect of HP1 free form was found at 45 mg/kg while HP1 loaded nanoemulsions reached the same effect at 30 mg/kg. Considering the same lipidic proportion in tested formulations, the maximum effect at lower concentrations could be attributed to better solubilization of the compound when loaded in nanoemulsions.

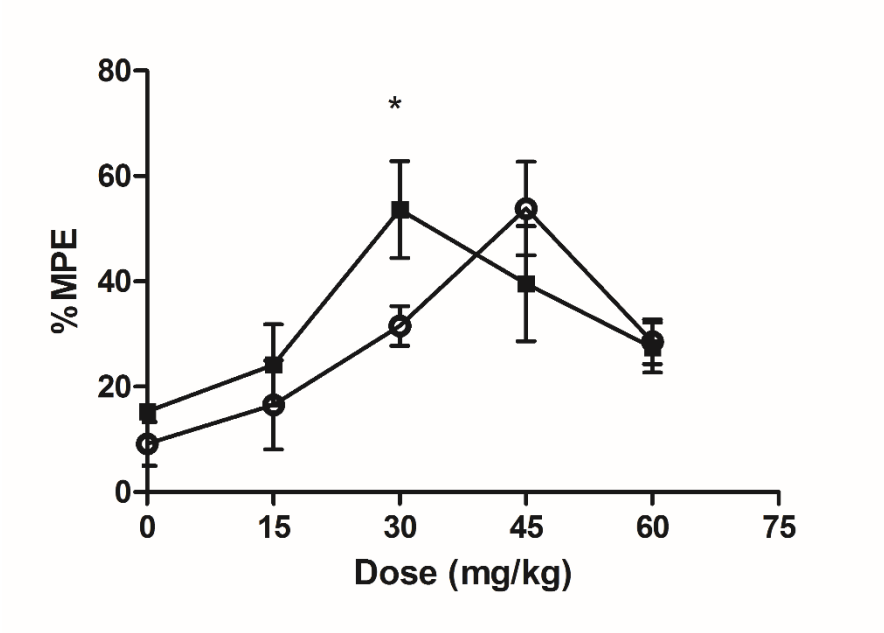

Figure 3. Dose reponse curve of % of Maximum Possible Antinociceptive Effect (%MPE) of HP1 free form (○) or loaded in nanoemulsions (■). The results are presented as mean ± standard error (n=9 mice/group). Significantly different values were detected by one-way ANOVA followed by Student Newmann Keuls test. *$p<0.05$ compared to vehicle free HP1 at 30 mg/kg.

The benzopyran HP1 demonstrated antinociceptive action in a dose dependent way supporting the data presented by HAAS et al. (2010). In that study, the authors showed that the effect presented by HP1 is mediated by the opioid system, since it was blocked by naloxone, an antagonist of this system. Scientific evidences point to the benzopyran skeleton, that presents an interesting pharmacological potential. In this context, many molecules having this nucleus, natural or synthetic, have demonstrated

relevant pharmacological activities: tonabersat (SB-220453) with anticonvulsivant properties (PARSONS et al., 2001), compounds with 7-hydroxi-2H-1-benzopyran, 2,3-diaryl and halogenated benzopyrans with anti-inflammatory potential and antipyretic activities by cicloxigenases inhibition (PRASANNA et al., 2004; EISSA et al., 2009) and even more, cannabinoids as Δ^9-tetrahydrocannabinol (THC) (WELCH et al., 2009)

The dose-response bell-shaped curve presented by benzopyran HP1, free form and loaded in nanoemulsion, is characteristic of some peculiar opioid analgesics such as buprenorphine and its metabolite norbuprenorphine (MARTIN et al., 1976; HUANG et al., 2001). This behavior has been attributed to the partial agonism of mu (μ) opioid receptor (MARTIN et al., 1976) and interaction with supraspinal opiod like receptors (ORL-1) which could provoque an effect pro-nociceptive or, at least, oppose opioid receptor mediated antinociception at higher doses (MOGIL and PASTERKNAK, 2001). Furthermore, buprenorphine has also the capacity of activates the delta (Δ) opioid receptor as an antagonic ligand (SADEE et al., 1982), whilist, the main opposite effect in the opioid system are due to interactions with mu (μ) and ORL-1 receptors (LUFT and COWAN, 2004). Therefore, the dose response curve presented by HP1 might be attributed with interactions with different opioid receptors, in a relation of agonism/antagonism dose dependant.

The use of nanotechnology based formulations as release systems is well documentated in literature. The analgesic compounds morphine (WANG et al., 2008) and acetylsalycilic acid (TANG et al., 2012) have already been incorpored in nanomulsions in order to increase the absorption, and thus, pharmacodynamic activity. Besides, better oral absorption of lipophilic compounds loaded in nanoemulsions has been demonstrated (TIWARi and AMIJI, 2006; PARVEEN et al., 2011; SARI et al., 2015).

Some physico-chemical mechanisms have been proposed to describe this effect. The first explanation is the fact that small negatively charged nanoparticles could go through the mucus layer of epithelial cells and, then, be absorbed by intestin (CONE, 2009). Besides, the lipid droplet superficial area increases with the decrease of particle size leading to an increase of lipid digestion by gastrointestinal enzymes (LI et al., 2011). Additionally, the bioavailability of lipophilic compounds could be diminished if the lipidic phase consist of indigestible lipid. In formulations with digestible lipids (like

MCT), the compound can be solubilized by micelles, mixed micelles, vesicles and free fatty acids as result of lipid digestion and thus, be absorbed by intestinal cells (CHAKRABORTY et al., 2009; QIAN et al., 2012).

In this context, HP1 loaded nanoemulsions produced the same effect than free form, but in a lower doses. This reduction in doses sugggests that HP1 when loaded in a nanoemulsion system might has a better solubilization in the gastrointestinal tract. Other impacts of a lipophilic compound nanoemulsification are described in literature as increasing of residence time in gastrontestinal tract, intestinal limphatic pathways stimulus, intestinal permeability alteration and/or reduction in the efflux transporters and metabolism activities (KOTTA et al., 2012). As can be seen in figure 3, the dose-response curve of free compound is given in higher doses and this fact could be atributted to a defficient solubilization of free molecule when administred only with aid of surfactants.

Finally, the active doses (HP1 free form and loaded in nanoemulsions) were evaluated in the rota rod test, to discard the possible motor impairement caused by them in the hot plate (Figure 4). The results demonstrated that the benzopyran HP1 free form and loaded in nanoemulsions did not show motor impairment in animals ($F_{treatment}$ (7.97)=6.392.$p<0.001$; $F_{interaction}$(7.97)=18.941. $p<0.001$), using haloperidol as positive control.

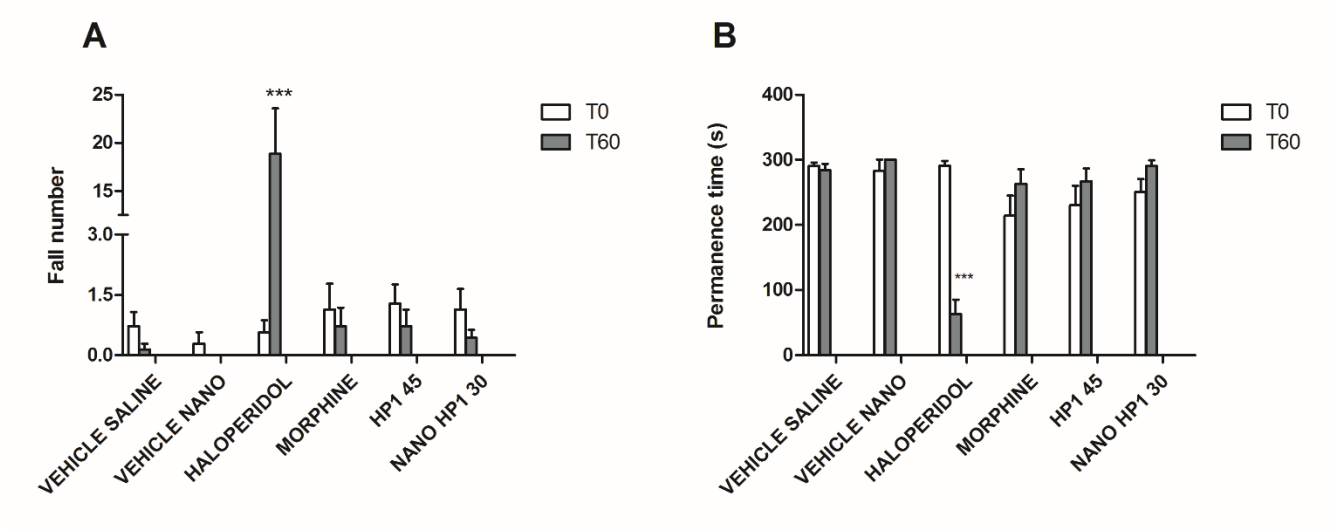

Figure 4. Effect of benzopyran HP1 free form (45 mg/kg) and loaded in nanoemulsion (30 mg/kg); haloperidol (positive control) and morphine (positive control of antinoception) in Rota Rod apparatus. The fall number (A) and the permanence time (B) were evaluated. The results are presented as mean ± standard error (n=7 mice/group). Significantly different values were detected two way repeated measures ANOVA followed by Student Newmann Keuls test *** $p<0.001$ comparing T0 with T60.

Conclusions

The results show the feasibility of incorporating HP1 into nanoemulsions. The antinociceptive effect of HP1 loaded in nanoemulsions was the same of free compound, but in lower doses (45 to 30 mg/kg). These results corroborate with literature data, where many studies demonstrate absorption enhancement and thus, improvement of biopharmaceutical aspects, such as bioavailability when a compound is loaded in nanoemulsions. However, assessing how nanoemulsions behave across the grastrointesinal tract and how the compound is released of this systems is needed in order to permit a better understanding of how this molecule exerts its antinociceptive action in a nanoemulsion form.

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References

AMERICAN PAIN SOCIETY. Principles of Analgesic Use in the Treatment of Acute Pain and Cancer Pain. 6.ed. Glenview IL*:* American Pain Society; 2008.

AMRESH, G.; SINGH, P.N.; RAO, C.H. Antinociceptive and antiarthritic activity of *Cissampelos pareira* roots. **Journal of Ethnopharmacology***, v*. 111, p. 531-536, 2007.

BOUCHEMAL, K.; BRIANÇON, S.; PERRIER, E.; FESSI, H. Nanoemulsion formulation using spontaneous emulsification: solvent, oil and surfactant optimization. **International Journal of Pharmaceutics**, v. 280, p. 241-251, 2004.

BRUNTON, L.L.; BLUMENTHAL, D.K.; MURRI, N.; DANDAN, R.H.; KNOLLMANN, B.C. **Goodman and Gilman's The Pharmacological Basis of Therapeutics**. 12th ed. New York: McGraw-Hill, 2011.1808p.

CALIXTO, J.B.; BEIRITH, A.; FERREIRA, J.; SANTOS, A.; CECHINEL FILHO, V.; YUNES, R. Naturally occurring antinociceptive substances from plants. **Phytotherapy Research**, v. 14, p. 401-418, 2000.

CHAKRABORTY, S.; SHUKLA, D.; MISHRA, B.; SINGH, S. Lipid – An emerging platform for oral delivery of drugs with poor bioavailability. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 73, p. 1-15, 2009.

CONE, R.A. Barrier properties of mucus. **Advanced Drug Delivery Reviews**, v. 61, p. 75-85, 2009.

COUNCIL FOR INTERNATIONAL ORGANIZATION OF MEDICAL SCIENCES AND THE INTERNATIONAL COUNCIL FOR LABORATORY ANIMAL SCIENCE. Guiding principles for biomedical research involving animals, 2012.

DAHAN, A.; HOFFMAN, A. Enhanced gastrointestinal absorption of lipophilic drugs. In: TOUITOU, E; BARRY, B.W.(editors). **Enhancement in Drug Delivery**. New York: CRC Press Taylor & Francis Group, 2007, p. 11-31.

DUNHAM, N.; MIYA, T. A note on a simple apparatus for detecting neurological deficit in rats and mice. **Journal of the American Pharmaceutical Association**, v.XLVI, p. 208-209, 1957.

EISSA, A.A.M.; FARAG, N.A.H.; SOLIMAN, G.A.H. Synthesis, biological evaluation and docking studies of novel benzopyranone congeners for their expected activity as anti-inflammatory, analgesic and antipyretic agents. **Bioorganic & Medicinal Chemistry**, v. 17, p. 5059-5070, 2009.

FERRAZ, A.B.F.; BORDIGNON, S.A.L.; STAATS, C.; SCHRIPSEMA, J.; von POSER GL. Benzopyrans from *Hypericum polyanthemum*. **Phytochemistry**, v. 57, p. 1227-1230, 2001.

HAAS J.S.; VIANA, A.F.; HECKLER, A.P.; VON POSER G.L.; RATES, S.M.K. The antinociceptive effect of a benzopyran (HP1) isolated from *Hypericum polyanthemum* in mice hot-plate test is blocked by naloxone. **Planta Medica**, v. 76, p.1419-1423, 2010.

HENSCHKE, N.; KAMPER., S.; MAHER, C. The epidemiology and economic consequences of pain. **Mayo Clinic Proceedings**, v. 90, p. 139-147, 2015.

HUANG, P., KEHNER, G.B.; COWAN, A.; LIU-CHEN, L.Y. Comparison of pharmacological activities of buprenorphine and norbuprenorphine: norbuprenorphine is a potent opioid agonist. **Journal of Pharmacology and Experimental Therapeutics**, v. 297, p. 688–69, 2001.

INTERNATIONAL ASSOCIATION FOR THE STUDY OF PAIN (IASP), **IASP taxonomy**. <http://www.iasp-pain.org/Taxonomy#Pain>. Updated October 20, 2014. Accessed May 5, 2016.

KOTTA, S.; KHAN, A.; PRAMOD, K.; ANSARI, S.; SHARMA, R.; ALI, J. Exploring oral nanoemulsions for biovaliability enhancement of poorly water soluble drugs. **Expert Opinion on Drug Delivery**, v. 9, p. 585-598, 2012.

KUMAR, N. WHO Normative Guidelines on Pain Management. **World Health Organization**, 2007.

LAWRENCE, M.J. Microemulsions as drug delivery vehicles. **Current Opinion in Colloid and Interface Science** , v. 1, p. 826-832, 1996.

LI, Y.; HU, M.; McCLEMENTS, D.J. Factors affecting lipase digestibility of emulsified lipids using an in vitro digestion model: proposal for a standardised pH-stat method. **Food Chemistry**, v. 126, p. 498-505, 2011.

LENNERNÄS, H. Animal data: The contributions of the Ussing chamber and perfusion systems to predicting human oral drug delivery *in vivo*. **Advanced Drug Delivery Reviews**, v. 59, p.1107-1120, 2007.

LUFTY, K.; COWAN, A. Buprenorphine: A unique drug with complex pharmacology. **Current Neuropharmacology**, v. 2, p. 395-402, 2004.

MARTIN, W.R.; EADES, C.G.; THOMPSON, J.A.; HUPPLER, R.E.; GILBERT, P.E The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal dog. **Journal of Pharmacology and Experimental Therapeutics**, v. 197, p.517–53, 1976.

McBETH, J.; JONES, K. Epidemiology of chronic musculoskeletal pain. **Best Practice & Research: Clinical Rheumatology,** v. 21, p. 403-425, 2007.

McCLEMENTS, D. Edible nanoemulsions: fabrication, properties, and functional performance. **Soft Matter**, v. 7, p. 2297-2316, 2011.

McLEMENTS, D. Nanoemulsion-based oral delivery systems for lipophilic bioactive components: nutraceuticals and pharmaceuticals. **Therapeutic Delivery***,* v. 4, p. 841-857, 2013.

MOGIL, J.S.; PASTERNAK, G.W. The molecular and behavioral pharmacology of the orphanin FQ/nociceptin peptide and receptor family**. Pharmacological Reviews**, v. 53, p. 381– 415, 2001.

NÖR, C. ; ALBRING, D. ; FERRAZ, A.B.F. ; SCHRIPSEMA, J. ;, PIRES, V. ; SONNET, P. ; GUILLAUME, D. ; von POSER, G.L. Phloroglucinol derivatives from four

Hypericum species belonging to the Trigynobrathys section. **Biochemical Systematics and Ecology**, v. 32, p. 517-519, 2004.

PARSONS, A.A.; BINGHAM, S.; RAVAL, P.; READ, S.; THOMPSON, M.; UPTON, N. Tonabersat (SB-220453) a novel benzopyran with anticonvulsant properties attenuates trigeminal nerve-induced neurovascular reflexes. **British Journal of Pharmacology**, v. 132, p. 1549-1557, 2001.

PARVEEN, R.; BABOOTA, S.; ALI, J.; AHUJA, A.; VASUDEV, S.S.; AHMAD, S. Effects of silymarin nanoemulsion against carbon tetrachloride-induced hepatic damage. **Archives of Pharmacal Research**, v. 34, p. 767-774, 2011.

POUTON, C.W. Formulation of poorly water-soluble drugs for oral administration: physicochemical and physiological issues and the lipid formulation classification system. **European Journal of Pharmaceutical Sciences**, v. 29, p. 278-287, 2006.

PRASANNA, S.; MANIVANNAN, E.; CHATURVEDI, S.C. Quantitative structure-activity relationship analysis of a series of 2.3-diaryl benzopyran analogues as novel selective cyclooxygenase-2 inhibitors. **Bioorganic & Medicinal Chemistry Letters**, v. 14, p. 4005-4011, 2004.

QIAN, C.; DECKER, E.A.; XIAO, H.; McCLEMENTS, D.J. Nanoemulsion delivery systems: influence of carrier oil on beta-carotene bioaccessibility. **Food Chemistry**, v. 135, p. 1440-1447, 2012.

RAJPOOT, P.; PATHAK, K.; BALI, V. Therapeutic applications of nanoemulsion based drug delivery systems: a review of patents in last two decades. **Recent Patents on Drug Delivery and Formulation**, v. 5, p. 163-172, 2011.

SADEE, W.; ROSENBAUM, J.S.; HERZ, A. Buprenorphine: differential interaction with opiate receptor subtypes *in vivo*. **Journal of Pharmacology and Experimental Therapeutics**, v. 223, p. 157–162, 1982.

SARI, T.P.; MANN, B.; KUMAR, R.; SIGH, R.R.B.; SHARMA, R., BHARDWAJA, M.; ATHIRA, S. Preparation and characterization of nanoemulsion encapsulating curcumin. **Food Hydrocolloid**, v. 43, p. 540-546, 2015.

TANG, S.; SIVAKUMAR, M.; MIN-HWEI, A.; SHRIDHARAN, P. Anti-inflammatory and analgesic activity of novel oral aspirin-loaded nanoemulsion and nano multiple emulsion formulations generated using ultrasound cavitation. **International Journal of Pharmaceutics**, v. 430, p. 299-306, 2012.

TIWARI, S.; AMIJI, M. Improved oral delivery of paclitaxel following administration in nanoemulsion formulations. **Journal of Nanoscience and Nanotechnology**, v. 9, p. 3215-3222, 2006.

TRELLE, S.; REICHENBACH, S.; WANDEL, S.; HILDEBRAND, P.; TSCHANNEN, B.; ADLAKA, R.; EGGER, M.; JÜNI P. Cardiovascular safety on non-steroidal anti-inflammatory drugs: network meta-analysis. **British Medical Journal**, v. 342, p. c7086, 2011.

YU, W.; TABOSA DO EGITO, E.S.; BARRATT, G.; DEVISSAGUET, J.P.; PUISIEUX, F.A. Novel approach to the preparation of injectable emulsions by spontaneous emulsification process. **International Journal of Pharmaceutics**, v. 15, p. 139-146, 1993.

WANG, J.J.; HUNG, C.F.; YEH, C.H.; FANG, J.Y. The release and analgesic activities of morphine and its ester prodrug, morphine propionate, formulated by water-in-oil nanoemulsions. **Journal of Drug Targeting**, v. 16, p. 295-301, 2008.

WELCH, S.P. Interaction of the cannabinoid and opioid systems in the modulation of nociception. **International Review of Psychiatry**, v. 21, p. 143-151, 2009.

WOODCOCK, J. A difficult balance – pain management, drug, safety and the FDA. **The New England Journal of Medicine**, v.361, p. 2105-2107, 2009.

Supplementary Data

Material and Methods

HP1 identification

In order to identify the benzopyran HP1 spectroscopic and spectrometric methods were performed.

Magnetic Nuclear Resonance (^{1}H and ^{13}C) was performed on a Varian MR400 spectrometer (at 400 MHz to ^{1}H and 100 MHz to ^{13}C). Spectra were recorded in $CDCl_3$ (Sigma Aldrich, St Louis, United States). Mass spectra was acquired in a positive-ion mode on a Waters Q-TOF (Waters Corp., United States).

Analitical methodology validation

Chromatographic conditions

The chromatographical analysis was performed using a High Performance Liquid Chromatograph Shimadzu® managed by the Software Labsolutions (1,24 SP2 version), with two pumps system Shimadzu® (LC-6AD), automatic injector Shimadzu® (10AD) and UV/Vis dual detector Shimadzu® (SPD 20AV). The separations were carried out with an isocratic system composed by acetonitrile (60%) and water (40%) using a Waters Nova-Pack C18 column (4 µm, 3,9 mm x 150mm) adapted to a guard column Waters Nova-Pack C18 60Å (3,9 mm x 20 mm). The flow rate was 1mL/min and the injection volume 20 µL. The detector sensibility was 1AUFS and the detection was performed in λ=230 nm in 25ºC.

Analytical validation

The methodology to quantify the benzopyran HP1 was validated following the normatives of ICH (The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) (Document Q2A, 1996). The evaluated parameters were linearity, precision, robustness and limit of detection and quantification.

To the linearity curves, methanolic solutions of benzopyran HP1 were prepared in seven concentrations (125; 93.75; 31.25; 15.62; 7.81; 3.91 e 1.955 µg/mL). The solutions were injected in triplicate. The average of injections was obtained after peak integration corresponding to HP1. After integration, a graphic was constructed plotting area versus concentration to obtain the calibration curves. The standard deviation (SD) and the relative standard deviation (RSD) were considered to each solution. The curves were repeated in three different experimental days.

The limits of detection (DL) and quantification (QL) were determined according the calibration curve, following the equations:

$$DL=3,3 \times sd/lc$$

$$QL=10 \times sd/lc$$

Where sd represents the intercept standard deviation and lc the inclination average of each curves.

Intermediate precision (interday and intraday) of chromatographic procedure was assessed by determining the RSD of values obtained after three injections in three different days. Three concentrations were evaluated within the calibration curve (125; 15.62 e 1.955 µg/mL). The robustness was assessed alternating few modifications in the oven temperature. Three concentrations were evaluated (125; 15.62 e 1.955 µg/mL) at three temperatures (20, 25 e 30 ºC). The injections were made in triplicate.

Results and Discussion

HP1 identification

The results obtained by spectroscopic (Figures 1 and 2) and spectrometric (Figure 3) analyzes were compared with authentic samples.

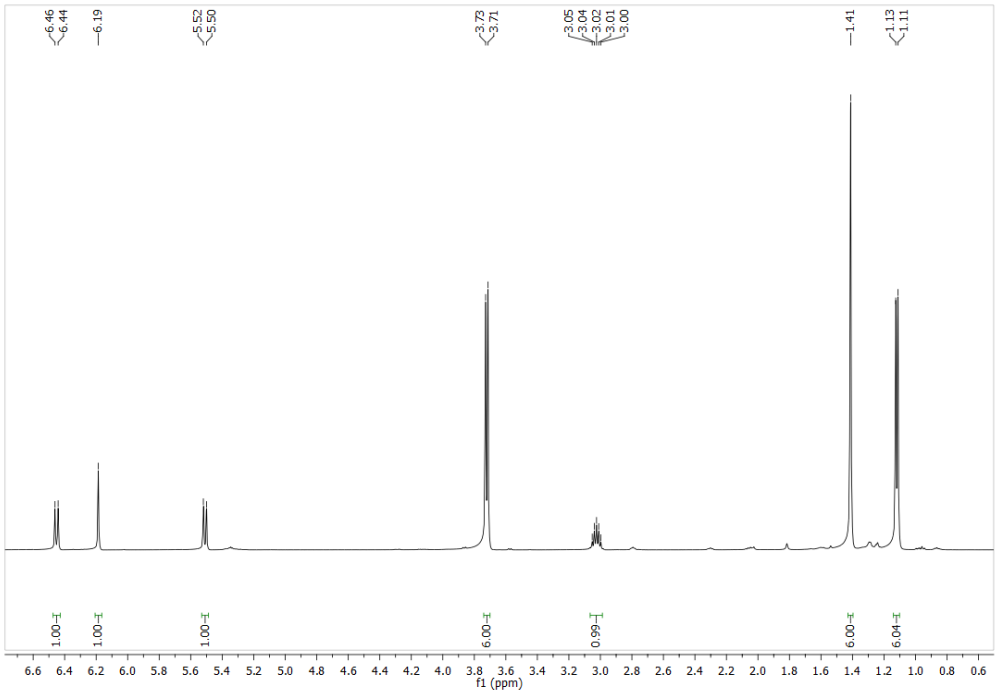

Figure 1. ^{1}H Nuclear Magnetic Ressonance of HP1

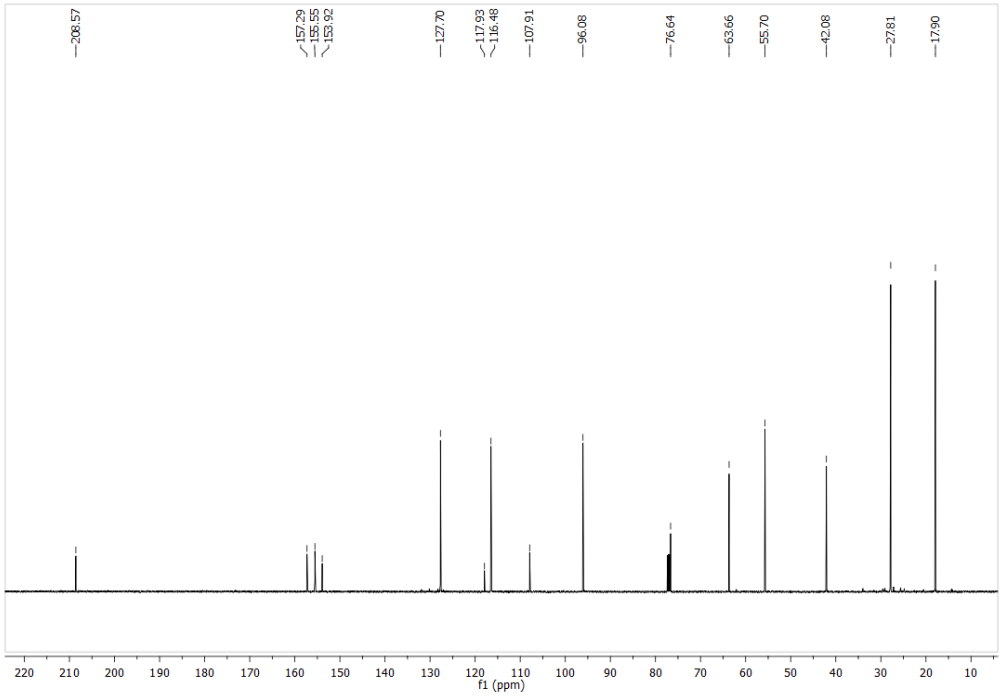

Figure 2. ^{13}C Nuclear Magnetic Ressonance of HP1

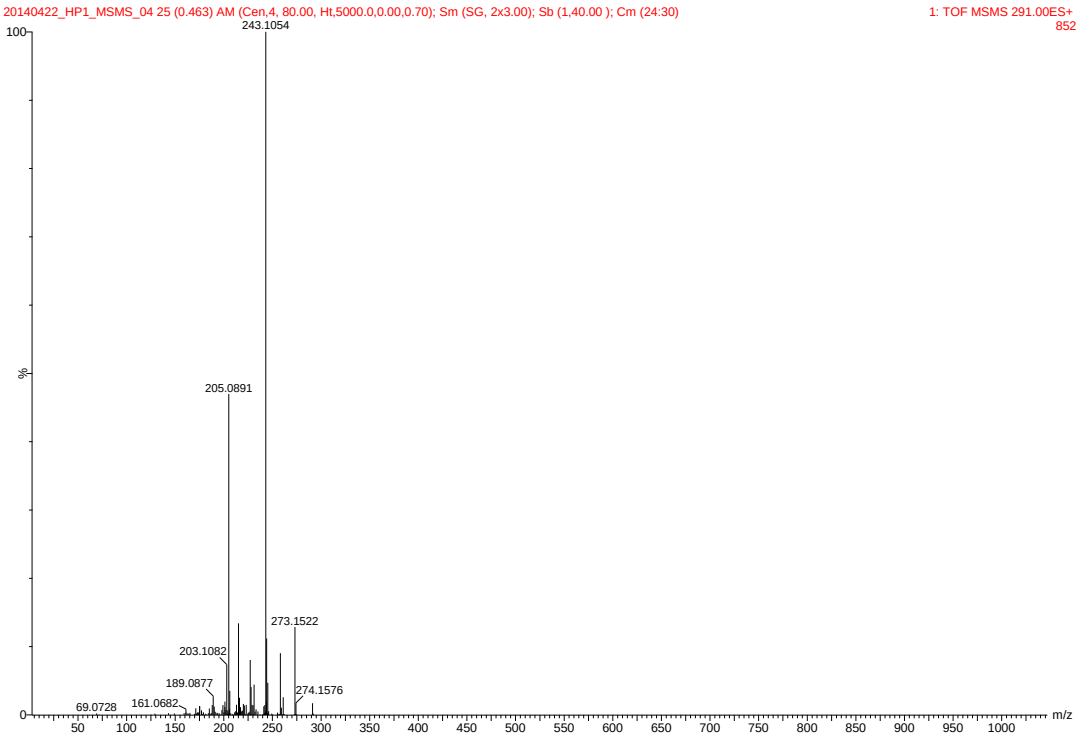

Figure 3. Mass spectra of HP1

Validation of an HPLC method for HP1 determination

Concerning the validation procedure, the HP1 retention time was approximately 8.4 minutes with intraday variation coefficient 0.48. The total time of analysis, 15 minutes, was suitable with total elution of compound. The chromatographic profile could be visualized in figure 4.

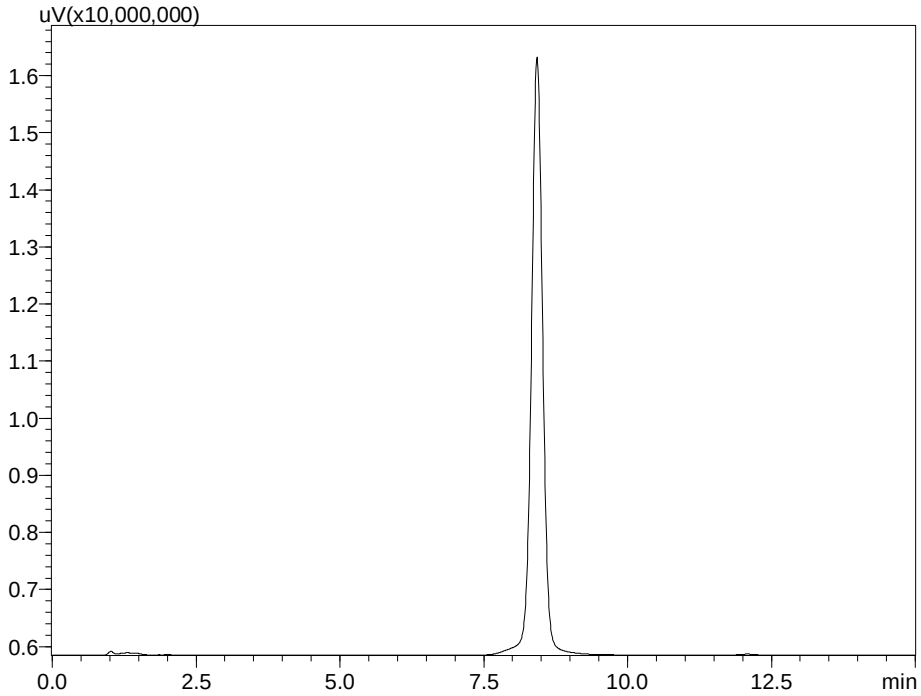

Figure 4. Chromatographic profile of benzopyran HP1 using the proposal method in this study.

The linearity of method was performed at seven concentrations, between 1.995 and 125 µg/mL. The table 1 represents the used concentrations to built the linearity curve. The graphical respresentation of curve can be visualized in figure 5.

Table 1. Peak areas in the analysed concentrations by HPLC

Concentration (µg/mL)	Área ± SD	RSD (%)
125	11363559 ± 45697	0.32
93.75	8435034± 123846	1.47
31.25	2920170 ± 31758.2	1.08
15.62	1537170 ± 20159.5	1.31
7.81	779377.9 ± 14496	1.85
3.1	385465.2 ± 4977.8	1.29
1.995	224943.3 ±3528.6	1.56

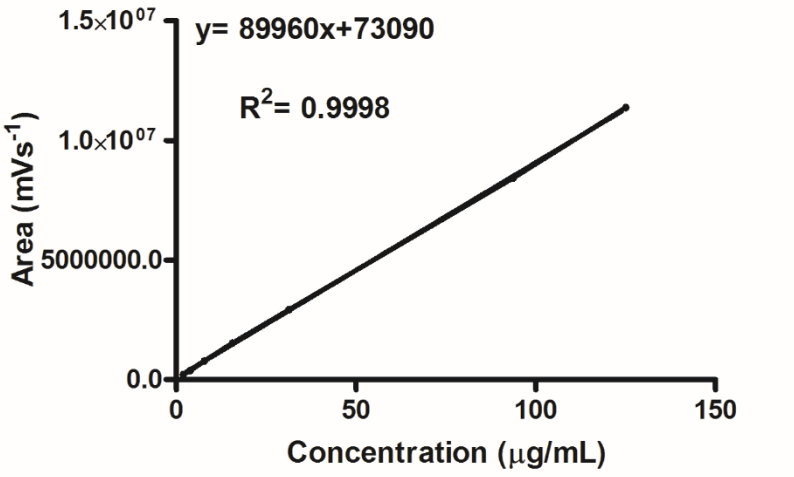

Figure 5. Calibration curve of the benzopyran HP1 by HPLC.

In the linear regression, the determination coefficient (R^2) that represents the association grade between the independent variable (x) and the dependent variable (y) must be closer to 1, which means strong correlation between the variables. The determination coefficient 0.9998 shows that the correlation between benzopyran concentration and equipament response is suitable. Evaluating the linear regression, significative F values to $\alpha<0.0001$ (80480) were found, proving the linear relation of variables.

The detection limit (DL) is expressed as the smaller analyte concentration that could be detected by the proposal method, while the quantification limit (QL) is the smaller concentration that could be quantified with suitable accuracy and precision (Document Q2-A, ICH, 1996). Using the equations presented in the material and methods section, were found values of 0.28 µg/mL and 0.944 µg/mL to DL and QL, respectively. These values demonstrated that the proposed method is enough sensitive to analyze the benzopyran HP1.

In order to evaluate the method precision the repeatibility (intraday precision) and intermediary precision (interday precision) assay were performed. The results were presented in table 2.

Table 2. Peak areas of benzopyran HP1 in the precision tests

	Conc[a]. (µg/mL)	**Day 1**	**Day 2**	**Day 3**	**Mean ± SD**	**RSD (%)**
Interday precision	125	11327862	11343581	11496882	1138944.7 ± 93377.41	0.82
	15.62	1517011	1544175	1533183	1531456.3 ± 13644.07	0.89
	1.955	224943.3	229855.7	221572	225457 ± 4165.65	1.85
		Test 1	**Test 2**	**Test 3**	**Mean ± SD**	**RSD (%)**
Intraday precision	125	11330710	11430570	1122307	1137862.3 ± 104260.7	0.91
	15.62	1526125	153125	1501782	1517010.67 ± 13273.44	0.87
	1.955	200461	197724	198872	199019 ± 1374.41	0.69

[a]Concentration

The variations observed in the results could be considered appropriate once the percentual relative standart deviation values are lower than 2%. In accordance to these results, it is possible to affirm that the method is precise (ICH, 1996).The robustness

was evaluated based on little modification in the oven temperature. The results are presented in table 3.

Table 3. Robusteness presented by the proposal method in function of temperature variation

Conc.[a] (µg/mL)	Peak area 20ºC	Peak area 25ºC	Peak area 30ºC	Peak area Mean ± SD	RSD (%)
125	11628959	11514196	11463848	11535668± 84624.07	0.73
15.62	15172829	1533183	1565155	1557056 ± 21027.44	1.35
1.955	223012.70	221572	222033.30	222206 ± 735.69	0.33

[a]Concentration

References

ICH. International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use. **Validation of Analytical Procedures: Text and Methodology Q2(R1)**, 1996.

5 Capítulo 3 – Avaliação da permeabilidade intestinal de HP1 livre e incorporado em nanoemulsões triglicerídicas

Os resultados apresentados neste capítulo foram obtidos nos Laboratórios de Farmacognosia e Institut Galien, Université Paris Sud, sob orientação dos professores **Dra. Gilsane Lino von Poser**, **Dr. Gilles Ponchel**, respectivamente.

A administração oral de compostos é a via com maior aceitação pelo paciente, principalmente, pela facilidade de administração. No entanto, essa via apresenta problemas relacionados à baixa absorção intestinal e, por conseguinte, biodisponibilidade. A baixa absorção é resultante das propriedades físico-químicas dos compostos, fatores fisiológicos e fatores relacionados a forma de dosagem (WASHINGTON, 2001; AGÜEROS et al., 2011).

As propriedades físico-químicas dos compostos e a fisiologia do trato gastrointestinal afetam a permeação de moléculas na membrana das células intestinais. Fatores como solubilidade, coeficiente de partição (Log D, Log P), pKa, peso molecular, formação de agregados, tamanho de partícula, pH no lúmen intestinal, área absortiva, fluxo sanguíneo intestinal e ação de enzimas influenciam na absorção por essa via (UNGELL, 1997; UNGELL e ABRAHAMSSON, 2001). Apesar desses fatores estarem fortemente envolvidos, a solubilidade em água e a permeabilidade frente a células intestinais, são os fatores determinantes da alta ou baixa absorção de compostos (WASHINGTON, 2001), sendo que aqueles com baixa solubilidade em água apresentam a absorção mais deficiente.

A fim de contornar a problemática de absorção de compostos com baixa solubilidade em água, os mesmos têm sido incorporados em sistemas de liberação lipídicos que podem contornar o problema de baixa absorção aumentando a solubilidade e /ou a dispersão no ambiente gastrointestinal aquoso. Ainda, esse tipo de formulação apresenta impacto positivo na permeabilidade intestinal o que por consequência, melhora sua absorção (CARRIÈRE, 2015). Esses sistemas mantém o composto em um a forma coloidal dispersa evitando que o mesmo precipite nos fluidos intestinais, para então ser absorvido na forma de micelas, micelas mistas ou vesículas em conjunto com os sais biliares da digestão (CHAKRABORTY et al., 2009).

Há diversas técnicas utilizadas para avaliar permeabilidade intestinal *in vitro*. Destacam-se os modelos que utilizam membranas artificiais (*PAMPA*) (KANSY et al., 2001), modelos de cultura celular (Caco-2) (BALIMANE e CHONG, 2005) e modelos *ex-vivo* utilizando tecidos intestinais (*Ussing Chambers*) (UNGELL et al, 1997). O modelo mais amplamente utilizado é o modelo de cultura celular (Caco-2), embora o modelo *ex-vivo* de *Ussing chambers* apresente algumas vantagens como expressão de transportadores de membrana, manutenção da produção de muco intestinal e

dados experimentais que podem ser comparados diretamente quando utilizados o mesmo modelo animal *in vivo* (MAZAFERRO et al., 2012).

5.1 Absorção intestinal de compostos

A via oral é a via preferencial para administração de compostos em virtude das vantagem que a mesma oferece em relação as outras vias (não invasiva, menor custo, menos efeitos adversos em relação ao local de administração, etc). A importância dessa via de administração é refletida no fato de que >60% das novas entidades moleculares recebidas pelo FDA (Food and Drug Administration) para a aprovação, em 2014, eram formulações destinadas a via oral (MULLARD, 2015).

Compostos administrados pela via oral devem atravessar o trato gastrointestinal antes de alcançar os capilares que levam a veia porta. Esse sistema compeende vários órgãos com funções distintas:

(1) Boca ou cavidade bucal: onde está a saliva composta de muco, água, sais e enzimas (amilase e lipase);

(2) Faringe e esôfago: agem como um caminho até o estômago;

(3) Estômago: onde ocorre a digestão. Contém suco gástrico composto de ácido clorídrico e algumas enzimas digestivas como pepsina e a lipase gástrica;

(4) Intestino delgado: é dividido em três seções (duodeno, jejuno e íleo), onde a maior parte da aborção ocorre com a passagem de compostos diretamente para a circulação sistêmica, ou então, primeiramente para a circulação linfática e após, para o sangue.

Os enterócitos são células epiteliais presentes no intestino envolvidas na absorção de compostos sejam eles endógenos ou exógenos. Essas células expressam transportadores dependentes de trisfosfato de adenosina (ATP) nas membranas apical e basolateral que participam da absorção e/ou efluxo de moléculas (HUGHES et al., 2008). Apesar de haver especificidade de substratos para os transportadores, esta é bem menor do que aquela encontrada para enzimas. No entanto, alguns casos devem ser considerados como especificidade para peptídeos

(PEPT) e moléculas ionizadas (PMAT, OTCN, OCT) (THE INTERNATIONAL TRANSPORTER CONSORTIUM, 2010; ESTUDANTE et al., 2013).

Além dos transportadores já mencionados, receptores presentes no sistema nervoso entérico (como opioides e canabinoides) (MASSA et al., 2005; HOLZER, 2009) também podem estar presentes na membrana dos enterócitos. O receptor canabinoide CB_1 por exemplo, pode ser encontrado na membrana dessas células epiteliais, principalmente aquelas presentes no cólon (IZZO e SHARKEY, 2010). Entretanto, ainda não está claro se esse tipo de receptor pode ter envolvimento com o transporte de substâncias para a corrente sanguínea, porém sabe-se que na presença de agonistas canabinoides, a permeabilidade intestinal se mostra aumentada em virtude da redução de expressão de proteínas das *tight junctions*, e consequente abertura das mesmas (MACCARRONE et al., 2015).

Ao chegar no intestino delgado, as moléculas podem ser absorvidas por transporte passivo ou ativo (via transportadores ATP dependentes). A difusão passiva compreende dois fenômenos: paracelular, onde o composto difunde através de poros aquosos presentes nas *tight junctions* (entre duas células epiteliais) e transcelular, onde a difusão se dá através da borda em escova dos enterócitos. O transporte ativo, por sua vez, ocorre através dos transportadores mencionados anteriormente (HURST et al., 2007).

Antes de alcançar a membrana dos enterócitos, compostos devem atravessar a barreira mucosa que protege as células epiteliais. Nessa barreira, muitos compostos ou sistemas de liberação podem ficar presos e serem assim removidos pela renovação dessa camada (ocorre aproximadamente entre 47 e 270 minutos) (LEHR et al., 1991; DÜNNHAUPT et al., 2015). A camada mucosa é composta de proteínas, carboidratos, lipídeos e altas quantidades de água (EHEHALT et al., 2004; HANSSON, 2012). As principais proteínas formadoras dessa camada são as mucinas, glicoproteínas secretadas pelas células calciformes dos intestinos delgado e grosso (ALLEN et al., 1998; KIM e HO, 2010). Essas glicoproteínas possuem carga negativa devido aos seus carboidratos (resíduos de sulfato e ácido sialílico) (LARSSON et al., 2009; PEARSON e BROWNLEE, 2010).

(5) Intestino grosso: armazena temporariamente substâncias não digeridas;

(6) Pâncreas e fígado: apesar de não pertencerem ao trato gastrointestinal propriamente dito, estas glândulas exócrinas contribuem substancialmente no processo de absorção intestinal de substâncias. O pâncreas secreta enzimas digestivas (proteases, lipases e amilases) a fim de digerir componentes de alimentos (carboidratos, gorduras, proteinas, etc) e bicarbonato de sódio para neutralizar os ácidos estomacais. O fígado, por sua vez, produz a bile que contém os sais biliares e fosfolipídeos que agem como surfactantes para emulsificar lipídeos, aumentando a absorção.

5.2 Absorção intestinal de compostos lipofílicos

Compostos lipofílicos possuem baixa solubilidade em água o que leva a baixa dissolução nos fluidos intestinais e absorção dessas moléculas pela via oral. Além disso, a baixa solubilidade também pode ocasionar variabilidade e falta de proporcionalidade nas doses administradas (PORTER e CHARMAN, 2001).

Nas últimas décadas, a administração oral de compostos lipofílicos tem tomado uma nova dimensão com o advento dos sistemas de liberação lipídicos, como carreadores de compostos pouco solúveis em água (POUTON, 2006). Dentre esses sistemas destacam se as nanoemulsões definidas como sistemas isotrópicos dispersos com tamanho de gotícula entre 50-500 nm (SOLANS et al., 2003), termodinamicamente estáveis .

Após a administração oral de nanoemulsões, a lipase gástrica inicia o processo de digestão dos triglicerídeos. Simultaneamente, a agitação mecânica (propulsão e retropropulsão) do estômago facilita a formação de uma emulsão composta pelos fluidos gástricos aquosos e produtos da digestão lipídica. Após, no intestino delgado, os triglicerídeos não digeridos são transformados em diglicerídeos, monoglicerídeos e ácidos graxos livres pela ação da lipase pancreática e co-lipase. Assim, a presença de lipídeos exógenos no intestino delgado faz estimula a secreção de sais biliares, fosfolipídeos e colesterol pelo fígado. Dessa forma, os produtos da digestão lipídica são incorporados em estruturas coloidais, incluindo micelas e vesículas unilamelares ou multilamelares formadas na presença dos sais biliares. Essas espécies coloidais são então absorvidas por transporte ativo ou passivo (CHAKRABORTY et al., 2009).

Além disso, é importante salientar que sistemas de liberação lipídicos como nanoemulsões que apresentam carga negativa, expressa em termos de potencial zeta, tem mais facilidade de atravessar a camada mucosa pois não sofrem o fenômeno de atração eletrostática com as glicoproteínas presentes no muco e assim chegam mais facilmente na borda das células epiteliais do que sistemas positivamente carregados que podem ser imobilizados no muco por interações iônicas (CRATER e CARRIER, 2010).

5.3 Permeabilidade intestinal

A perrmeabilidade intestinal em conjunto com solubilidade, taxa de dissolução e trânsito gastrointestinal, é uma das variáveis biofarmacêuticas que determina a extensão de absorção intestinal de compostos, tanto em humanos como em animais (LENNERNAS et al., 1992; AMIDON et al., 1995).

É importante considerar que a permeabilidade intestinal depende de múltiplos mecanismos de transporte, paralelos ou não, como: difusão passiva (transcelular), transporte ativo e efluxo (mediados por carreadores dependentes de ATP). Além disso, compostos também podem permear pela via paracelular, apesar de estudos *in vivo* demonstrarem que essa via contribui muito pouco para a absorção de compostos farmacológicamente ativos (FAGERHOLM et al., 1996).

O jejuno é a região do intestino delgado onde ocorre a absorção da maior parte dos compostos. Esse fato se dá por essa região do intestino possuir maior área superficial e por ser o local onde a maioria dos transportadores ativos está localizada (LENNERNAS et al., 1992; SUN et al., 2002).

O método mais comumente utilizado para avaliar permeabilidade intestinal *in vitro* é aquele que utiliza células Caco-2 (derivadas de adenocarcinoma humano de cólon) (BALIMANE e CHONG, 2005). Essas células sofrem, em cultura, diferenciação espontânea e se tornam polarizadas (com uma superfície apical e outra basolateral), assumindo assim características de enterócitos com *tight junctions* e exibindo funções inerentes às células epiteliais do intestino. Apesar dessa diferenciação, os transportadores de membrana são expressos por essas células em quantidades muito menores quando comparada a situação *in vivo* (SUN et al., 2002). Além disso, não há

produção de muco por essas células fazendo com que sua influência não possa ser estudada (DEFERME et al., 2008). Modelos de co-cultura celular (por exemplo Caco2/HT29) tem sido propostos (WIKMAN-LARHED e ARTURSSON, 1995). Entretanto, o uso de tecido vivo e intacto se mostra mais realístico, como por exemplo o modelo de *ussing chambers*.

O modelo de *ussing chambers* é eficiente para investigações de transporte e metabolismo de compostos *ex vivo*. Este modelo utiliza porções teciduais de intestino que são montadas entre dois compartimentos de difusão celular (UNGELL, 1997). Os compartimentos mucoso e seroso são normalmente mantidos em tampão Krebs-Ringer bicarbonato que é continuamente gaseificado com uma mistura O_2:CO_2 (95:5) para prover tensão de oxigênio suficiente para manter a viabilidade do tecido (DEFERME et al., 2002). O composto a ser testado é então adicionado no lado mucoso ou seroso para estudar o sentido absortivo ou secretório. Ainda, parâmetros eletrofisiológicos são monitorados ao longo do experimento. Dessa forma, a diferença de potencial reflete o gradiente de voltagem gerado pelo tecido, a resistência transepitelial, a integridade tecidual e a corrente de curto circuito os fluxos iônicos ao longo do epitélio (POLENTARUTTI et al., 1999).

As maiores aplicações da técnica de *ussing chambers* são a determinação do transporte transepitelial de compostos em combinação com metabolismo intestinal (ROGERS et al., 1987; ANNAERT et al., 2000), estudo de diferenças regionais na absorção intestinal (NEJDFORS et al., 2000; HWANG et al., 2002; BAJKA et al., 2003), e o envolvimento de transportadores intestinais na absorção de compostos (MOLS et al., 2005; ZAKELJ et al., 2006). No entanto, esse método não foi desenvolvido como uma ferramenta de *high throughput screening*, mas se utilizado da forma correta, oferece diversas vantagens em relação a outros utilizados para o mesmo fim (UNGELL, 2002).

Para começar há uma boa correlação com a permeabilidade aparente do jejuno humano, *in vivo* (LENNERNÄS et al., 1997), para compostos de baixa e alta permeabilidade, principalmente quando o transporte passivo está envolvido. Ainda, a técnica pode ser utilizada em diferentes regiões do trato gastrointestinal, avaliando assim características regionais de absorção de compostos (UNGELL et al., 1997; POLENTARUTTI et al., 1999). Este método também é muito útil na avaliação de

mecanismos de absorção (UNGELL, 2002). Por fim, poder ser utilizados tecidos humanos, porções de biópsia e porções intestinais de animais (SÖDERHOLM et al., 1998; SJÖSTRÖM et al., 2000; UNGELL, 2002).

Dessa forma, os resultados apresentados neste capítulo exploram os parâmetros que regem a permeabilidade intestinal de HP1 na sua forma livre e quando incorporado em nanoemulsões, pelo método de *Ussing chambers*. Os resultados estão apresentados na forma de um manuscrito científico.

MANUSCRITO 3- Intestinal permeation enhancement of benzopyran HP1 when loaded in triglyceride nanoemulsions.

Manuscrito em preparação

Intestinal permeation enhancement of benzopyran HP1 when loaded in triglyceride nanoemulsions.

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Abstract

The benzopyran HP1 is a substance known to exert antinociceptive action in mice (p.o). Although HP1 pharmacological potential, its poor water solubility leads to poor intestinal permeation and absorption. To overcome these hindrances, alternatives as incorporation in nanostructured lipidic release systems have been used. Thus, the aims of this study were to evaluate the intestinal permeability of HP1 (free form and loaded in nanoemulsions) in the Ussing chamber model (*ex vivo*) to have a better understanding of how this molecule can be absorbed following oral delivery. The Ussing chambers experiments demonstrated significant differences in the permeation fluxes through the rat jejunum intestinal membrane, with higher values in the absorptive direction, indicating that active transporters were, at least partially, involved in the HP1 transport across intestine. Since HP1 structure looks like a cannabinoid ligand, the implication of intestinal cannabinoid receptor (CB_1) in HP1 absorption was investigated using different intestinal portions: jejunum and colon. No differences were found when jejunum tissue was used. In contrast, in colonic tissue the results presented an interesting effect, with a possible interaction between HP1 and CB_1 receptor. Regarding the release system, HP1 permeation loaded in nanoemulsions was assessed. The nanoemulsion apparent permeability was 4 times higher than HP1 free form, thus improving its absorption. Together, these results help to understand the absorption process of benzopyran HP1 (free form and loaded in nanoemulsions). Ussing chambers are an interesting model in pre-clinic studies since some robust correlations have been established between permeability obtained in rats and humans for both absorption mechanisms: carrier-mediated or passive diffusion. Applied to HP1,

these correlations suggest that this molecule as a fairly good probability to be efficiently delivered to humans, provided an adequate formulation based in lipid release systems and able to ensure an adequate dissolution profile in the gastrointestinal tract.

Keywords: Benzopyran HP1, intestinal permeability, cannabinoid receptor, nanoemulsion

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Introduction

The genus *Hypericum* (Hypericaceae) represents a source of compounds with antinociceptive activity (BONKANKA et al., 2011; BRIDI et al., 2016; RAZIQ et al., 2016). Among them, benzopyran HP1 from *Hypericum polyanthemum* Klotzsch ex Reichardt, is a substance known to exert this action in mice by oral route (HAAS et al., 2010; MEIRELLES et al., 2016 not published data).

Scientific evidences pointed out that the benzopyran skeleton derivatives could be responsible by pharmacological activities due to a synergism between the systems opioid and cannabinoid (CICHEWICZ, 2004; DESROCHES and BEAULIEU, 2010). Some molecules with this type of structure have demonstrated antinociceptive activity such as Δ^9-tetrahydrocannabinol (THC) (WELCH et al., 2009) and sculetin (RZODKIEWICZ et al., 2015).

Cannabinoids and opioids produce analgesia through a G-protein system coupled mechanims, that could even be co-localized in the same neuron (CICHEWICZ, 2004). Furthermore, studies have demonstrated that the analgesic effect of the most famous cannabinoid molecule, THC, is at least in part, mediated by opioid receptor, indicating a connection between this two signaling pathways (MASON et al., 1999; WELCH and EADES, 1999). HAAS et al (2010) have demonstrated that HP1 exerts its antinociceptive activity by opioid system and since the connection between opioid and cannabinoid pathways has already been described, the hypothesis of HP1 be a substract of CB_1 receptor on intestinal epithelial cells (MACCARRONE et al., 2015) aiming oral absorption should be further investigated.

The oral administration is the dominant delivery route because offers many advantages such as being painless and easily self administrable resulting in a high patient acceptance (BERNKOP-SCHNÜRCH, 2013). In spite of these advantages, currently drug developments projects are impaired because of poor gastrointestinal absorption, and then, poor biavailability (LENNERNÄS, 2007). In accordance with this fact, several marketed drugs presents problems of low permeability, rapid metabolism and poor safety and tolerability (HODJSON, 2001). It is estimated that approximately 40% of approved drugs and 90% of drugs in development are classified as poorly water soluble (<100 µg/mL) (TAKAGI et al., 2006). Therefore, since the water solubility is a

critical determinant to drug absorption, a poor dissolution rate could implicates in low permeability, thus, alternatives that improves this aspect are necessary.

Recently, we have demonstrated that a triglyceride nanoemulsion is capable of improving the antinociceptive activity of benzopyran HP1 by oral route (MEIRELLES et al., 2016 not published data) and the better explanation is the higher solubilization and, and consequently, absorption by epthelial cells from small intestine.

Lipid based drug delivery systems, as nanoemulsions, have gained attention in drug delivery of poorly water soluble (eg. HP1) drugs due to their ability to improve the solubility of these drugs (JANNIN et al., 2008). The absorption of drug from these systems depends on many factors including particle size, degree of emulsification and lipolysis and rate of dispersion (POUTON, 2000).

Thus, the aim of this study was to evaluate the intestinal permeability of HP1 (free form and loaded in nanoemulsions) in the Ussing chamber model (*ex vivo*) to have a better understanding of how this molecule can be absorbed and exert its antinociceptive activity following oral delivery. Moreover, an *in vitro* digestion method was performed to know the impact of enzymatic lipolysis in the nanoemulsion. Finally, the influence of intestinal mucus in the nanoemulsion intestinal passage was achieved.

Material and Methods

Reagents

HP1 was isolated from aerial parts from *Hypericum polyanthemum* according Meirelles et al., 2016 (not published data). Sulfobutyl éther β-cyclodextrin was donated by Captisol® (California, United States). Pancreatin from porcine pancreas and rimonabant hydrochloride were purchased from Sigma Aldrich, France. Acetonitrile and Methanol HPLC grade were obtained from VWR reagents (Fontenay-sous-bois, France). Egg lechitin (Lipoid® E80) was kindly donated by Lipoid (Steinhausen, Switzerland) and medium chain triglycerides (Miglyol® 812) were acquired from Sasol (Hamburg, Germany). Krebs Bicarbonate Ringer Buffer at pH 7.4 with or without 1%

of sulfobutyl éther.β-cyclodextrin and 2% of pancreatin was prepared according the protocol described below.

Nanoemulsion preparation

Triglyceride nanoemulsion was prepared by means of spontaneous emulsification method (YU et al., 1993; BOUCHEMAL et al., 2003). The oil core (medium chain triglycerides (8%), egg lechitin (2%) and benzopyran HP1 (3.0 mg mL^{-1})) and aqueous components (polyssorbate 80 (1%)) were dissolved in an ethanol:acethone (50:50) mixture and ultrapure water, respectively. The oil phase was shed, slowly, under moderate magnetic stirring, on the aqueous phase. The solvent excess was eliminated under reduced pressure at 50ºC until de final volume required.

Physico-chemical characterization of nanoemulsion

The mean particle size and polydispersion index were detected through dynamic light scattering, in Malvern Zetasizer ZS (3000HS Zetasizer, Malvern Instruments, Worcestershire, UK) equipment. The zeta potential was determined according the particles eletroforetic mobility in the same equipment. The results were expressed as mean of three determinations of three different nanoemulsions.

Intestinal permeation studies

Krebs Ringer Bicarbonate Buffer

The studies were carried out in Krebs Ringer Bicarbonate Buffer (KBr Buffer) (UNGELL et al., 1992) with slightly modifications. The following composition was used: NaCl (108 mM), KCl (4.7 mM), Na_2HPO_4 (1.8 mM), KH_2PO_4 (0.4 mM), $NaHCO_3$ (15 mM), $MgSO_4$ (1.2 mM), $CaCl_2$ (1.25 mM) and *D*-glucose (11.5 mM), added of 1% of sulfobutyl éther β-cyclodextrin and 2% of pancreatin.

Ex-vivo experiments by "Ussing Chambers"

Male Wistar rats (200-250 g) were sacrificed by CO_2 gradient and then, jejunum or colon were excised, rinsed with cold Krebs Bicarbonate Buffer and cut into segments of 2-3 cm without Peyer´s Patches.

HP1 free form permeation experiments

The jejunum portions (1 cm^2) were mounted in "Ussing chambers" with modified Krebs Bicarbonate Buffer in the acceptor compartiment (mucosal or serosal side, depending on the direction to be analyzed). The sample was then placed in the respective compartiment, mucosal side when the absorptive direction was analyzed and serosal side when the secretory direction was analyzed. The system was maintained at 37ºC and continuously oxigenated with O_2/CO_2 (95:5%). At pre estabilished time intervals (0, 30, 60, 90,120 and 180 minutes) aliquots of 500 µL were withdrawal from the acceptor compartment and replaced by the same amounts of fresh buffer in order to maintain the mass balance. Aliquots were also withdrawal from the donator compartment at the beginning and at the end of experiment to monitor any changes in the concentration during the experiment. These assays were conducted in jejunum and colon. The experiments were repeated in the absorptive direction at 4ºC, where the activity of ATP-dependent transporters is disabled and the absorption occur only by passive diffusion. The samples were then analyzed by HPLC means, using a Waters 515 pump and a Waters 717 plus autosampler. UV absorbance at 230nm was monitored with a Waters 486 absorbance detector. Free HP1 (0.344 mM) was analyzed in both directions.

After that, the influence of the cannabinoid system in the transport of HP1 across intestinal membrane (jejunum and colon) was assessed by adding CB_1 inverse agonist, rimonabant, 30 minutes before addition of HP1 in Ussing chamber. This time of 30 minutes is enough to rimonabant bind in the sites of CB_1 receptor in order to investigate its influence in HP1 transport.

Furthermore, the apparent permeability was calculated following the equation:

$$\mathbf{Papp = (dQ/Dt) \times (1/AC_0)}$$

where (dQ/Dt) is the flow of HP1 across the intestinal membrane, A is the surface of membrane and C_0 is initial concentration of HP1. In order to standarize the conditions Papp was calculed between 30 and 120 minutes of experiment, obeying the curves linearity.

HP1 nanoemulsion permeation experiments

The experimental design was the same of HP1 free form, except that only the absorptive direction at 37ºC was analyzed. Moreover, a set of experiments with a semi-permeable membrane (cut-off 40,000 g/mol) was also conducted in order to evaluate the mucus influence in HP1 nanoemulsion passage across the intestinal membrane. In addition, the influence of CB_1 inverse agonist, rimonabant, in the nanoemulsion passage was investigated in the absorptive direction at 37ºC.

Electrical parameters

During all experiments, electrical parameters were evaluate in order to ensure the tissue viability. A four electrode system was used to achieve electrical measurements. Transmucosal potential difference (PD) was recorded between two KCl saturated bridges connected to a MDVC-2C voltage clamp (BioRad®, France) via calomel electrodes filled with KCl satured solution (2M). Potential difference was short-circuited during the experiment by a short-circuit current (Isc) via agar bridges placed in each half-cell and adapted to platinum electrodes connected to an automatic MDCV-2C voltage clamp. Delivered Isc (short-circuit current) was corrected for fluid resistance and recorded at pre-set times. The transmucosal epithelial resistance (%TEER) was calculed according the Ohm´s law:

$$\mathbf{TEER = \Delta U/10,}$$

$$\mathbf{\Delta U = U_{10} - U_0}$$

where, U= Transmucosal potential difference and $U_{10=}$ Transmucosal potential difference when a current of 10µA is applied.

In vitro nanoemulsion lipolysis

In vitro lipolysis experiments were conducted according GRIFFIN et al., 2014 with slightly modifications. 0.5 g of nanoemulsion was dispersed in 45 mL of digestion buffer (50 mM tris maleate, 150mM NaCl, 5 mM $CaCl_2$, pH = 7.5) added of 5 mM of taurocholic acid and 1.25 mM phosphatidylcholine (conditions simulating fasted state intestinal conditions). Digestion was initiated by addition of 5 mL of pancreatin extract (2% w/v), freshly prepared as described by SEK et al., 2002. Lipolysis was followed over 90 min at 37 ºC using a pHameter seven multi (Mettler Toledo®), and mantaining the pH at 7.5 using 0.2 M NaOH. Aliquots of 5 mL were withdrawal from the digestion medium at time zero (before addition of pancreatin solution) and at pre-set time intervals (5, 10, 15, 30, 60 and 90 minutes) after addition of pancreatin solution. Samples were subsequently centrifuged using a Optima LE 80K Beckman coulter ultracentrifuge (Beckman Instruments, Palo Alto, USA) equipped with 90 Ti rotor for 90 min at 37ºC and 30000 rpm in order to separate the sample into two phases: an intermediate aqueous phase (containing bile salts, fatty acids and monoacyglicerols) and an upper oily phase (containing undigested triacylglycerols and/or diacylglycerols). Each layer was then, diluted with a mixture of acetonitrile:water and evaluated for HP1 content using HPLC.

Ex vivo mucoadhesion

Ex vivo mucoadhesion on rat intestinal mucosa was performed according the method described by DURRER et al., 1994 a, b.

Male Wistar rats (200-250 g) were sacrificed by CO_2 gradient and then, jejunum was removed, rinsed with cold Krebs Bicarbonate Buffer and cut into segments of 4 cm^2 without Peyer´s Patches. Each segment was opened longwise and mounted into aluminum plates carefully in order to not remove the mucus layer. A second aluminum plate, with 2 cm^2 cleft in the center, was then fixed over the first plate to the purpose of fix the slice there. Following the experiment, 0.5 mL of a nanoemulsion preparation (200 µg mL^{-1}) in modified Krebs Bicarbonate Buffer was added in the plates (2 cm^2 cleft) at room temperature and the supernatant was withdrawal in pre-set times (1, 3, 5, 15, 30 and 60 minutes), diluted with a mixture of acetonitrile:water and analyzed by

HPLC. The difference between the amount of HP1 in the initial solution and in the supernatant is the HP1 quantity adhered in the mucus layer.

Results and Discussion

Ussing chambers experiments are a very convenient model for both attempting to predict oral absorption intensity and elucidating absorption mechanisms directly in the animal tissues, which are to be used in further pharmacokinetics studies (BERGGREN et al., 2004; van DER KERKHOF et al., 2006). This method is recurrently compared with those of monolayers of human colonic adenocarcinoma-derived cell line (Caco-2), the most frequently used (VARMA and PACHANGNULA, 2005; AGÜEROS et al., 2009). In fact, the use of a living tissue gives a result more realistic because of it is capable of express all membrane transporters, the mucus production is maintained during all experiment and the results could be directly correlated to *in vivo* experiments that will be conducted in the same animal model (MAZZAFERRO et al., 2012).

This model is widely used to investigate intestinal transport of drugs as well as regional variations in transport whereas studies in human tissues are limited, due to a shortage of tissue specimens (LENNERNÄS et al., 2007). Furthermore, highly correlations between permeability in rat jejunum tissue and corresponding human data can be found for drug intestinal permeability with both carrier-mediated and passive diffusion absorption mechanisms (LENNERNÄS et al., 1997; 2007). Nevertheless, it is important to note that bioavailability cannot be predicted by the Ussing chamber model, only permeability (CAO et al., 2006).

Therefore, permeation studies of HP1 (free form and loaded in nanoemulsions) were achieved by Ussing chambers method in Wistar rats.

Owing to low water solubility of benzopyran HP1 (5 µ/mL), 1% of sulfobutyl éther β-cyclodextrin was added to KBr Buffer to facilitate solubilization. Additionally, 2% of pancreatin was also added to buffer in order to mimic as much as possible the conditions of the intestinal environment. Therefore, the effect of KBr buffer added of sulfobutyl éther β-cyclodextrin and pancreatin on the tissue integrity was tested by

measuring the tissue resistance during the experiment (Figure. 1). The results showed that the buffer was not capable of end up with tissue integrity.

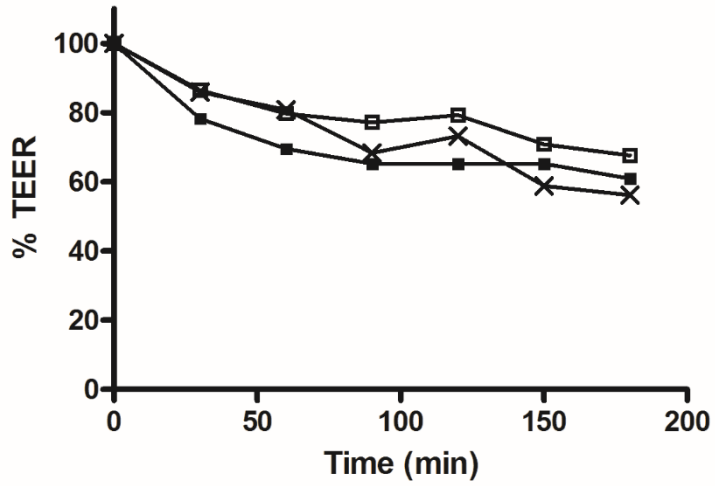

Figure 1. Effect of modified KBr buffer (■) and HP1 free form (solubilized in modified KBr buffer) in the absorptive (□) and secretory (×) directions on the TEER (Transmucosal Eletrical Resistance) of jejunum mucosa of Wistar Rats mounted in Ussing Chambers at pH 7.4. Data expressed as mean ±SD (n=4).

Significative differences between permeation fluxes and percentage of molecule that can cross the intestinal tissue were evidenced to HP1 free form. Between 30 and 120 minutes the apparent permeability in the mucosal-serosal direction (absorptive) was 2.90 ± 0.58 x 10^{-6} cm s^{-1} while the value found to serosal-mucosal direction (secretory) was 1.20 ± 0.37 x 10^{-6} cm s^{-1}, indicating that uptake active transporters were at least partially involved in the transport of the molecule across intestine. These results were confirmed when the experiments were performed at 4ºC, with a two folds decrease of the apparent permeability to 1.10 ± 0.31 x 10^{-6} cm $s^{-1,}$ under conditions where the absorption occur only by passive diffusion (ATP dependent transporters disabled) (MARDONES et al., 2004).

Hence, these investigations have demonstrated that active transport of HP1 is as important as passive mechanisms. Since its structure looks like a cannabinoid molecule such as THC (Δ^9- tetrahydrocannabinol) and there is a strong correlation between the patwhways opioid and cannabionoid (CICHEWICZ, 2004), the implication of the intestinal receptor CB_1 in HP1 absorption was investigated using different intestinal portions: jejunum and colon. This different portions were used because all other experiments were done using jejunum portions and there are evidences that this type of receptor is more expressed in epithelial colonic cells (WRIGHT et al., 2005; MARQUÉZ et al., 2009).

No differences were found in HP1 free form permeation in jejunum tissue (Figure 2A). On the other hand, in colonic tissue the CB_1 inverse agonist rimonabant had an interesting effect (Figure 2B). At the first 30 minutes, all HP1 disponible could cross the colonic epithelial cells, after that, the passage remained at a constant rate. This fact might be explained by the possible interaction of HP1 with CB_1 receptor after 30 minutes. Studies have shown that agonists of cannabinoid system (CB_1) could enhance molecule permeability by reducing the expression of tight junction proteins and antagoinists have the opposite effect (MACCARRONE et al., 2015). The results obtained to HP1 free form in colonic epithelial cells could be hypothesized as a competition between HP1 and rimonabant by CB_1 receptor. When CB_1 inverse agonist is not present, lower HP1 quantitites could cross the intestinal tissue, probably due to an interaction or antagonistic or just physical-chemical with this receptor. Nevertheless, this possible interaction can not explain the results found in jejunum tissue, which leads to the possibility of a unknown drug transporter be participating of HP1 absorption.

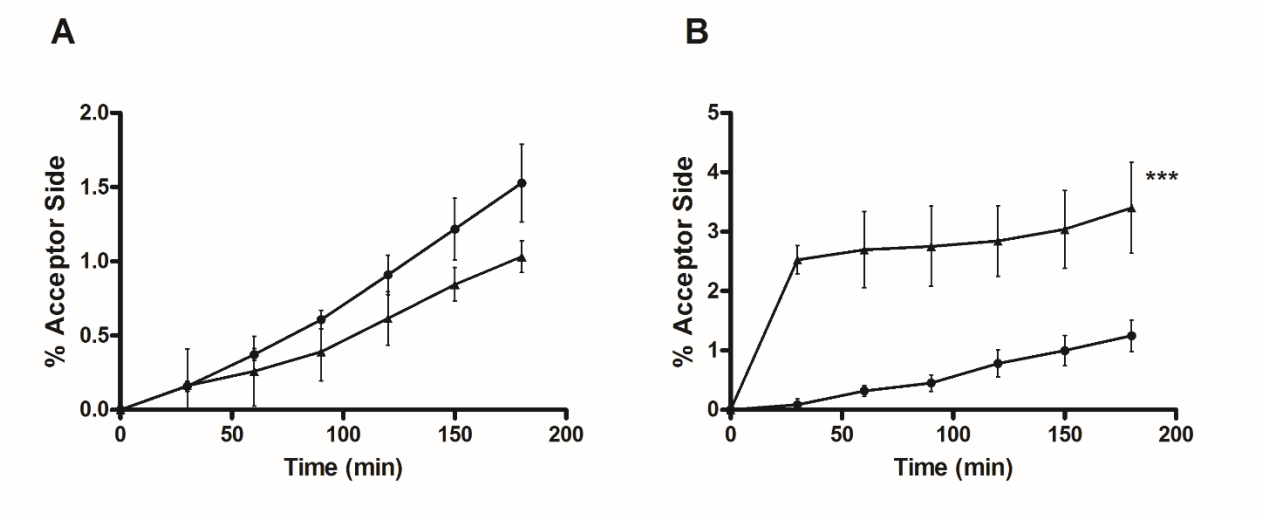

Figure 2. Amount of HP1 absorbed across the jejunum tissue (A) and colonic tissue (B) in absence (•) and in presence (▲) of CB_1 inverse agonist, rimonabant. Data expressed as mean ±SD (n=4). Significative different values were detected by t-test. ***p<0.001.

In order to explain the better antinociceptive results observed for trigliceryde nanoemulsion than to HP1 free form (MEIRELLES et al., 2016, not published data), the permeability of HP1 loaded in a triglyceride nanoemulsion was also assessed. The physicochemical characterization demonstrated size average of 150.44 ± 6.9 nm and zeta potential of -18.10 ± 6.5 mV corroborating with our previous results.

Initially, *in vitro* digestion of triglyceride nanoemulsion was evaluated to demonstrate the effect of pancreatic enzymes in the release system. The amount of free fatty acids released from the nanoemulsion was about 60% as showed figure 3A. When HP1 amounts were quantified in the oil and aqueous colloidal phases, the results were according the amount of free fatty acids released in the digestion media (Figure 3B). It is important rebound that poorly water soluble drugs might precipitate in the gastric and intestinal fluids without the presence of specific vehicles as lipidic systems. This type of system helps lipophilic drugs to remain dispersed in a colloidal solution throughout its transit in the gastrointestinal tract. The lipidic components of a triglyceride nanoemulsion and their lipolysis products are probably re-organized in complex structures with bile lipids (bile acids, phospholipids and colesterol) before be absorbed in the brush border of intestinal cells (CARRIÈRE, 2015). These results

demonstrate the importance of nanoemulsions as HP1 release systems and helps to clarify the mechanisms of HP1 absorption when loaded in these systems.

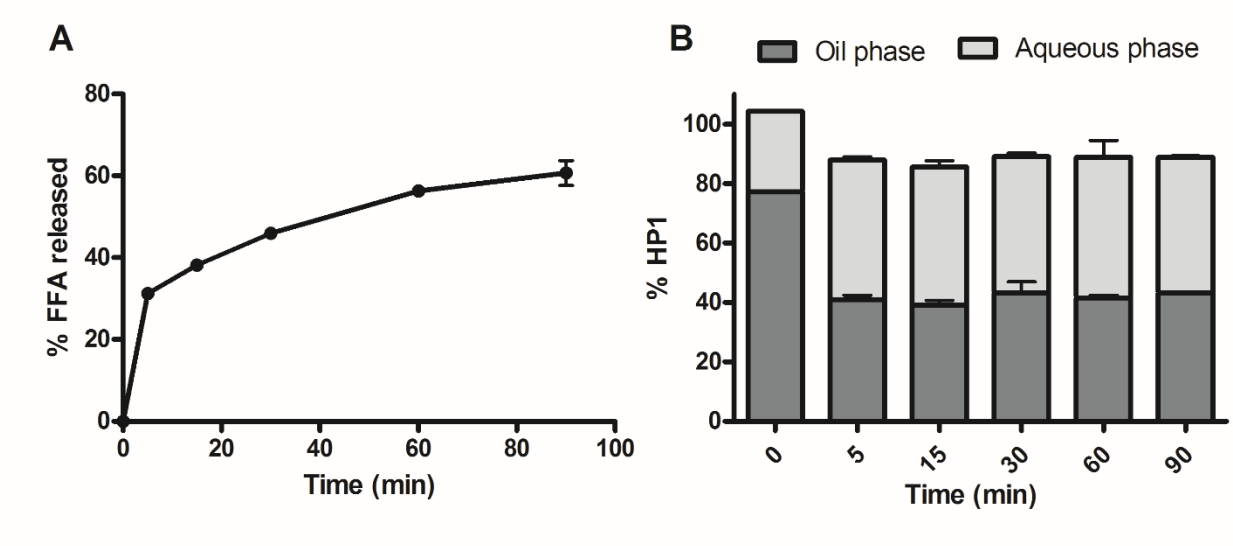

Figure 3. Amount of free fatty acids (FFA %) released from triglyceride nanoemulsions (A) and distribuition of HP1 (%) between the oily and the aqueous colloidal phase (B) over 90 minutes of *in vitro* lipolysis. Data expressed as mean ±SD (n=3).

The effect of triglyceride nanoemulsions in the HP1 permeability was then evaluated. The figure 4 shows that the percentage of HP1 in the acceptor side is much higher when it is loaded in nanoemulsions than when it is in the free form. Moreover, the HP1 apparent permeabilitity (Papp) has increased from 2.90 ± 0.58 x 10^{-6} cm s^{-1} to 11.47 ± 2.03 cm s^{-1} when loaded in nanoemulsions, an enhacement ratio about 5.26. These results helps to clarify those obtained in the antinociception study (Meirelles et al., 2016, not published data), where HP1 loaded in nanoemulsions presented an antinociceptive effect in lower doses, most likely by an enhancement of intestinal permeability.

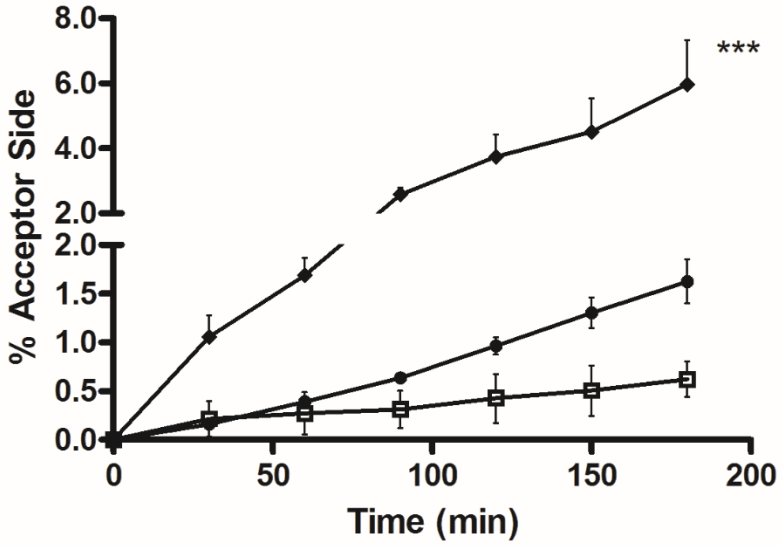

Figure 4. Amount of HP1 absorbed across the jejunum tissue in free form at absorptive direction at 37 º C (●) and 4 ºC (□) and when loaded in triglyceride nanoemulsions at 37 ºC (◆). Data expressed as mean ±SD (n=4). Significative different values were detected by t-test. **p<0.01.

After reach the brush border of intestinal cells, drug candidates must overcome a layer of high viscoelasticity and adhesive mucus. This mucus protects mucosal surfaces in intestinal tract and many drug delivery systems could be caught there by steric hindrances or adhesion processes. Subsequently, this systems could be removed by mucus turnover (NETSOMBOON and BERKNOP-SCHNÜRCH, 2016).

Some strategies to avoid the back diffusion of drug delivery systems have been applied such as employment of negatively charged systems. The mucus gel barrier is composed by cross linked entagled mucin fibers, sloughled cells, bacteria, lipids, salts, proteins, macromolecules and cellular debris (MOGHISSI et al., 1960; COLES et al., 1984; JOHANSSON et al., 2011) and exhibits a negative net charge (HASSAN and GALLO, 1990). Thus, negatively charged systems can move easily within the mucus, whereas positively charged systems are immobilized due to ionic interactions. Since HP1 nanoemulsion has a negative charge (-18.10 ± 6.5 mV) experiments with a semi-permeable membrane over the mucosal side, avoiding the direct contact between nanoemulsion droplets negatively charged and intestinal mucus were done to confirm if this system could be entrapped in the mucus layer.

As can be seen in figure 5, any trace of HP1 was found in the acceptor side when loaded in a nanoemulsion system. Unexpectedly, very few quantities of HP1 free form have the ability to cross the intestinal membrane when the semi-permeable membrane was present (cut-off membrane: 40.000 g/mol and MW HP1= 290.34 g/mol) indicating that both HP1 in both conditions (free form and loaded in nanoemulsions) have in the mucus interaction a determinant factor to be absorbed. It is important to note that the result obtained for HP1 free form is probably due to the high lipophilicity of molecule that needs to be associated with mucus components to be absorbed.

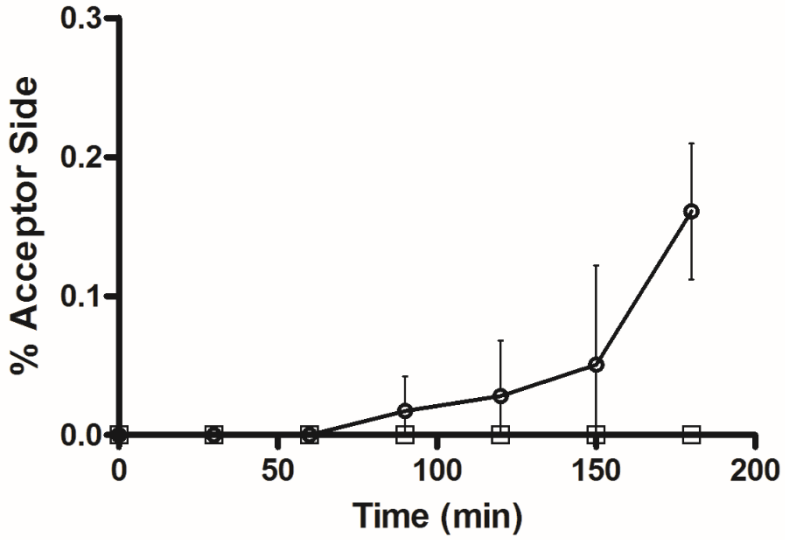

Figure 5. Amount of HP1 (free form (○) and loaded in nanoemulsions (□)) absorbed across the jejunum tissue with a semi-permeable membrane over the mucosal avoiding mucus interaction .Data expressed as mean ±SD (n=4).

To confirm these results, mucoadhesion experiments *ex vivo* were performed. In 30 minutes, about 50 % of nanoemulsion was adhered in intestinal mucus (Figure 6). Despite HP1 nanoemulsion having a negative charge, it is capable of be adhered in the mucus layer due to the diffusion process until epithelial cells. The mucoadhesion is important to retain the dosage form at the site of action and thus, provide an enhancement of absorption. It is important to highlight that mucoadhesion must occur in a shortly period since the intestinal mucus turnover (estimated between 47 and 270 minutes) (LEHR et al., 1991) could remove the delivery system from intestinal

environment. The fact that in 30 minutes, the HP1 nanoemulsion mucoadhesion have already reached a plateau of 60% is benefic ever since it happens before the start of intestinal mucus turnover and hence, works to improve the contact time and at the end, the system absorption.

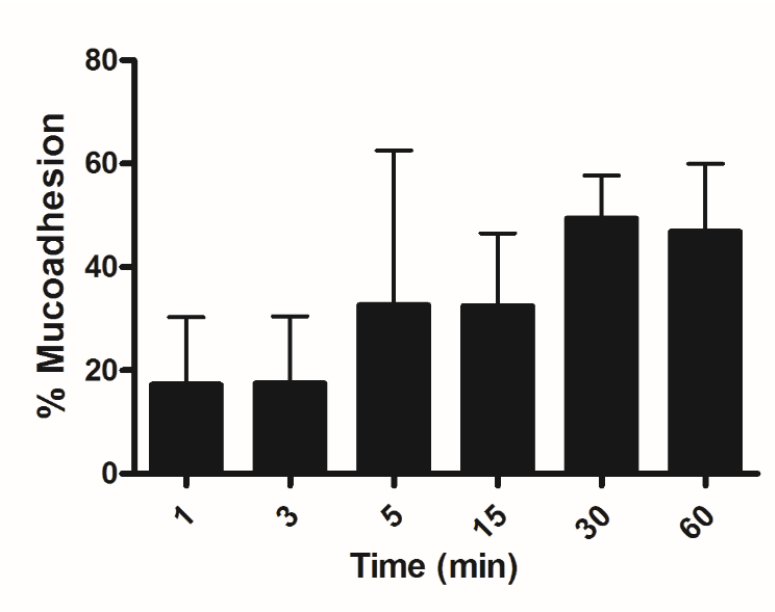

Figure 6. % of HP1 nanoemulsion mucoadhesion at different times. Data expressed as mean ±SD (n=4).

Conclusions

To sum up, the active transport of benzopyran HP1 free form is as important as passive transport and there is a probability of intestinal cannabinoid system be involved in this transport. However, the existence of a specific intestinal transporter of HP1 must be further investigated. Regarding lipidic release systems, HP1 when loaded in nanoemulsions, presents a higher apparent permeability by the dispersion in colloidal solution increasing the solubility and thus, permeability. Together, these results help to clarify those obtained in our previous study concerning antinociceptive activity, where HP1 incorpored in nanoemulsions demonstated a lower effective doses.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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References

AGÜEROS, M.; RUIZ-GATÓN, L.; VAUTHIER, C.; BOUCHEMAL, K.; ESPUELAS, S.; PONCHEL, G.; IRACHE, J. Combined hydroxypropyl-[beta]-cyclodextrin and poly (anhydride) nanoparticles improve the oral permeability of paclitaxel. **European Journal of Pharmaceutical Sciences**, v. 38, p. 405–413, 2009.

BERGGREN, S.; HOOGSTRAATE, J.; FAGERHOLM, U.; LENNERNAS, H. Characterization of jejunal absorption and apical efflux of ropivacaine, lidocaine and bupivacaine in the rat using *in situ* and *in vitro* absorption models. **European Journal of Pharmaceutical Sciences**, v. 21, p. 53–560, 2004.

BERNKOP-SCHNÜRCH, A. Reprint of: Nanocarrier systems for oral drug delivery: Do we really need them? **European Journal of Pharmaceutical Sciences**, v. 50, p. 2-7, 2013.

BONKANKA, C.X.; SÁNCHEZ-MATEO, C.; RABANAL, R.M. Antinociceptive activity of *Hypericum grandifolium* Choisy in mice. **Journal of Natural Medicines**, v.65, p. 122-128, 2011.

BOUCHEMAL, K.; BRIANÇON, S.; PERRIER, E.; FESSI, H. Nanoemulsion formulation using spontaneous emulsification: solvent, oil and surfactant optimization. **International Journal of Pharmaceutics***,* v. 280, p. 241-251, 2004;.

BRIDI, H.; CCANA-CCAPATINTA, G.V.; STOLZ, E.; MEIRELLES, G.; BORDIGNON, S.; RATES, S.; von POSER, G. Dimeric acylphloroglucinols from *Hypericum austrobrasiliense* exhibiting antinociceptive activity in mice. **Phytochemistry**, v. 122, p. 178-83, 2016.

CAO, X.; GIBBS, S.T.; FANG, L.; MILLER, H.A.; LANDOWSKI, C.P.; SHIN, H.C.; LENNERNAS, H.; ZHONG, Y.; AMIDON, G.L.; YO, L.X.; SUN, D. Why is it challenging to predict intestinal drug absorption and oral bioavailability in human using rat model, **Pharmacological Research**, v. 23, p. 1675-1686, 2006.

CARRIÈRE, F. Impact of gastrointestinal lipolysis on oral lipid-based formulations and bioavailability of lipophilic drugs. **Biochimie**, v. 125, p. 297-305, 2015.

CICHEWICZ, D. Synergistic interactions between cannabinoid and opioid analgesics. **Life Sciences**, v. 74, p. 1317-1324, 2004.

COLES, S.; BHAKSAR, K.; O´SULLIVAN, D.; NEILL, K.; REID, L. Airway mucus: composition and regulation of its secretion by neuropeptides *in vitro*. **Ciba Foundation Symposium**, v. 109, p. 40-60, 1984.

DESROCHES, J.; BEAULIEU, P. Opioids and cannabinoids interactions: involvement in pain management. **Current Drug Targets**, v.11, p. 462-473, 2010.

DURRER, C.; IRACHE, J.; PUISIEUX, F.; DUCHENE, D.; PONCHEL, G. Mucoadhesion of latexes. I. Analytical methods and kinetic studies. **Pharmaceutical Research**, v.11, p. 674–679, 1994a.

DURRER, C.; IRACHE, J.; PUISIEUX, F.; DUCHENE, D.; PONCHEL, G. Mucoadhesion of latexes. II. Adsorption isotherms and desorption studies**.** **Pharmaceutical Research**, v.11, p. 680–683, 1994b.

GRIFFIN, B.; KUENTZ, M.; VERTZONI, M.; KOSTEWICZ, E.; FEI, Y.; FAISAL, W.; STILLHART, C.; O´DRISCOLL, C.; REPPAS, C.; DRESSMAN, J. Comparison of *in vitro* tests at various levels of complexity for the prediction of *in vivo* performance of lipid-based formulations: Case studies with fenofibrate. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 86, p. 427-437, 2014.

HAAS, J.; VIANA, A.; HECKLER, A.; von POSER, G.; RATES, S. The antinociceptive effect of a benzopyran (HP1) isolated from *Hypericum polyanthemum* in mice hot-plate test is blocked by naloxone. **Planta Medica**, v. 76, p. 1419-1423, 2010.

HASSAN, E.; GALLO, J. A simple rheological method for the *in vitro* assessment of mucin-polymer bioadhesive bond strength. **Pharmaceutical Research**, v. 7, p. 491-495, 1990.

HERLING, A.W.; KILP, S.; JURETSCHKE, H.P.; NEUMANN-HAEFELIN, C.; GERL, M.; KRAMER, W. Reversal of visceral adiposity in candy-diet fed female Wistar rats by the CB1 receptor antagonist rimonabant. **International Journal of Obesity** (Lond), v. 32, p. 1363-1372, 2008.

HODGSON J. ADMET—turning chemicals into drugs. **Nature Biotechnology**, v. 19, p. 722-726, 2001.

IKONEN, E. Cellular cholesterol trafficking and compartmentalization. **Nature Reviews Molecular Cell Biology**, v. 9, p. 125-138, 2008.

JANNIN, V.; MUSAKHANIAN, J.; MARCHAUD, D. Approaches for the development of solid and semi-solid lipid-based formulations. **Advanced Drug Delivery Reviews**, v. 60, p. 734-746, 2008.

JOHANSSON, M.; AMBORT, T.; PELASEYED, D.; SCHÜTTE, J.; GUSTAFSSON, A.; SUBRAMANI, D.B.; HOLMÉN-LARSSON, J.M.; THOMSSON, K.A.; BERGSTRÖM, J.H.; van DER POST, S.; RODRIGUEZ-PIÑEIRO, A.M.; SJÖVALL, H.; BÄCKSTRÖM, M.; HANSSON, G.C. Composition and functional role of the mucus layers in the intestine. **Cellular and Molecular Life Sciences**, v. 68, p. 3635-3641, 2011.

LEHR, C.; POELMA, F.; JUNGINGER, H.; TUKKER, J. An estimate of turnover time of intestinal mucus gel layer in the rat in situ loop. **International Journal of Pharmaceutics**, v. 70, p. 235–40, 1991.

LENNERNÄS H. Human jejunal effective permeability and its correlation with preclinical drug absorption models. **Journal of Pharmacy and Pharmacology**, v. 49, p. 627-638, 1997.

LENNERNÄS, H. Animal data: The contributions of the Ussing Chamber and perfusion systems to predicting human oral drug delivery *in vivo*. **Advanced Drug Delivery Reviews**, v. 59, p. 1107-1120, 2007.

MACCARRONE, M.; BAB, I.; BÍRÓ, T.; CABRAL, G.; DEY, S.; DI MARZO, V.; KONJE, J.; KUNOS, G.; MECHOULAM, R.; PACHER, P.; SHARKEY, K.; ZIMMER. A. Endocannabinoid signaling at the periphery: 50 years after THC. **Trends in Pharmacological Sciences**, v. 36, p. 277-296, 2015.

MARDONES, P.; ANDRINOLO, D.; CSENDES, A.; LAGOS, N. Permeability of human jejunal segments to gonyautoxins measured by the Ussing chamber technique. **Toxicon**, v. 44, p. 521-528, 2004.

MARQUÉZ, L.; SUÁREZ, J.; IGLESIAS, M.; BERMUDEZ-SILVA, F.; RODRÍGUEZ DE FONSECA, F.; ANDREU, M. Ulcerative colitis induces changes on the expression of the endocannabinoid system in the human colonic tissue. **PLoS One**, v. 4, e6893, 2009.

MASON, D.J.; LOWE, J.; WELCH, S. Cannabinoid modulation of dynorphin A: correlation to cannabinoid-induced antinociception. **European Journal of Pharmacology**, v. 378, p. 237–248, 1999.

MAZZAFERRO, S.; BOUCHEMAL, K.; SKANJI, R.; GUEUTIN, C.; CHACUN, H.; PONCHEL G. Intestinal permeation enhancement of docetaxel encapsulated into methyl-β-cyclodextrin/poly(isobutylcyanoacrylate) nanoparticles coated with thiolated chitosan. **Journal of Controlled Release**, v. 162, p. 568-574, 2012.

MOGHISSI, K.; NEUHAUS, O.; STEVENSON, C. Composition and properties of human cervical mucus. I. Electrophoretic separation and identification of proteins. **Journal of Clinical Investigation**, v.39, p. 1358-1363, 1960.

NETSOMBOON, K.; BERKNOP-SCHNÜRCH, A. Mucoadhesive vs. mucopenetrating particulate drug delivery. **European Journal of Pharmaceutics and Biopharmaceutics,** v. 98, p. 76-89, 2016.

POUTON, C.W. Lipid formulations for oral administration of drugs: nonemulsifying, self-emulsifying and ‘self-microemulsifying drug delivery systems. **European Journal of Pharmaceutical Sciences**, v. 11, p. S93–8, 2000.

RAZIQ, N.; SAEED, M.; SHAHID, M.; MUHAMMAD, N.; KHAN, H.; GUL, F. Pharmacological basis for the use of *Hypericum oblongifolium* as a medicinal plant in the management of pain, inflammation and pyrexia. **BMC Complementary and Alternative Medicine**, doi: 10.1186/s12906-016-1018-z, 2016.

RZODKIEWICZ, P.; GASINSKA, E.; MASLINSKI, S.; BUJAKSKA-ZADROZNY, M. Antinociceptive properties of esculetin in non-inflammatory and inflammatory models of pain in rats. **Clinical and Experimental Pharmacology and Physiology**, v. 42, p. 213-1219, 2015.

SEK, L.; PORTER, C.; KAUKONEN, A.; CHARMAN, W. Evaluation of the *in vitro* digestion profiles of long and medium chain glycerides and the phase behaviour of their lipolytic products. **Journal of Pharmacy and Pharmacology**, v. 54, p. 29-41, 2002.

SIMONS K, IKONEN E. Functional rafts in cell membranes. **Nature**, v. 387, p. 569-572, 1997.

TAKAGI, T.; RAMACHANDRAN, C.; BERMEJO, M.; YAMASHITA, S.; YU, L.X.; AMIDON, G.L A provisional biopharmaceutical classification of the top 200 oral drug products in the United States, Great Britain, Spain, and Japan. **Molecular Pharmaceutics**, v. 3, p. 631–643, 2006.

UNGELL, A.; ANDREASSIN, A.; LUNDIN, K.; UTTER, L. Effects of enzymatic inhibition and increased paracellular shunting on transport of vasopressin analogues in the rat. **Journal of Pharmaceutical Sciences**, v. 81, p. 640-645, 1992.

van DE KERKHOF, E.; UNGELL, A.; SJOBERG, A.; DE JAGER, M.; HILGENDORF, C.; DE GRAAF, I.; GROOTHUIS, G. Innovative methods to study human intestinal drug metabolism *in vitro*: precision-cut slices compared with Ussing chamber preparations, **Drug Metabolism and Disposition**, v. 34, p. 1893-1902, 2006.

VARMA, M.; PANCHAGNULA, R. Enhanced oral paclitaxel absorption with vitamin E-TPGS: effect on solubility and permeability *in vitro*, *in situ* and *in vivo.* **European Journal of Pharmaceutical Sciences**, v. 25, p. 445-453, 2005.

YU, W.; TABOSA DO EGITO, E.S.; BARRATT, G.; DEVISSAGUET, J.P.; PUISIEUX, F.A. Novel approach to the preparation of injectable emulsions by spontaneous

emulsification process. **International Journal of Pharmaceutics**, v. 15, p. 139-146, 1993.

WELCH, S.P.; EADES, M. Synergistic interactions of endogenous opioids and cannabinoid systems. **Brain Research**, v. 848, p.183–190, 1999.

WELCH, S.P. Interaction of the cannabinoid and opioid systems in the modulation of nociception. **International Review of Psychiatry**, v. 21, p. 143-151, 2009.

WRIGHT, K.; ROONEY, N.; FEENEY, M.; TATE, J.; ROBERTSON, D.; WELHAM, M. Differential expression of cannabinoid receptors in the human colon: cannabinoids promote epithelial wound healing. **Gastroenterology**, v. 129, p. 437−453, 2005.

6 DISCUSSÃO GERAL

De acordo com as evidências científicas acerca do potencial biológico de espécies de *Hypericum* do Sul do Brasil, vários pesquisadores têm direcionado seus estudos à análise fitoquímica e exploração de atividades biológicas de extratos e produtos isolados destas espécies.

Assim, este trabalho objetivou complementar estudos já iniciados com determinadas espécies nativas do Rio Grande do Sul. Inicialmente, o estudo de atividade antifúngica contra fungos emergentes foi aprofundado, com base no trabalho de BARROS e colaboradores (2013). Após, de acordo com o estudo de HAAS e colaboradores (2010) se deu início a investigações com formulações nanotecnológicas contendo o benzopirano HP1, reconhecido por sua ação antinociceptiva.

Como já discutido ao longo deste trabalho, apesar de novos medicamentos terem sido inseridos na terapêutica antifúngica, o número de infecções causadas por fungos leveduriformes emergentes, principalmente do gênero *Candida* está crescendo cada vez mais (PFALLER et al., 2014). Dado esse fato, alternativas que almejem a terapia multialvos, ou seja, diferentes compostos com diferentes mecanismos de ação são cada vez mais importantes.

Conforme observado nesta tese, a fração lipofílica de *Hypericum carinatum* apresentou atividade antifúngica moderada. A ação observada pode ser atribuída à presença de floroglucinóis diméricos (uliginosina B) e benzofenonas (carifenonas A e B), característicos desse tipo de fração. Alguns autores atribuem metabólitos destas classes como responsáveis pela atividade antifúngica presente em frações e extratos lipofílicos de *Hypericum* (DALL'AGNOL et al., 2003; FENNER et al., 2005).

Em virtude dos resultados observados para *H. carinatum*, estudos de associação da fração lipofílica com fluconazol foram realizados. O fluconazol foi escolhido para esse estudo por ser amplamente utilizado no sistema público de saúde, ser de baixo custo e fácil acesso (RENAME, 2014).

Os resultados obtidos nos testes de associação entre a fração lipofílica de *H. carinatum* e fluconazol foram interessantes e concordaram pelas duas técnicas utilizadas. Pelo método do *checkerboard*, a fração lipofílica de *H. carinatum* foi capaz de diminuir a CIM de fluconazol para todos os fungos leveduriformes testados, ocorrendo a reversão da resistência para *C. krusei* e *C. famata* (de 32 µg/mL para 8 µg/mL para *C. krusei* e de 8 µg/mL para 2 µg/mL para *C. famata*). Para *C. parapsilosis*

e *Cryptococcus neoformans,* o fluconazol na sua CIM foi capaz de provocar dano celular maior do que a associação, não justificando o uso da mesma. Os resultados da análise isobolar corroboram com aqueles obtidos no *checkerboard*, havendo sinergismo para *C. krusei* e um possível efeito aditivo para *C. parapsilosis*. Esses resultados são relevantes a medida que cepas de fungos antes sensíveis aos agentes antifúngicos, estão cada vez mais se tornando resistentes.

O uso indiscriminado de agentes antimicrobianos tem resultado no surgimento de cepas resistentes de diversas espécies fúngicas (HUANG e CAO, 2012), fazendo com que o tratamento dessas infecções seja cada vez mais desafiador (PFALLER, 2012). A resistência clínica de espécies de *Candida* ao fluconazol é extensamente documentada (CLANCY et al., 2005; PFALLER et al., 2006; TORTORANO et al., 2006), sendo que atualmente estima-se que existam aproximadamente 30 espécies capazes de provocar infecções sistêmicas, principalmente em pacientes imunossuprimidos (PFALLER e DIEKEMA, 2004; 2007; PFALLER et al., 2010; 2012; ZHANG et al., 2015).

A resistência ao fluconazol é encontrada em maior proporção em espécies de *Candida* não-*albicans* (CLEVELAND et al., 2012). Adicionalmente, há espécies que apresentam resistência intrínseca como *C. krusei* e aquelas que adquirem facilmente os genes de resistência, como *C. glabrata* (CHAPELAND-LECLERC et al., 2010). Esses dados vão ao encontro da patogenicidade desconhecida de espécies de *Candida* não-*albicans* e o uso indiscriminado de fluconazol, restringindo as opções terapêuticas para o tratamento de infecções causadas por estes micro-organismos.

Os fármacos azólicos, entre eles o fluconazol, inibem o crescimento fúngico por interferir na biossíntese do ergosterol na membrana celular (WHITE et al., 1998; KANAFANI e PERFECT, 2008). Para as benzofenonas, o possível mecanismo de ação reside no fato que alguns compostos dessa classe são capazes de bloquear o complexo enzimático citocromo P-450 e assim impedir a formação do ergosterol da membrana fúngica (PODUST et al., 2007). Ainda, a literatura científica relata o mecanismo de ação para compostos fenólicos em geral, presentes em frações lipofílicas, tais como alteração no dimorfismo do fungo (ZHANG et al., 2011), abertura de canais iônicos de membrana (RAO et al., 2010) e ruptura da membrana (GALLUCI et al., 2014). Baseados nesses mecanismos, a fração lipofílica de *H. carinatum* pode estar auxiliando a entrada de fluconazol na célula fúngica por algum dos mecanismos citados, visto que a fração sozinha demonstrou ação apenas moderada.

Os resultados obtidos neste trabalho reforçam a importância de terapias multialvos no combate de fungos leveduriformes emergentes. Há vários estudos na literatura que comprovam a importância desse tipo de associação (ROCHA DA SILVA, 2014; PIPPI et al., 2015; KATRAGKOU et al., 2015). Não há dúvidas de que a terapia combinada entre a fração lipofílica de *H. carinatum* e fluconazol é benéfica, porém mais estudos devem ser realizados para determinar a natureza dessa interação e qual o mecanismo de ação da mesma. Por se tratar de uma fração, é muito difícil prever qual composto está agindo ou se a mistura deles é responsável pelo efeito. Portanto, análises dos compostos isolados das frações, sozinhos e em combinação, se fazem necessárias para em um futuro padronizar essa associação que foi capaz de reverter a resistência de alguns fungos ao fluconazol.

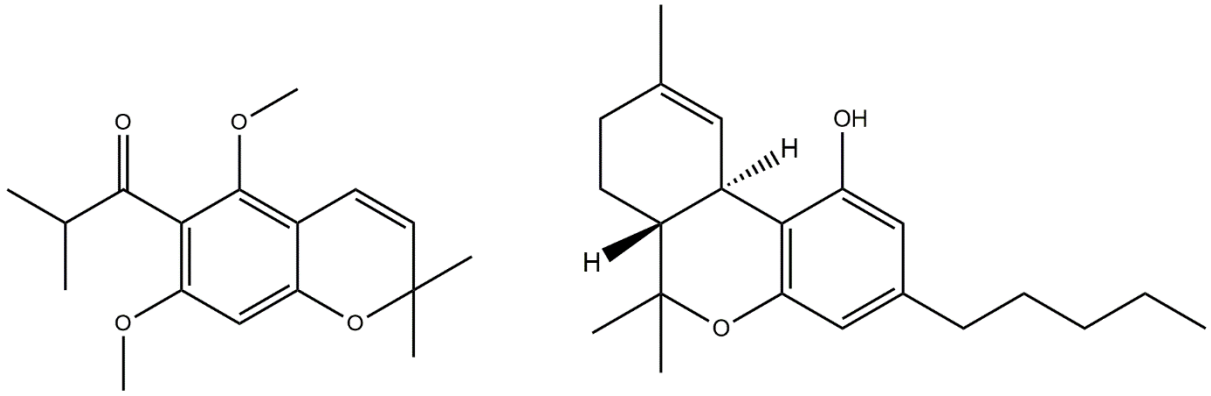
Estudos com extratos de espécies vegetais são de grande valia com o intuito de alcançar um perfil de atividade biológica para determinada classe de metabólitos majoritários para cada espécie. Porém, atualmente, o advento de novas tecnologias está direcionando cada vez mais para trabalhos com compostos isolados de plantas e que futuramente possam ser utilizados com protótipos de fármacos ou mesmo como fármacos.

Nesse contexto, HAAS e colaboradores (2010) avaliaram o perfil antinociceptivo de três benzopiranos isolados de *Hypericum polyanthemum*. Neste estudo foi observado que o benzopirano HP1 possui ação antinociceptiva mediada pelo sistema opioide. Entretanto, apesar desse efeito importante, a baixa solubilidade em água deste composto dificulta a avaliação do mesmo em estudos mais aprofundados acerca desta atividade.

O benzopirano HP1 foi hábil ao exercer sua ação antinociceptiva pela via oral (45 mg/kg). Os resultados obtidos nesta tese corroboram com aqueles demonstrados anteriormente (HAAS et al., 2010), onde esta molécula apresenta uma curva dose resposta característica em forma de sino ou U invertido. Dentre os fármacos opioides conhecidos atualmente, apenas a buprenorfina e seu respectivo metabólito, a norbuprenorfina apresentam esse tipo de comportamento (LUFTY et al., 2003). Como explanado no capítulo 2, esse fármaco apresenta farmacologia complexa sendo agonista potente, porém parcial do receptor mi (μ) opioide, além de ser agonista do receptor do tipo opioide (ORL-1) quando em altas concentrações (KHANNA e PILLARISETTI, 2015). A ativação desse receptor no corno dorsal tem efeito analgésico, porém a ativação cerebral pode bloquear a antinocicepção (DAVIS, 2012),

caracterizando a curva em forma de sino. Esse tipo de comportamento pode estar envolvido na farmacologia do benzopirano HP1, visto que grande parte dos fármacos opioides apresentam curva dose resposta sigmoidal e não em forma de sino. Apesar disso, mais evidências devem ser analisadas a fim de confirmar essa hipótese.

Além de todo o envolvimento do sistema opioide, deve se levar em consideração a relação entre os sistemas opioide e canabinoide visto que, estruturalmente, a molécula do benzopirano HP1 possui relação com ligantes do sistema canabinoide (Figura 5.1).



1 **2**

Figura 5.1 Relação estrutural entre o benzopirano HP1 (**1**) e o ligante canabinoide Δ^9-tetrahidrocanabinol (THC) (**2**)

Fármacos ligantes dos sistemas opioide (mi (μ)) e canabinoide (CB_1) possuem perfis farmacológicos semelhantes demonstrando efeito antinociceptivo, sedação, hipotensão, depressão motora, entre outros (MANZANARES et al., 1999; MASSI et al., 2001; MALDONADO e VALVERDE, 2003; CORCHERO et al., 2004). Além disso, canabinoides podem modular a função opioide em vários níveis, desde associação direta receptor-receptor até alterações a nível de liberação de peptídeos endógenos e/ou compartilhamento de sinais de transdução. Evidências dessas interações podem ser observadas em estudos que apresentam casos de tolerância cruzada, potenciação mútua e comunicação entre os receptores (CICHEWICZ e McCARTHY, 2003; MALDONADO e VALVERDE, 2003; ROBERTS et al., 2006; COX et al., 2007; WELCH, 2009).

Dada a interação entre esses dois sistemas, nesta tese foi investigada a possível participação do sistema canabinoide intestinal na absorção do benzopirano HP1. Evidências sugerem que além da presença no sistema nervoso entérico como os receptores opioides, os receptores canabinoides também podem estar presentes na membrana das células epiteliais intestinais, principalmente nas do cólon (IZZO e SHARKEY, 2010).

Os resultados demonstraram que quando o receptor canabinoide (CB_1) está bloqueado, a nível de cólon, há uma maior taxa de absorção do benzopirano HP1. Estas evidências sugerem que possa ocorrer uma competição entre rimonabanto (agonista inverso canabinoide) e HP1 pela ligação ao receptor. Na ausência do agonista inverso, uma fração de HP1 pode se ligar a esse receptor e assim se tornar indisponível para ser absorvida pela célula intestinal. No entanto, não se pode atribuir o transporte intestinal de HP1 ao sistema canabinoide, visto que a ligação pode ser de antagonismo ou por simples interação físico química.

Como evidenciado nesse estudo, o transporte ativo é tão importante quanto o transporte passivo na absorção intestinal do benzopirano HP1, porém o mecanismo pelo qual o transporte ativo ocorre não foi possível de ser elucidado. Entre os transportadores intestinais já relatados na literatura (THE INTERNATIONAL TRANSPORTER CONSORTIUM, 2010; ESTUDANTE et al., 2013), a molécula de HP1 não possui os requisitos mínimos necessários, estruturalmente, para ligar-se em nenhum deles e por esta razão, o envolvimento do sistema canabinoide foi investigado. É importante ressaltar que o receptor canabinoide (CB_1) não se trata de um transportador propriamente dito, como os demais, e por isso mais estudos devem ser realizados a fim de elucidar qual o tipo de interação ocorre entre HP1 e o receptor canabinoide (CB_1). Além disso, o transporte ativo de HP1 pode ser realizado através de transportadores ainda não elucidados.

Além de estudos de ação antinociceptiva e permeabilidade intestinal do benzopirano HP1 na sua forma livre, nesta tese também foram desenvolvidos estudos utilizando sistemas de liberação lipídicos a fim de contornar o problema da baixa solubilidade em água de HP1.

Nas últimas décadas, a administração oral de moléculas com baixa solubilidade em água tomou uma nova dimensão com o surgimento sistemas de liberação lipídicos que visam aumentar a solubilidade e, por conseguinte, a biodisponibilidade dessas moléculas (POUTON et al., 2006). Essas formulações incluem soluções ou

suspensões oleosas, emulsões, micro ou nanoemulsões (POUTON, 2000). No mercado farmacêutico já há fármacos incorporados com sucesso nesses sistemas de liberação, tais como efavirenz (Sustiva®), saquinavir (Fortovase®), ritonavir (Norvir®) e clofazamine (Lamprene®).

As propriedades únicas dessas formulações, sua diversidade físico–química e a biocompatibilidade com membranas biológicas fazem com que sejam boas candidatas como carreadoras de compostos lipofílicos. Entre os sistemas de liberação lipídicos, as nanoemulsões chamam atenção por sua estabilidade e facilidade de penetração nas mucosas do intestino delgado, que em última análise, facilita a absorção do composto que as mesmas carreiam (RAJPOOT et al., 2011).

Os resultados obtidos nesta tese estão alicerçados no fato que as nanoemulsões aumentam a solubilidade de compostos lipofílicos e assim, melhoram a absorção dos mesmos. O fato do benzopirano HP1 quando incorporado em nanoemulsões apresentar dose efetiva menor que quando administrado na sua forma livre corrobora com diversos estudos na literatura que demonstram redução da dose ativa de um composto quando inserido em um sistema nanoestruturado (GANTA et al., 2010; CHHABRA et al., 2011; RAGELLE et al.,2012).

A absorção de compostos lipofílicos inseridos em nanoemulsões ocorre pela liberação do mesmo do núcleo oleoso e incorporação em micelas mistas formadas com os sais biliares da digestão lipídica. Essa absorção ocorre em duas fases: digestiva e absortiva.

A fase digestiva requer a hidrólise dos triacilglicerois da fase oleosa, inicialmente pela lipase gástrica, em produtos mais polares como os monoacilglicerois e ácidos graxos livres que chegam a primeira porção do intestino, o duodeno, juntamente com o conteúdo gástrico (CAREY et al., 1983). No intestino, o baixo pH do conteúdo gástrico provoca a liberação do hormônio secretina da mucosa duodenal diretamente na corrente sangüínea. Assim, o pâncreas produz e secreta bicarbonato de sódio e as enzimas lipase e co-lipase a fim de facilitar a digestão dos triacilglicerois ainda não digeridos no estômago e neutralizar o pH dos fluidos intestinais (BORGSTROM, 1980; BERNBACK et al., 1989) Parcialmente ionizados, os ácidos graxos livres e monoacilglicerois agem como potentes agentes emulsificantes e promovem a ligação do complexo lipase/co-lipase na superfície da nanoemulsão. Visto que esse complexo enzimático é solúvel em água, é capaz de se ligar na

interface óleo/água da nanoemulsão e agir promovendo a hidrólise da fase oleosa (KOZLOVM e HELFRICH, 1992; EMBLETOM e POULTON, 1997). Por fim, o processo digestivo termina com a formação de micelas mistas ou vesículas formadas pelos produtos da digestão (monoacilglicerois e ácidos graxos livres) com os sais biliares presentes no intestino, e prontas para serem absorvidas (OLLIVON et al., 1988; PATERNOSTRE et al,1998) . Essa fase é considerada crítica pois há uma mudança de ambiente que circunda a formulação e se a mesma não contiver os componentes adequados pode levar a precipitação da molécula alvo e consequente piora na absorção (GRIFFIN et al., 2014).

Na fase absortiva, as espécies coloidais (micelas mistas, vesículas e ácidos graxos livres) são absorvidas pelas células epiteliais do intestino por difusão passiva, difusão facilitada e/ou transporte ativo. No citosol, a proteína ligante de ácidos graxos transporta essas espécies coloidais da membrana apical até o retículo endoplasmático liso (CHAKRABORTY et al., 2009). Assim, um gradiente de concentração facilita a absorção dos ácidos graxos em processo mediado por carreador (STREMMEL, 1988). Após, as espécies coloidais são transportadas, via sistema porta, para corrente sanguínea e podem permanecer dessa forma por um longo período até atingirem o sistema alvo (HWANG e MAUK, 1977).

O aumento da permeabilidade do benzopirano HP1, quando inserido em nanoemulsões (manuscrito 3) pode explicar a diminuição da dose ativa em estudos de ação antinociceptiva (manuscrito 2). Essa melhora se deve, provavelmente, a uma melhor solubilização da molécula com a formação de espécies coloidais, evidenciadas nos experimentos de lipólise *in vitro*, e, por conseguinte melhor absorção pelas células epiteliais do intestino, sem o risco de precipitações.

Por fim, cabe ressaltar que antes de chegar a superfície das células intestinais os compostos tanto na sua forma livre quanto quando inseridos em sistemas de liberação, devem atravessar a camada de muco que protege essas células.

O benzopirano HP1 (livre ou inserido em nanoemulsões) é dependente de interações com a camada mucosa para alcançar a superfície das células intestinais. Visto que o mesmo é uma molécula bastante lipofílica, necessita de interações com os componentes do muco, provavelmente formando complexos com proteínas e carboidratos, para então alcançar a superfície das células intestinais e ser absorvido.

Em contrapartida, as nanoemulsões também necessitam de interação com o muco para serem absorvidas. Esses sistemas apresentaram mucoadesão em cerca de 30 minutos, o que permite com que as enzimas do trato gastrointestinal ajam e liberem o conteúdo do núcleo oleoso para formar as micelas mistas e vesículas para a absorção como explanado anteriormente.

Os resultados apresentados nesta tese são de grande importância no que diz respeito a estudos com formulações contendo compostos isolados de plantas. O objeto de estudo, benzopirano HP1, foi isolado pela primeira vez no ano de 2001 (FERRAZ et al., 2001) e desde então, tem sido muito investigado, apresentando diversas atividades biológicas.

7 CONCLUSÕES GERAIS

Este estudo demonstrou o quão ricas em compostos com atividades biológicas importantes são as espécies de *Hypericum* nativas do Sul do Brasil. Este trabalho foi dividido em dois capítulos com enfoques diferenciados, porém ambos dando continuidade a trabalhos prévios produzidos no grupo de pesquisa.

Os resultados apresentados no primeiro capítulo comprovaram que a associação de dois produtos no combate a fungos leveduriformes resistentes a fármacos comumente utilizados é benéfica. A explicação mais provável para a redução na concentração inibitória mínima do fluconazol quando associado com a fração lipofílica de *H. carinatum* está no fato de que metabólitos presentes na fração podem estar, de alguma maneira, possibilitando o maior acesso do fármaco à célula fúngica, conseguindo assim erradicar o micro-organismo.

Entretanto, é preciso avaliar qual composto da fração é responsável pela ação antinfúngica ou se essa atividade provém de um possível efeito sinérgico entre os metabólitos presentes nesta fração. Após, é necessário verificar se a associação entre compostos isolados da fração e fluconazol é positiva, e assim, prever o possivel mecanismo de ação da mistura.

O segundo capítulo apresentou a incorporação do benzopirano HP1, isolado de *H. polyanthemum*, em nanoemulsões visando maior absorção deste composto pelo organismo e melhora na ação antinociceptiva. Além disso, nesse capítulo também foram apresentados estudos de permeação intestinal tanto de HP1 na sua forma livre quanto contido em nanoemulsões. Os resultados corroboraram com vários estudos da literatura, onde há diminuição da dose ativa de um composto lipofílico quando incorporado em sistemas de liberação lipídicos, por uma maior eficiência de solubilização e assim absorção.

Em relação a estudos de permeabilidade intestinal do benzopirano HP1 na sua forma livre, os resultados demonstram que o processo de transporte ativo é tão importante quanto o transporte passivo na absorção dessa molécula pelas células intestinais. No entanto, o correto transportador ativo não foi possível de ser elucidado, ocorrendo a possibilidade de envolvimento do sistema canabinoide intestinal.

Em conjunto, os resultados dos dois capítulos desta tese demonstram o alto potencial farmacológico de espécies de *Hypericum* e abrem possibilidades para novas perspectivas de estudos com essas plantas.

8 PERSPECTIVAS

Com base nos resultados apresentados nesta tese as perspectivas são:

- Avaliar a ação antinociceptiva do benzopirano HP1 (livre e incorporado em nanoemulsões) em diferentes tempos, duas e três horas, a fim de verificar a duração do efeito antinociceptivo. Os resultados apresentados foram obtidos em uma hora;
- Verificar qual o tipo de ligação do benzopirano HP1 com o receptor canabinoide (CB_1) para poder então, fazer uma correlação com a ação antinociceptiva via sistema opiode;
- Investigar possíveis transportadores ativos envolvidos na absorção intestinal do benzopirano HP1.

9 REFERÊNCIAS

AGÜEROS, M.; ESPUELAS, S.; ESPARZA, I.; CALLEJA, P.; PEÑUELAS, I.; PONCHEL, G.; IRACHE, J. Cyclodextrin-poly(anhydride) nanoparticles as new vehicles for oral drug delivery. **Expert Opinion in Drug Delivery**, v. 8, p. 721-734, 2011.

ALLEN, A.; HUTTON, D.A.; PEARSON, J.P. The MUC2 gene product: a human intestinal mucin. **International Journal of Biochemistry and Cell Biology**, v. 30. p. 797-801, 1998.

ALMEIDA, R.N.; NAVARRO, D.S.; BARBOSA-FILHO, J.M. Plants with central analgesic activity. **Phytomedicine**, v. 8, p. 310-322, 2001.

AMERICAN PAIN SOCIETY. Principles of Analgesic Use in the Treatment of Acute Pain and Cancer Pain. 6.ed. Glenview Ill: **American Pain Society,** 2008, 120 p.

AMIDON, G.L. ; LENNERNÄS, H. ; SHAH, V.P. ; CRISON, J.R. A theoretical basis for a biopharmaceutic drug classification: the correlation *in vitro* drug product dissolution and *in vivo* bioavailability. **Pharmaceutical Research**, v. 12, p. 413-420, 1995.

ANNAERT, P.; TUKKER, J.J.; VAN GELDER, J.; NAESENS, L.; DE CLERCQ, E.; VAN DEN MOOTER, G.; KINGET, R.; AUGUSTIJNS, P. *In vitro*, *ex vivo*, and *in situ* intestinal absorption characteristics of the antiviral ester prodrug adefovir dipivoxil. **Journal of Pharmaceutical Sciences**, v. 89, p 1054-1062, 2000.

BAJKA, B.H.; GILLESPIE, C.M.; STEEB, C.B.; READ, L.C.; HOWARTH, G.S. Applicability of the Ussing chamber technique to permeability determinations in functionally distinct regions of the gastrointestinal tract in the rat. **Scandinavian Journal of Gastroenterology**, v. 38, p. 732-741, 2003.

BALI, V.; ALI, M.; ALI, J. Novel nanoemulsion for minimizing variations in bioavailability of ezetimibe. **Journal of Drug Targeting**, v. 18, p. 7506-7519, 2010.

BALIMANE, P.V.; CHONG, S. Cell culture-based models for intestinal permeability: A critique. **Drug Discovery Today,** v.10, p.335–343, 2005.

BARKER, K.S.; CRISP, S.; WIEDERHOLD, N.; LEWIS, R.E.; BAREITHER, B.; ECKSTEIN, J.; BARBUCH, R.; BARD, M.; ROGERS, P.D. Genome-wide expression profiling reveals genes associated with amphotericin B and fluconazole resistance in experimentally induced antifungal resistant isolates of *Candida albicans*. **Antimicrobial Agents and Chemotherapy**, v. 54, p. 376-385, 2004.

BARROS, F.M.C.; PIPPI, B.; DRESCH, R.R.; DAUBER, B.; LUCIANO, S.C.; APEL M.A.; FUENTEFRIA, A.M.; von POSER, G.L. Antifungal and antichemotatic activities and quantification of phenolic compounds in lipophilic extracts of *Hypericum* spp. native to South Brazil. **Industrial Crops and Products**, v. 44, p. 294-299, 2013.

BARROS, E.; BARROS, H.M.T. **Medicamentos na Prática Clínica**. Porto Alegre: Artmed, 2010, 936p.

BENYAMIN, R.; TRESCOT, A.; DATTA, S.; BUENAVENTURA, R.; ADLAKA, R.; SEHGAL, N. Opioid complications and side effects. **Pain Physician**, v. 11, p. 105-120, 2008.

BERENBAUM, M. What is synergy? **Pharmacological Reviews**, v.41, p. 93-141, 1989.

BERNBACK, S.; BLACKBERG, L.; HERNELL, O. Fatty acids generated by gastric lipase to promote human milk triacylglycerol digestion by pancreatic colipase–dependent lipase. **Biochimica et Biophysica Acta**, v.1001, p. 286-293, 1989.

BLOMS-FUNKE, P.; GILLEN, C.; SCHUETTLER, A.J.; WNENDT, S. Agonistic effects of the opioid buprenorphine on the nociceptin/OFQ receptor. **Peptides**, v. 21, p. 1141-1146, 2000.

BORGSTROM, B. Importance of phospholipids, pancreatic phospholipase A2 and fatty acid for the digestion of dietary fat: *In vitro* experiments with the porcine enzymes. **Gastroenterology**, v. 78, p. 954-962, 1980.

BOUCHER, A.; TAM, V. Mathematical formulation of additivity for antimicrobial agents. **Diagnostic Microbiology and Infectious Disease**, v. 55, p. 319-325, 2006.

BRUNTON, L.L.; BLUMENTHAL, D.K.; MURRI, N.; DANDAN, R.H.; KNOLLMANN, B.C. **Goodman and Gilman's The Pharmacological Basis of Therapeutics**, New York: McGraw-Hill, 2011, 1808p.

BUSSMANN, R.W.; MALCA-GARCÍA, G.; GLENN, A.; SHARONA, D.; CHAIT, G.; DÍAZ, D.; POURMAND, K.; JONAT, B.; SOMOGY, S.; GUARDADO, G.; AGUIRRE, C.; CHAN, R.; MEYER, K.; KUHLMAN, A.; TOWNESMITH, A.; EFFIO-CARBAJAL, J.; FRÍAS-FERNANDEZ, F.; BENITO M. Minimum inhibitory concentrations of medicinal plants used in northern Peru as antibacterial remedies. **Journal of Ethnopharmacology**, v. 132, p. 101–108, 2010.

BUTLER, M.S. Natural products to drugs: natural product-derived compounds in clinical trials. **Natural Products Reports**, v. 25, p. 575-516, 2008.

BYRNES, E.J.; BARTLETT, K.K.; PERFECT, J.R. *Cryptococcus gattii*: an emerging fungal pathogen infecting humans and animals. **Microbes Infection**, v. 13, p. 895-907, 2011.

CALIXTO, J.B.; BEIRITH, A.; FERREIRA, J.; SANTOS, A.; CECHINEL FILHO, V.; YUNES, R. Naturally occurring antinociceptive substances from plants. **Phytotherapy Research,** v. 14, p. 401-418, 2000.

CAREY, M.C; SMALL, D.M; BLISS, C.M. Lipid digestion and absorption. **Annual Review of Physiology**, v. 45, p. 651-677, 1983.

CARRIÈRE, F. Impact of gastrointestinal lipolysis on oral lipid-based formulations and bioavailability of lipophilic drugs. **Biochimie**, v. 125, p. 1-9, 2015.

CENTERS FOR DISEASE CONTROL AND PREVENTION (CDC). Emergence of *Cryptococcus gattii* - Pacific Northwest, 2004-2010. **Morbidity and Mortality Weekly Report**, v. 59, p. 865-868, 2010.

CHAKRABORTY, S.; SHUKLA, D.; MISHRA, B.; SINGH, S. Lipid – An emerging platform for oral delivery of drugs with poor bioavailability. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 73, p. 1-15, 2009.

CHAPELAND-LECLERC, F.; HENNEQUIN, C.; PAPON, N.; NOEL, T.; GIRARD, A. Acquisition of flucytosine, azole, and caspofungin resistance in *Candida glabrata* bloodstream isolates serially obtained from a hematopoietic stem cell transplant recipient. **Antimicrobial Agents and Chemotherapy**, v. 54, p.1360–1362, 2010.

CHEN, S.C.; PLAYFORD, E.G.; SORRELL, T.C. Antifungal therapy in invasive fungal infections. **Current Opinion in Pharmacology**, v.10, p. 522-530, 2010.

CHHABRA, G.; CHUTTANI, K.; MISHRA, A.; PATHAK, K. Design and development of nanoemulsion drug delivery system of amlodipine besilate for improvement of oral bioavailability. **Drug Development and Industrial Pharmacy**, v. 27, p. 907-916, 2011.

CICHEWICZ, D.L; MCCARTHY, E.A. Antinociceptive synergy between Δ^{9}-tetrahydrocannabinol and opioids after oral administration. **Journal of Pharmacology and Experimental Therapeutics**, v. 304, p. 1010-1015, 2003.

CICHEWICZ, D. Synergistic interactions between cannabinoid and opioid analgesics. **Life Sciences**, v. 74, p. 1317-1324, 2004.

CLANCY, C.J.; YU, V.L.; MORRIS, A.J.; SNYDMAN, D.R.; NGUYEN, M.H. Fluconazole MIC and the fluconazole dose/MIC ratio correlate with therapeutic response among patients with candidemia. **Antimicrobial Agents and Chemotherapy**, v. 49, p. 3171-3177, 2005.

CLEVELAND, A.A.; FARLEY, M.M.; HARRISON, L.H.; STEIN, B.; HOLLICK, R. Changes in incidence and antifungal drug resistance in candidemia: results from population-based laboratory surveillance in Atlanta and Baltimore, 2008–2011. **Clinical Infectious Disease**, v. 55, p. 1352-1361, 2012.

CLINICAL LABORATORY STANDARD INSTITUTE. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard **M27-S4. CLSI**, Villanova, Pennsylvania, 2012.

COLOMBO, A.L.; NUCCI, M.; PARK, B.J. Epidemiology of candidemia in Brazil: a nationwide sentinel surveillance of candidemia in eleven medical centers. **Journal of Clinical Microbiology**, v. 44, p. 2816-1822, 2006.

CORCHERO, J.; MANZANARES, J.; FUENTES, J.A. (2004) Cannabinoid/ opioid crosstalk in the central nervous system. **Critical Reviews in Neurobiology**, v. 16, p. 159–172, 2004.

COX, M.L.; HALLER, V.L.; WELCH, S.P. Synergy between Δ^{9}- tetrahydrocannabinol and morphine in the arthritic rat. **European Journal of Pharmacology**, v. 567, p. 125-130, 2007.

COX, G.; PERFECT, J. Microbiology and epidemiology of *Cryptococcus neoformans* infection. **Uptodate**, 2013.

CRAGG, G.M.; NEWMANN, D.J. Natural Products as Sources of New Drugs from 1981 to 2014. **Journal of Natural Prducts**, v. 70, p. 629-661, 2016.

CRATER, J.S.; CARRIER, R.L. Barrier properties of gastrointestinal mucus to nanoparticle transport. **Macromolecular Bioscience**, v. 10, p. 1473-1483, 2010.

CROCKETT, S.L.; ROBSON, N. Taxonomy and chemotaxonomy of the genus *Hypericum*. **Medicinal and Aromatic Plant Science and Biotechnology**, v. 5, p. 1-13, 2011.

CUENCA-ESTRELLA, M. Combinations of antifungal agents in therapy–what value are they? **Journal of Antimicrobial and Chemotherapy**, v. 54, p. 854-869, 2004.

DALL'AGNOL, R.; FERRAZ, A.; BERNARDI, A.P.; ALBRING, D.; NÖR, C.; SARMENTO, L.; LAMB, L.; HASS, M.; von POSER, G.L.; SCHAPOVAL, E.E.S. Antimicrobial activity of some *Hypericum* species. **Phytomedicine**, v. 10, p. 141-147, 2003.

DAUDT, R.; von POSER, G.L.; NEVES, G.; RATES, S.M.K Screening for the antidepressant activity of some species of *Hypericum* from South Brazil. **Phytotherapy Research**, v. 14, p. 344-246, 2000.

DAVIS, M.P. Twelve reasons for considering buprenorphine as a front- line analgesic in the management of pain. **Journal of Supportive Oncology**, v. 10, p 209-219, 2012.

DEFERME, S.; ANNAERT, O.; AUGUSTIJNS, P. *In Vitro* Screening Models to Assess Intestinal Drug Absorption and Metabolism. **Drug Absorption Studies Volume VII of the series Biotechnology: Pharmaceutical Aspects** pp 182-215, 2008.

DESAI, P.P.; DATE, A.A.; PATRAVALE, V.B. Overcoming poor oral bioavailability using nanoparticle formulations – opportunities and limitations. **Drug Discovery Today**, v. 9, p. 87-95, 2012.

DULGER, G.; DULGER, B. Antifungal activity of *Hypericum havvae* against some medical *Candida* yeast and *Cryptococcu*s species. **Tropical Journal of Pharmaceutical Research**, v. 13, p. 405-408, 2014.

DUM, J.; BLASIG, J.; HERZ, A. Buprenorphine: demonstration of physical dependence liability. **European Journal of Pharmacology**, v. 70, p. 293-300, 1981.

DÜNNHAUPT, S.; KAMMONA, O.; WALDNER, C.; KIPARISSIDES, C.; BERKNOP-SCHNÜRCH, A. Nano-carrier systems: Strategies to overcome the mucus gel barrier. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 96, p. 447-453, 2015.

EHEHALT, R.; WAGENBLAST, J.; ERBEN, G.; LEHMANN, W.D.; HINZ, U.; MERLE, U.; STREMMEL, W. Phosphatidylcholine and lysophosphatidylcholine in intestinal mucus of ulcerative colitis patients, a quantitative approach by nanoelectrospray-tandem mass spectrometry, **Scandinavian Journal of Gastroenterology**, v. 39, p. 737-742, 2004.

EKSTEROWICZ, N.; QUINLAN-COWELL, A.; VANDERVEER,B.; MENEZ, J. Acute pain management. In: St .Marie, B. (Ed.) **Core Curriculum for Pain Management Nursing**. St. Louis: Saunders, 2010, p. 343.

EMBLETON, J.K; POUTON, C.W. Structure and function of gastro-intestinal lipases, **Advanced Drug Delivery Reviews**, v. 25, p. 15-32, 1997.

ESPINEL-INGROFF, A.; ALLER, I.A.; CANTON, E.; CASTAÑON-OLIVARES, R.; CHOWDHARY, A.; CORDOBA, S.; CUENCA-ESTRELLA, M., FOTHERGILL, A.; FULLER, J.; GOVENDER, N.; HAGEN, F.; IIINAIT-ZARAGOZI, M.T.; JOHNSON, E.; KIDD, S.; LASS-FLÖRL, C.; LOCKHART, S.R.; MARTINS, M.A.; MEIS, J.F.; MELHEM, M.S.C.; OSTROSKY-ZEICHNER, L.; PELAEZ, T.; PFALLER, M.A.; SCHELL, W.A.; ST-GERMAIN, G.; TRILLES, L.; TURNIDGE, J. *Cryptococcus neoformans-Cryptococcus gattii* species Complex: as International study of a wild-type susceptibility endopoint distribuitions and epidemiological cutoff values for fluconazole, itraconazole, posaconazole and voriconazole. **Antimicrobial Agents and Chemoterapy**, v. 56, p. 6898-5906, 2012.

ENSIGN, L.M.; CONE, R.; HANES, J. Oral drug delivery with polymeric nanoparticles: The gastrointestinal mucus barriers. **Advanced Drug Delivery Reviews**, v. 64, p. 557-570, 2012.

ESTUDANTE, M.; MORAIS, J.; SOVERAL, G.; BENET, L. Intestinal drug transporters: An overview. **Advanced Drug Delivery Reviews**, v. 65, p. 1340-1356, 2013.

EUROPEAN COMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TEST, Antifungal Agents Breakpoints tables for interpretation of MICs, versão 7, 2014.

FAGERHOLM, U.; JOHANSSON, M.; LENNERNÄS, H. The correlation between rat and human small intestinal permeability to drugs with different physico-chemical properties. **Pharmaceutical Research**, v. 13, p. 13-35, 1996.

FENNER, R.; SORTINO, M.; RATES, S.M.K.; DALL'AGNOL, R.; FERRAZ, A.; BERNARDI, A.P.; ALBRING, D.; NÖR, C.; von POSER, G.; SCHAPOVAL, E.; ZACCHINO, S. Antifungal activity of some Brazilian *Hypericum* species. **Phytomedicine**, v. 12, p. 236–240, 2005.

FERNÁNDEZ-FERNÁNDEZ, C.; CALLADO, L.F.; GIRÓN, R.; SÁNCHEZ, E.; ERDOZAIN, A.M.; LÓPEZ-MORENO, J.A.; MORALES, P.; RODRÍGUEZ DE FONSECA, F.; FERNÁNDEZ-RUIZ, J.; GOYA, P.; MEANA, J.J.; MARTÍN, M.I.; JAGEROVIC, N. Combining rimonabant and fentanyl in a single entity: preparation and pharmacological results. **Drug Design, Development and Therapy**, v. 8, p. 263-277, 2014.

FERRAZ, A.B.F.; BORDIGNON, S.A.L.; STAATS, C.; SCHRIPSEMA, J.; von POSER. G.L. Benzopyrans from *Hypericum polyanthemum*. **Phytochemistry**, v. 57, p. 1227-1230, 2001.

FERRAZ, A.B.F.; SCHRIPSEMA, J.; POHLMANN, A.R.; von POSER, G.L. Uliginosin B from *Hypericum myrianthum*. **Biochemical Systematics and Ecology**, v. 30, p. 989-991, 2002.

FLORES V.G.; TOVAR R.M.; ZALDIVAR P.G., MARTINEZ E.A. Meningitis due to *Cryptococcus neoformans*: treatment with posaconazole. **Current HIV Research**, v. 10, p. 620-623, 2012.

FUCHS., F.D., WANNMACHER, L., FERREIRA, M.B.C. **Farmacologia clínica: Fundamentos da terapêutica racional**, Rio de Janeiro:Guanabara Koogan, 2004, 1074p.

GALLUCCI, M.N.; CAREZZANO, M.E.; OLIVA, M.M.; DEMO, M.S.; PIZZOLITTO, R.P.; ZUNINO, M.P.; ZYGADLO, J.A.; DAMBOLENA, J.S. *In vitro* activity of natural phenolic compounds against fluconazole-resistant *Candida* species: a quantitative structure–activity relationship analysis. **Journal of Applied Microbiology**, v. 116, p. 795-804, 2014.

GANTA, S.; DESHPANDE, D.; KORDE, A.; AMIJI, M. A review of multifunctional nanoemulsion systems to overcome oral and CNS drug delivery barriers. **Molecular Membrane Biology**, v. 27, p. 260-273, 2010.

GNERRE, C.; von POSER, G.L.; FERRAZ, A.; VIANA, A.; TESTA, B.; RATES. S.M. Monoamine oxidade inhibitory activity of some *Hypericum* species native to South Brazil. **Journal of Pharmacy and Pharmacology**, v. 53, p. 1273-1279, 2001.

GOLDBERG, D.S.; McGEE, S.J. Pain as a global public health priority. **BMC Public Health**, v. 11, p. 770-775, 2011.

GRIFFIN, B.; KUENTZ, M.; VERTZONI, M.; KOSTEWICZ, E.; FEI, Y.; FAISAL, W.; STILLHART, C.; O´DRISCOLL, C.; REPPAS, C.; DRESSMAN, J. Comparison of *in vitro* tests at various levels of complexity for the prediction of *in vivo* performance of lipid-based formulations: Case studies with fenofibrate. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 86, p. 427-437, 2014.

GUINEA, J.; SANCHEZ-SOMOLINOS, M.; CUEVAS, O. Fluconazole resistance mechanisms in *Candida krusei*: the contribution of efflux pumps. **Medical Mycology**, v. 44, p. 575–578, 2006.

GULLO A. Invasive fungal infections: the challenge continues. **Drugs**, v. 69, p. 65-73, 2009.

HAAS, J.S.; VIANA, A.F.; HECKLER, A.P.; von POSER, G.L.; RATES, S.M.K. The antinociceptive effect of a benzopyran (HP1) isolated from *Hypericum polyanthemum* in mice hot-plate test is blocked by naloxone. **Planta Medica,** v. 76, p. 1419-1423, 2010.

HANSSON, G.C. Role of mucus layers in gut infection and inflammation. **Current Opinion in Microbiology**, v. 15, p. 57-62, 2012.

HEEL, R.C.; BROGDEN, R.N.; SPEIGHT, T.M.; AVERY, G.S. Buprenorphine: a review of its pharmacological properties and therapeutic efficacy. **Drugs**, v. 17, p. 81-110, 1979.

HERBRECHT, R.; DENNING, D.W.; PATTERSON, T.F. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. **The New England Journal of Medicine**, v. 347, p. 408-415, 2002.

HOHMANN, A.G.; BRILEY, E.M.; HERKENHAM, M. Pre and postsynaptic distribution of cannabinoid and mu opioid receptors in rat spinal cord. **Brain Research**, v. 822, p. 17–25, 1999.

HOLZER, P. Opioid receptors in the gastrointestinal tract. **Regulatory Peptides**, v. 155, p. 11-17, 2009.

HORN, D.L.; NEOFYTOS, D.; ANAISSIE, E.J. Epidemiology and outcomes of candidemia in 2019 patients: data from the prospective antifungal therapy alliance registry. **Clinical Infectious Disease**, v. 48, p. 695–703, 2009.

HOWLETT, A.C.; BARTH, F.; BONNER, T.I.; CABRAL, G.; CASELLAS. P.; DEVANE, W.A.; FELDER, C.C.; HERKENHAM, M.; MACKIE, K.; MARTIN, B.R.; MECHOULAM, R.; PERTWEE, R.G. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. **Pharmacological Reviews**, v. 54, p. 161-202, 2002,

HUANG, M.; KAO, K.C. Population dynamics and the evolution of antifungal drug resistance in *Candida albicans*. **FEMS Microbiology Letters**, v. 333, p. 85-93, 2012.

HUGHES, J.D.; BLAGG, J.; PRICE, D.A.; BAILEY, S.; DECRESCENZO, G.A.; DEVRAJ, R.V.; ELLSWORTH, E.; FOBIAN, Y.M.; GIBBS, M.E.; GILLES, R.W. Physicochemical drug properties associated with *in vivo* toxicological outcomes. **Bioorganic & Medicinal Chemistry Letters**, v. 18, p. 4872–4875, 2008.

HURST, S.; LOI, C.M.; BRODFUEHRER, J.; EL-KATTAN, A. 2007. Impact of physiological, and biopharmaceutical factors in absorption and metabolism mechanisms on the drug oral bioavailability of rats and humans. **Expert Opinion on Drug Metabolism and Toxicology**, v. 3, p. 469-489, 2007.

HWANG, K.J; MAUK, M.R. Fate of lipid vesicles in vivo: a gamma-ray perturbed angular correlation study. **Proceedings of the National Academy of Sciences of the United States of America (PNAS)**, v. 74, p. 4991-4995, 1977.

HWANG, K.K.; JIANG, L.; REN, Y.; MARTIN, L.L.; MARTIN, N.F. Site-specific absorption of M100240 and MDL 100,173 in rats evaluated using Sweetana-Grass diffusion chamber technology. **Journal of Pharmacological and Toxicological Methods**, v. 48, p.97–101, 2002.

INTERNATIONAL ASSOCIATION FOR THE STUDY OF PAIN (IASP), IASP taxonomy. Disponível: <http://www.iasp-pain.org/Taxonomy#Pain>. Atualizada 20 de Outubro de 2014. Acesso em 5 de maio de 2016

IZZO, A.; SHARKEY, K. Cannabinoids and the gut: New developments and emerging concepts. **Pharmacology and Therapeutics**, v. 126, p. 21-38, 2010.

JAYASURIYA, H.; McCHESNEY, J.D.; SWANSON, S.; PEZZUTO, J. Antimicrobial and cytotoxic activity of rottlerin-type compounds from *Hypericum drummondii*. **Journal of Natural Products**, v. 52, p. 325-331, 1989.

JOHNSON, M.D.; MACDOUGALL, C.; OSTROSKY-ZEICHNER, L.; PERFECT, J.; REX, J. Combination antifungal therapy. **Antimicrobial Agents and Chemothererapy**, v. 48, p. 693-715, 2004.

KANAFANI, Z.A.; PERFECT, J.R. Antimicrobial resistance: resistance to antifungal agents: mechanisms and clinical impact. **Clinical Infectious Diseases**, v. 46, p. 120-128, 2008.

KANSY, M.; FISCHER, H.; KRATZAT, K.; SENNER, F.; WAGNER, B.; PARRILLA, I. Highthroughput artificial membrane permeability studies in early lead discovery and development. In: TESTA, B.; van DE WATERBEEMD, H.; FOLKERS, G.; GUY, R (Eds). **Pharmacokinetic Optimization in Drug Research. Biological, Physicochemical and Computational Strategies**. Hoboken: Wiley-Interscience, 2001. p. 447–464.

KATRAGKOU, A.; MCCARTHY, M.; ALEXANDER, E.L.; ANTACHOPOULOS, C.; MELETIADIS, J.; JABRA-RIZK, M.A.; PETRAITIS, V.; ROILIDES, E.; WALSH, T.J. *In vitro* interactions between farnesol and fluconazole, amphotericin B or micafungin against *Candida albicans* biofilms. **Antimicrobial Agents and Chemotherapy**, v. 70, p. 470-478, 2015.

KHANNA, I.; PILARISETTI, S. Buprenorphine – an attractive opioid with underutilized potential in treatment of chronic pain. **Journal of Pain Research**, v. 8, p. 859-870, 2015.

KIEFFER, B.L. Recent advances in molecular recognition and signal transduction of active peptides: receptors for opioid peptides. **Cellular and Molecular Neurobiology**, v. 15, p. 615–635, 1995.

KIEFFER, B.L.; GAVÉRIAUX-RUFF, C. Exploring the opioid system by gene knockout. **Progress in Neurobiology**, v. 66, p 285-306, 2002.

KIM, Y.S.; HO, S.B. Intestinal goblet cells and mucins in health and disease: recent insights and progress. **Current Gastroenterology Reports**, v. 12, p. 319-330, 2010.

KONTOYIANNIS, D.P.; LEWIS, R.E. Antifungal drug resistance of pathogenic fungi. **Lancet**, v. 359, p. 1135-1144, 2002.

KOTTA, S.; KHAN, A.; PRAMOD, K.; ANSARI, S.; SHARMA, R.; ALI, J. Exploring oral nanoemulsions for biovaliability enhancement of poorly water soluble drugs. **Expert Opinion on Drug Delivery**, v. 9, p. 585-598, 2012.

KOZLOVM, M.M; HELFRICH, W. Effects of a co-surfactant on the stretching and bending elasticities of a surfactant monolayer. **Langmuir**, v. 8, p. 2792– 2797, 1992.

KU, M.S.; DULIN, W., 2010. A biopharmaceutical classification-based right first time formulation approach to reduce human pharmacokinetic variability and project cycle time from first in human to clinical proof of concept. **Pharmaceutical Development & Technology**, v. 1, p. 1–18, 2010.

LARSSON, J.M.; KARLSSON, H.; SJÖVALL, H.; HANSSON, G.C. A complex, but uniform *O*-glycosylation of the human MUC2 mucin from colonic biopsies analyzed by nanoLC/MSn. **Glycobiology**, v. 19, p. 756-766, 2009.

LE NAOUR, M.; AKGÜN, E.; YEKKIRALA, A.; LUNZER, M.M.; POWERS, M.D; KALYUZHNY, A.E.; PORTOGHESE, P.S. Bivalent ligands that target μ opioid (MOP)

and cannabinoid 1 (CB_1) receptors are potent analgesics devoid of tolerance. **Journal of Medicinal Chemistry**, v. 56, p. 5505-5513, 2013.

LEHR C.; POELMA, F.; JUNGINGER, H.; TUKKER, J. An estimate of turnover time of intestinal mucus gel layer in the rat in situ loop. **International Journal of Pharmaceutics***,* v. 70, p. 235–40, 1991.

LENNERNÄS, H.; AHRENSTEDT, O.; HÄLLGREN, R.; KNUTSON, L.; RYDE, M.; PAALZOW, L.K. Regional jejunal perfusion, a new in vivo approach to study oral drug absorption in man. **Pharmaceutical Research**, v. 9, p. 1243-1251, 1992.

LEWIS, R.E. Current Concepts in antifungal pharmacology. **Mayo Clinic Proceedings**, v. 86, p. 805-817, 2011.

LIU, J.; ZHAI, W.; YANG, Y.; SHI, J.; LIU, Q.; LIU, G.; FANG, N.; LI, J.; GUO, J. GABA and 5-HT systems are implicated in the anxiolytic-like effect of spinosin in mice. **Pharmacology, Biochemistry and Behavior**, v. 128, p. 41-49, 2015.

LIZASOAIN, I.; LEZA, J.C.; LORENZO, P. Buprenorphine: bell-shaped dose-response curve for its antagonist effects. **General Pharmacology**, v. 22, p. 297-300, 1991.

LOCKHART, S.R.; IQBAL, N.; BOLDEN, C.B.; DEBESS, E.E.; MARSDEN-HAUG, N.; WORHLE, R.; THAKUR, R.; HARRIS, J.R. Epidemiologic cutoff values for triazole drugs in *Cryptococcus gattii*: correlation of molecular type and *in vitro* susceptibility. **Diagnostic Microbiology and Infectious Disease**, v. 73, p. 144-148, 2012.

LOESER, J.D.; TREEDE, R.D. The Kyoto protocol of IASP basic pain terminology. **Pain**, v. 137, p. 473-477, 2008.

LUFTY, K.; EITAN, K.; BRYANT, C.; YANG, Y.; SALIMINEJAD, N.; WALWYN, W.; KIEFFER, B.; TAKESHIMA, H.; CAROLL, I.; MAIDMENT, N.; EVANS, C. Buprenorphine induced antinociception is mediated by μ-opioid receptors and compromised by concomitant activation of opioid receptor-like receptors. **The Journal of Neuroscience**, v. 23, p. 10331-10337, 2003.

MACCARRONE, M.; BAB, I.; BÍRÓ, T.; CABRAL, G.; DEY, S.; DI MARZO, V.; KONJE, J.; KUNOS, G.; MECHOULAM, R.; PACHER, P.; SHARKEY, K.; ZIMMER, A. Endocannabinoid signaling at the periphery: 50 years after THC. **Trends in Pharmacological Sciences**, v. 36, p. 277-296, 2015.

MALDONADO, R.; VALVERDE, O. Participation of the opioid system in cannabinoid-induced antinociception and emotional-like responses. **European Neuropsychopharmacology**, v. 13, p. 401-410, 2003.

MALINGRE, M.M.; BEIJNEN, J.H.; SCHELLENS, J.H. Oral delivery of taxanes. **Investigational New Drugs,** v. 19, p. 155–162, 2001.

MANZANARES, J.; CORCHERO, J.; ROMERO, J.; FERNANDEZ-RUIZ, J.J.; RAMOS, J.A.; FUENTES, J.A. Pharmacological and biochemical interactions between opioids and cannabinoids. **Trends in Pharmacological Sciences**, v. 20, p. 287-294,1999.

MASSA, F.; STORR, M.; LUTZ, B. The endocannabinoid system in the physiology and pathophysiology of the gastrointestinal tract. **Journal of Molecular Medicine**, v. 83, p. 944-054, 2005.

MASSI, P.; VACCANI, A.; ROMORINI, S.; PAROLARO, D. Comparative characterization in the rat of the interaction between cannabinoids and opiates for their immunosuppressive and analgesic effects. **Journal of Neuroimmunology**, v. 117, p. 116–124, 2001.

MAZZAFERRO, S.; BOUCHEMAL, K.; SKANJI, R.; GUEUTIN, C.; CHACUN, H.; PONCHEL G. Intestinal permeation enhancement of docetaxel encapsulated into methyl-β-cyclodextrin/poly(isobutylcyanoacrylate) nanoparticles coated with thiolated chitosan. **Journal of Controlled Release**, v. 162, p. 568-574, 2012.

McCLEMENTS, D. Edible nanoemulsions: fabrication, properties, and functional performance. **Soft Matter**, v. 7, p. 2297-2316, 2011.

McLEMENTS, D. Nanoemulsion-based oral delivery systems for lipophilic bioactive components: nutraceuticals and pharmaceuticals. **Therapeutic Delivery***,* v. 4, p. 841-857, 2013.

MEUNIER, J.C.; MOLLEREAU. C.; TOLL, L.; SUAUDEAU, C.; MOISAND, C.; ALVINERIE, P.; BUTOUR, J.L.; GUILLEMOT, J.C.; FERRARA, P.; MONSARRAT, B.; MAZARGUIL, H.; VASSART, G.; PARMENTIER, M.; COSTENTIN, J. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. **Nature**, v. 377, p. 532-535, 1995.

MEUNIER, J.C. Nociceptin/orphanin FQ and the opioid receptor-like ORL1 receptor. **European Journal of Pharmacology**, v. 340, p. 1-5, 1997./

MICELI, M.H.; DIAZ, J.A.; LEE, S.A. Emerging opportunistic yeast infections. **Lancet Infectious Diseases**, v. 11, p. 142-151, 2011.

MOGIL, J.S.; PASTERNAK, G.W. The molecular and behavioral pharmacology of the orphanin FQ/nociceptin peptide and receptor family**. Pharmacological Reviews**, v. 53, p. 381– 415, 2001.

MOLS, R.; DEFERME, S.; AUGUSTIJNS, P. Sulfasalazine transport in *in vitr*o, *ex vivo* and *in vivo* absorption models: contribution of efflux carriers and their modulation by co-administration of synthetic nature-identical fruit extracts. **Journal of Pharmacy and Pharmacology**, v. 57, p. 1565-1573, 2005.

MULLARD, A. 2014 FDA drug approvals. **Nature Drug Discovery Reviews**, v. 14, p. 77-81, 2015.

MÜLLER, W.E. **St. John's wort and its active principles in depression and anxiety.** Alemanha: Birkhäuser Basel, 2005. 190 p.

NARANG, A.S.; DELMARRE, D.; GAO, D. Stable drug encapsulation in micelles and microemulsions. **International Journal of Pharmaceutics**, v. 345, p. 9-25, 2007.

NEJDFORS, P.; EKELUND, M.; JEPPSSON, B.; WESTRÖM, B.R. Mucosal *in vitro* permeability in the intestinal tract of the pig, the rat, and man: Species- and region

related differences. **Scandavian Journal of Gastroenterology**, v. 5, p. 501–507, 2000.

NISHIOKA.,Y.; YOSHINO, H. Lymphatic targeting with nanoparticulate system. **Advanced Drug Delivery Reviews**, v. 47, p. 55–64, 2001.

NÖR, C.; ALBRING, D.; FERRAZ, A.B.F.; SCHRIPSEMA, J.; PIRES, V.; SONNET, P.; GUILLAUME, D.; von POSER, G.L. Phloroglucinol derivatives from four *Hypericum species* belonging to the *Trigynobrathys* section. **Biochemical Systematics and Ecology**, v. 32, p. 517-519, 2004.

NUCCI, M.; MARR, K.A. Emerging fungal diseases. **Clinical Infectious Disease**, v. 41, p. 521–526, 2005.

OLLIVON, M.; EIDELMAN, O.; BLUMENTHAL, L.; WALTER, A. Micelle-vesicle transition of egg phosphatidylcholine and octylglucoside, **Biochemistry**, v. 27, p. 1695-1703, 1988.

PAPPAS, P.G.; KAUFFMAN, C.A.; ANDES, D.; BENJAMIN, D.K.; CALANDRA, T.F.;, EDWARDS, J.E.; FILLER, S.G.; FISHER, J.F.; KULLBERG, B.J.; OSTROSKY-ZEICHNER, L.; REBOLI, A.C.; REX, J.H.; WALSH, T.J.; SOBEL, J.D. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. **Clinical Infectious Disease**, v. 48, p. 503-535, 2009.

PARSONS, A.A.; BINGHAM, S.; RAVAL, P.; READ, S.; THOMPSON, M.; UPTON, N. Tonabersat (SB-220453) a novel benzopyran with anticonvulsant properties attenuates trigeminal nerve-induced neurovascular reflexes. **British Journal of Pharmacology**, v. 132, p. 1549-1557, 2001.

PATERNOSTRE, M.T.; ROUX, M.; RIGAUD, J.L Mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents. 1. Solubilization of large unilamellar liposomes (prepared by reverse-phase evaporation) by triton X-100, octyl glucoside, and sodium cholate. **Biochemistry**, v. 27, p. 2688-2677, 1988.

PEARSON, J.P.; BROWNLEE, I.A. The interaction of large bowel microflora with the colonic mucus barrier. **International Journal of Inflammation**, 321426, 2010.

PERFECT, J.R.; CASADEVALL, A. Cryptococcosis. **Infectious Disease Clinic of North America**, v. 16, p. 837-874, 2002.

PFALLER, M.A.; DIEKEMA, D.J. Rare and emerging opportunistic fungalpathogens: concern for resistance beyond *Candida albicans* and *Aspergillus fumigatus.* **Journal of Clinical Microbiology**, v. 42, p. 4419-4431, 2004.

PFALLER, M.A.; DIEKEMA, D.J.; COLOMBO, A.L.; KIBBLER, C. *Candida rugosa*, an emerging fungal pathogen with resistance to azoles: geographic and temporal trends from the ARTEMIS DISK antifungal surveillance program. **Journal of Clinical Microbiology**, v. 44, p. 3578-3582, 2006.

PFALLER, M.A.; DIEKEMA, D.J. Epidemiology of invasive candidiasis: a persistent public health problem. **Clinical Microbiology Reviews**, v. 20, p. 133–163, 2007.

PFALLER, M.; ANDES, D.; DIEKEMA, D.; ESPINEL-INGROFF, A.; SHEELAND, D. THE CLSI SUBCOMMITTEE FOR ANTIFUNGAL SUSCEPTIBILITY TESTING. Wild-type MIC distributions, epidemiological cutoff values and species-specific clinical breakpoints for fluconazole and *Candida*: Time for harmonization of CLSI and EUCAST broth microdilution methods. **Drug Resistance Updates**, v. 13, p. 180-195, 2010.

PFALLER, M.; NEOPHYTOS, D.; DIEKEMA, D.; AZIE, N.; MEIER-KRIESCHE, H.; QUAN, S.; HORN, D. Epidemiology and outcomes of candidemia in 3648 patients: data from the Prospective Antifungal Therapy (PATH Alliance®) registry. 2004-2008. **Diagnostic Microbiology and Infectious Disease**, v. 74, p. 323-331, 2012.

PFALLER, M.; ANDES, D.; DIEKEMA, D.; HORN, D.; ROTSTEIN, C.; FRANKS, B.; AZIE, N. Epidemiology and outcomes of invasive candidiasis due to non-*albicans* species of *Candida* in 2496 Patients: Data from the prospective antifungal therapy (PATH) registry 2004–2008. **PLos One**, v. 9, p. e101510, 2014.

PIPPI, B.; DALLA LANA, A.; MORAES, R.C.; GÜEZ, C.M.; MACHADO, M.; OLIVEIRA, L.F.S.; von POSER, G.L.; FUENTEFRIA, A.M. *In vitro* evaluation of the acquisition of resistance, antifungal activity and synergism of Brazilian red propolis with antifungal drugs on *Candida* spp. **Journal of Applied Microbiology**, v. 118, p. 839-50, 2015.

PLAPIED, L.; DUHEM, N.; DES RIEUX, A.; PRÉAT, V. Fate of polymeric nanocarriers for oral drug delivery. **Current Opinion in Colloid & Interface Science**, v. 16, p. 228-237, 2011.

PODUST, L.; von KRIES, J.; EDDINE, A.; KIM, Y.; YERMALITSKAYA, L.; KUEHNE, R.; OULLET, H.; WARRIER, T.; ALTEKÖSTER, M.; LEE, J-S.; RADEMANN, J.; OSCHKINAT, H.; KAUFMANN, S.; WATERMAN, M. Small-Molecule scaffolds for CYP51 inhibitors identified by high-throughput screening and defined by X-ray crystallography. **Antimicrobial Agents and Chemotherapy**, v. 51, p. 3915-3923, 2007.

POLENTARUTTI, B.I.; PETERSON, A.L.; SJÖBERG, A.K.; ANDERBERG, E.K.; UTTER, L.M.; UNGEL, A.L. Evaluation of excised rat intestinal segments in the Ussing chamber: Investigation of morphology, electrical parameters and permeability characteristics. **Pharmaceutical Research**, v. 16, p. 446-454, 1999.

PORTER, C.J.; CHARMAN, W.N. *In vitro* assessment of oral lipid based formulations. **Advanced Drug Delivery Reviews**, v. 50, p. S127-47, 2001.

PORTER, C.J.H.; TREVASKIS, N.L.; CHARMAN, W.N. Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs. **Nature Reviews Drug Discovery**, v. 6, p. 231-248, 2007.

POUTON, C.W. Lipid formulations for oral administration of drugs: nonemulsifying, self-emulsifying and 'self-microemulsifying' drug delivery systems. **European Journal of Pharmaceutical Sciences**, v. 11, p. S93–8, 2000.

POUTON, C.W. Formulation of poorly water-soluble drugs for oral administration: physicochemical and physiological issues and the lipid formulation classification system. **European Journal of Pharmaceutical Sciences**, v. 29, p. 278-287, 2006.

RAGELLE, H.; CRAUSTE-MANCIET, S.; SEGUIN, J.; BROSSARD, D.; CHERMAN, D.; ARNAUD, P.; CHABOT, G.G. Nanoemulsion formulation of fisetin improves bioavailability and antitumour activity in mice. **International Journal of Pharmaceutics**, v. 427, p. 452-459, 2012.

RAJPOOT, P.; PATHAK, K.; BALI, V. Therapeutic applications of nanoemulsion based drug delivery systems: a review of patents in last two decades. **Recent Patents on Drug delivery & Formulation**, v. 5, p. 163-172, 2011.

RAMAGE, G.; SAVILLE, S.P.; THOMAS, D.P.; LOPEZ-RIBOT J.L. *Candida* biofilms: an update. **Eucaryotic cell**, v. 4, p. 633-638, 2005.

RAO, A.; ZHANG, Y.; MUEND, S.; RAO, R. Mechanism of antifungal activity of terpenoid phenols resembles calcium stress and inhibition of the TOR pathway. **Journal of Antimicrobial Agents and Chemotherapy**, v. 54, p. 5062-5069, 2010.

RENAME. Relação nacional dos medicamentos essenciais. **Ministério da Saúde, Secretaria de Ciência, Tecnologia e Insumos Estratégicos, Departamento de Assistência Farmacêutica e Insumos Estratégicos**. 9.ed., Brasília: Ministério da Saúde, 2015, 228 p.

REX, J.H.; BENNETT, J.E.; SUGAR, A.M.; PAPPAS, P.G.; WASHBURN, R.G.; SCHELD, W.M.; KARCHMER, A.W.; DINE, A.P. Candidemia study group: National Institute of Allergy and Infectious Diseases Mycoses Study Group. A randomized trial comparing fluconazole with amphotericin B for the treatment of candidemia in patients without neutropenia. **The New England Journal of Medicine**, v. 331, p. 1325-1330, 1994.

ROCHA, L.; MARSTON, A.; KAPLAN, M.; STOECKLIEVANS, H.; THULL, U.; TESTA, B.; HOSTETTMANN, K. An antifungal c-pyrone and xantones with monoamine oxidadase inhibitory activity from *Hypericum brasiliense*. **Phytochemistry**, v. 36, p. 1381-1385, 1994.

ROCHA, L.; MARSTON, A.; POTTERAT, O.; KAPLAN, M.A.C.; STOECKLIEVANS, H.; THULL, U.; TESTA, B.; HOSTETTMANN, K. Antibacterial phloroglucinols and flavonoids from *Hypericum brasiliense*. **Phytochemistry**, v. 40, p. 1447-1452, 1995.

ROCHA DA SILVA, C.R.; DE ANDRADE NETO, J.B.; CAMPOS, R.; FIGUEIREDO, N.; SAMPAIO, L.; MAGALHÃES, H.; CAVALCANTI, B.; GASPAR, D.; DE ANDRADE, G.; LIMA, I.S.; VIANA, G.; MORAES, M.; LOBO, M.; GRANJEIRO, T.; NOBRE JÚNIOR, H. Synergistic Effect of the favonoid catechin,quercetin or epigallocatechin gallate with fluconazole induces apoptosis in *Candida tropicalis* resistant to fluconazole. **Antimicrobial Agents and Chemotherapy**, v. 58, p. 1468-1478, 2014.

ROGERS, S.M.; BACK, D.J.; ORME, M.L.E. Intestinal metabolism of ethinylestradiol and paracetamol *in vitro*: studies using Ussing chambers. **British Journal of Clinical Pharmacology**, v. 23, p. 7127-734, 1987.

ROSENBLUM, A.; MARSCH, L.; JOSEPH, H.; PORTENOY, R. Opioids and the Treatment of chronic pain: Controversies, current status, and future directions. **Experimental and Clinical Psychopharmacology**, v. 16, p. 405-416, 2008.

RUHNKE, M. Epidemiology of *Candida albicans* infections and role of non-*Candida-albicans* yeasts. **Current Drug Targets**, v. 7, p. 495–504, 2006.

SAAG, M.S.; GRAYBILL, R.J.; LARSEN, R.A. Practice guidelines for the management of cryptococcal disease. Infectious Diseases Society of America. **Clinical Infectious Disease**, v. 30, p. 710-718, 2000.

SALIO, C.; FISCHER, J.; FRANZONI, M.F.; MACKIE, K.; KANEKO, T.; CONRATH, M. CB1-cannabinoid and μ-opioid co-localization on postsynaptic target in the rat dorsal horn. **Neuro report**, v. 12, p. 3689–3692, 2001.

SARKER, D.K. Engineering of nanoemulsions for drug delivery. **Current Drug Delivery,** v. 2, p. 297–310, 2005.

SEGAL, B.H.; KWON-CHUNG, J.; WALSH, T.J.; KLEIN, B.S.; BATIWALLA, M.; ALMYROUDI, N.G.; HOLLAND, S.M.; ROMANI, L. Immunotherapy for fungal infections. **Clinical Infectious Disease**, v. 42, p. 507–515, 2006.

SHAPIRA, M.; GAFNI, M.; SARNE, Y. Long-term interactions between opioid and cannabinoid agonists at the cellular level: cross-desensitization and downregulation. **Brain Research**, v. 960, p. 190–200, 2000.

SHAPIRA, M.; VOGEL, Z.; SARNE, Y. Opioid and cannabinoid receptors share a common pool of GTP-binding proteins in co-transfected cells, but not in cells which endogenously co-express the receptors. **Cellular and Molecular Neurobiology**, v. 20, p. 291-304, 2003.

SILVA, S.; NEGRI, M.; HENRIQUES, M.; OLIVEIRA, R.; WILLIAMS, D.; AZEREDO, J. Silicone colonization by non-*Candida albican*s species in the presence of urine. **Journal of Medical Microbiology**, v. 59, p. 747-754, 2010.

SILVA, S.; NEGRI, M.; HENRIQUES, M.; OLIVEIRA, R.; WILLIAMS, D.; AZEREDO, J. *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. **FEMS. Microbiology Reviews**, v. 36, p. 288-305, 2012.

SJÖSTRÖM, M.; SJÖBERG, Å.; UTTER, L.; HYLTANDER, A.; KARLSSON, J.; STOCKMAN, E. Excised human intestinal segments as a mechanistic tool for verifying transport properties of drug candidates. Abstract AAPS. **Journal of Pharmaceutical Sciences**, supplementary, 2000.

SÖDERHOLM, J. D.; HEDAMN, L.; ARTURSSON, P.; FRANZEN, L.; LARSSON, J.; PANTZAR, N. Integrity and metabolism of human ileal mucosa in vitro in the Ussing chamber. **Acta Physiologica Scandinavica**, v. 162, p. 47-56, 1998.

SOCIEDADE BRASILEIRA EM ESTUDOS DA DOR (SBED), 2012.

SOLANS, C.; ESQUENA, J.; FORGIARINI, A.M.; MORALES, D.; IZQUIERDO, P.; AZEMAR, N.; GARCIA, M.J., 2003. Nano-emulsions: formation, properties and applications. **Surfactant Science series**, v.109, p. 525–554m 2003.

STOLZ, E.D.; VIANA, A.F.; HASSE, D.R.; von POSER, G.L.; DO REGO, J.; RATES, S.M.K. Uliginosin B presents antinociceptive effect mediated by dopaminergic and

opioid systems in mice. **Progress in Neuro-Psychopharmacology & Biological Psychiatry**, v. 39, p. 80–87, 2012.

STOLZ, E.D.; HASSE, D.R.; von POSER, G.L.; RATES, S.M.K. Uliginosin B, a natural phloroglucinol derivative, presents a multimediated antinociceptive effect in mice. **Journal of Pharmacology and Pharmacotherapeutics**, v. 66, p. 1774-1785, 2014.

STREMMEL, W. Uptake of fatty acids by jejunal mucosal cells is mediated by a fatty acid binding membrane protein. **Journal of Clinical Investigation**, v. 82, p. 2001-2010, 1988.

SUN, D.; LENNERNAS, H.; WELAGE, L.S.; BARNETT, J.L.; LANDOWSKI, C.P.; FOSTER, D.; FLEISHER, D.; LEE, K.D.; AMIDON, G.L. Comparison of human duodenum and Caco-2 gene expression profiles for 12,000 gene sequences tags and correlation with permeability of 26 drugs. **Pharmaceutical Research**, v. 19, p. 1400-1416, 2002.

TALLARIDA, R.J. 2006. An overview of drug combination analysis with isobolograms. **Journal of Pharmacology and Experimental Therapeutics**. v.319, p.1-7, 2006.

TIWARI, S.; AMIJI, M. Improved oral delivery of paclitaxel following administration in nanoemulsion formulations. **Journal of Nanoscience and Nanotechnology**, v. 9, p. 3215-3222, 2006.

TIWARI, S. Nanoemulsion formulations for tumor-targeted delivery. In: Mansoor, M. Amiji,M. (Eds.). **Nanotechnology for Cancer Therapy**. New York: CRC Press, 2007, p. 723-739.

THE INTERNATIONAL TRANSPORTER CONSORTIUM. Membrane transporters in drug development**. Nature Reviews. Drug Discovery**, v. 9, p. 215-236, 2010.

TORTORANO, A.; KIBBLER, C.; PEMAN, J.; BERNHARDT, H.; KLINGSPOR, L.; GRILLOT, R. Candidaemia in Europe: epidemiology and resistance. **International Journal of Antimicrobial Agents**, v. 27, p. 359-366, 2006.

TRELLE, S.; REICHENBACH, S.; WANDEL, S.; HILDEBRAND, P.; TSCHANNEN, B.; ADLAKA, R.; EGGER, M.; JÜNI, P. Cardiovascular safety on non-steroidal anti-inflammatory drugs: network meta-analysis. **British Medical Journal**, v. 342, c7086, 2011.

UNGELL, A.L. *In vitro* absorption studies and their relevance to absorption from the GI tract. **Drug Development and Industrial Pharmacy**, v. 23, p. 879–892, 1997.

UNGELL, A.L.; ABRAHAMSSON, B. Biopharmaceutical support in candidate drug selection. In: Pharmaceutical Preformulation and Formulation. A Practical Guide from Candidate Drug Selection to Commercial Dosage Formulation. Gibson, M (Ed) United States: Interpharm Press, 2001.

UNGELL, A.L. Transport studies using intestinal tissue *ex vivo*. In: Lehr, C.M. (Ed) Cell Culture Models of Biological Barriers. Taylor and Francis: London, 2002. p. 164–188.

UPTON, N.; BLACKBURN, T.P.; CAMPBELL, C.A.; COOPER, D.; EVANS, M.L.; HERDON, H.J.; KING, P.D.; RAY, A.M.; STEAN, T.O.; CHAN, W.N.; EVANS, J.M.; THOMPSON, M. Profile of SB-204269, a mechanistically novel anticonvulsant drug, in rat models of focal and generalized epileptic seizures. **British Journal of Pharmacology**, v. 121, p. 1679-1686, 1997.

van HECKE, O.; TORRANCE, N.; SMITH, B.H. Chronic pain epidemiology and its clinical relevance. **British Journal of Anhestesiology**, v. 111, p. 13-18, 2013.

van VURREN, S.F.; NAIDO, D. An antimicrobial investigation of plants used traditionally in southern Africa to treat sexually transmitted infections. **Journal of Ethnopharmacology**, v. 130, p. 552–558, 2010.

VIANA, A.F.; HECKLER, A.P.; FENNER, R.; RATES, S.M.K. Analgesic activity of *Hypericum caprifoliatum* and *Hypericum polyanthemum* (Guttiferae). **Brazilian Journal of Medical and Biological Research**, v. 36, p. 631–634, 2003.

VYAS, T.K.; SHAHIWALA, A.; AMIJI, M.M. Improved oral bioavailability and brain transport of saquinavir upon administration in novel nanoemulsion formulations. **International Journal of Pharmaceutics**, v. 347, p. 93-101, 2008.

von KROFF, M.; KOLODNY, A.; DEYO, R.; CHOU, R. Long-term opioid therapy considered. **Annals of Internal Medicine** , v.155, p. 325-328, 2011.

von POSER, G.L.; RECH, S.B.; RATES, S.M.K. Chemical and pharmacological aspects of southern Brazilian *Hypericum* species. In: Silva, J.A.T. (Org). **Floriculture, Ornamental and Plant Biotechnology: advances and topical issues** UK: Global Science Books, 2006, p. 510-516.

ZAKELJ, S.; STURM, K.; KRISTL, A. Ciprofloxacin permeability and its active secretion through rat small intestine *in vitro*. **International Journal of Pharmaceutics**, v. 313, p. 175-180, 2006.

ZARAGOZA, R.; PEMÁN, J. Opciones terapêuticas para el tratamento antifúngico en el paciente crítico. **Revista Iberoamericana de Micologia**, v. 29, p. 108-113, 2012.

ZHANG, Y.; BENET, L.Z. The gut as a barrier to drug absorption: combined role of cytochrome P450 3A and P-glycoprotein. **Clinical Pharmacokinetic**, v. 40, p.159–168, 2001.

ZHANG, L.; CHANG, W.; SUN, B.; GROH, M.; SPEICHER, A.; LOU, H. Bisbibenzyls, a new type of antifungal agents, inhibit morphogenesis switch and biofilm formation through upregulation of *DPP3* in *Candida albicans*. **PLoS One**. v. 6, p. e28953, 2011.

ZHANG, L.; XIAO, M.; WATTS, M.; WANG, H.; FAN, X.; KONG, F.; XU, Y. Development of fluconazole resistance in a series of *Candida parapsilosis* isolates from a persistent candidemia patient with prolonged antifungal therapy. **BMC Infectious Diseases**, v.15, p. 340, 2015.

WASHINGTON, C.; WASHINGTON, C.G.; WILSON, C.G. **Physiological Pharmaceutics: Barriers to Drug Absorption**. Taylor and Francis, London; 2001, 328p.

WIKMAN-LARHED, A.; ARTURSSON, P. Co-cultures of human intestinal goblet (HT29-H) and absorptive (Caco-2) cells for studies of drug and peptide absorption. **European Journal of Pharmaceutical Sciences**, v. 3, p. 171–193, 1995.

WHITE, T.C.; MARR, K.A.; BOWDEN, R.A. Clinical, cellular and molecular factors that contribute to antifungal drug resistance. **Clinical Microbiology Reviews**, v. 11, p. 382–402, 1998.

WELCH, S.P.; STEVENS, D.L. Antinociceptive activity of intrathecally administered cannabinoids alone, and in combination with morphine, in mice. **Journal of Pharmacology and Experimental Therapeutics**, v. 262, p. 10–18, 1992.

WELCH, S.P.; EADES, M. Synergistic interactions of endogenous opioids and cannabinoid systems. **Brain Research**, v. 848, p.183–190, 1999.

WELCH, S.P. Interaction of the cannabinoid and opioid systems in the modulation of nociception. **International Review of Psychiatry**, v. 21, p. 143-151, 2009.

WICKLINE, S.A.; LANZA, G.M. Nanotechnology for molecular imaging and targeted therapy. **Circulation**, v**.** 107, p. 1092–1095, 2003.

WNENDT, S.; KRUGER, T.; JANOCHA, E.; HILDEBRANDT, D.; ENGLBERGER, W. Agonistic effect of buprenorphine in a nociceptin/OFQ receptor-triggered reporter gene assay. **Molecular Pharmacology**, v. 56, p. 334-338, 1999.

WOODCOCK, J. A difficult balance – pain management, drug, safety and the FDA. **The New England Journal of Medicine**, v. 361, p. 2105-2107, 2009.

WUHRMAN, E.; COONEY, M. Acute Pain: Assesment and Treatment. **Medscape**, v. 3, p. 1-13, 2011.

YANG, S.C.; BENITA, S. Enhanced absorption and drug targeting by positively charged submicron emulsions. **Drug Development Research**, v. 50, p. 476–486, 2000.