UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Estudos químico e biológico de espécies de *Hypericum* das seções *Brathys* e *Trigynobrathys*

FRANCISCO MAIKON CORRÊA DE BARROS

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Estudos químico e biológico de espécies de *Hypericum* das seções *Brathys* e *Trigynobrathys*

Tese apresentada por Francisco Maikon Corrêa de Barros para obtenção do TÍTULO DE DOUTOR em Ciências Farmacêuticas

Orientador: Dra. Gilsane Lino von Poser

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“A VIDA É UMA PLANTA POR CUIDAR”

(Vinícius Brum e Mauro Ferreira)

RESUMO

O gênero *Hypericum* (Guttiferae = Clusiaceae) compreende 494 espécies acomodadas em 36 seções taxonômicas. Os representantes americanos pertencem, sobretudo, às seções *Brathys* e *Trigynobrathys*, as duas maiores com 87 e 52 espécies, respectivamente. Popularmente, estas plantas são utilizadas como antissépticos, diuréticos, digestivos e agentes de cicatrização. Quimicamente, apresentam tendência em acumular floroglucinóis diméricos, além de benzofenonas, benzopiranos, flavonoides e ácidos fenólicos, metabólitos cuja atividade antidepressiva, antinociceptiva, antimicrobiana, antiproliferativa e antioxidante são descritas. Considerando o potencial terapêutico, este trabalho objetivou a determinação dos principais compostos fenólicos presente nas flores de espécies *Hypericum* nativas do Sul do Brasil (*H. campestre*, *H. caprifoliatum*, *H. carinatum*, *H. connatum*, *H. linoides*, *H. myrianthum*, *H. polyanthemum* e *H. salvadorense*) e dos Páramos peruanos (*H. andinum* e *H. laricifolium*); a extração e análise dos compostos fenólicos de *H. carinatum* obtidos com CO_2 supercrítico e a investigação da atividade antifúngica e antiquimiotática dos extratos lipofílicos de *H. caprifoliatum*, *H. carinatum*, *H. linoides*, *H. myrianthum* e *H. polyanthemum.* Para os experimentos, foram coletadas (2008 - 2010) as partes aéreas das plantas em floração. Todas as coletas foram autorizadas pelos órgãos de proteção ambiental. Os métodos extrativos incluem maceração estática, ultrassom e CO_2 supercrítico (temperatura = 40, 50 ou 60ºC; pressão = 90, 120, 150 ou 200 bar). As análises por CLAE foram realizadas em coluna de fase reversa (C18), sistemas isocráticos compostos por acetonitrila, água e ácido trifluroacético e detecção ultravioleta (220, 270 ou 254 nm); Os picos foram identificados pela comparação dos tempos de retenção/co-injeção com padrões e quantificados pela curva de calibração dos compostos. Os ensaios antifúngico e antiquimiotático foram realizados pelo método de microdiluição em caldo e inibição da migração de neutrófilos, respectivamente. O floroglucinol dimérico uliginosina B (0,008 – 0,188%) e o flavonoide hiperosídeo (0,057 – 5,987%) foram os principais metabólitos detectados nas flores das espécies investigadas. *Hypericum caprifoliatum* e *H. andinum* apresentaram o maior rendimento destes compostos, respectivamente.

Sete espécies apresentaram japonicina A em concentrações de 0,003 a 0,087% (*H. myrianthum*). O rendimento de hiperbrasilol B variou de 0,006% em *H. laricifolium* a 0,011% em *H. caprifoliatum*. Os benzopiranos (HP1 = 0,200%, HP2 = 0,225% e HP3 = 0,327%) e as benzofenonas (carifenona A = 0,309% e carifenona B = 0,062%) ocorreram exclusivamente em *H. polyanthemum* e *H. carinatum*. As máximas quantidades de ácido clorogênico, isoquercitrina, quercitrina e guaijaverina foram observadas, respectivamente, em *H. campestre* (1,458%), *H. andinum* (1,161%), *H. carinatum* (0,231%) e *H. laricifolium* (0,404%). Tais resultados fornecem suporte adicional para o significado quimiotaxonômico dos derivados diméricos de floroglucinol. Temperatura (40°C = 3,04%, 50°C = 2,21% e 60°C = 1,05%) e pressão (90 bar = 0,95%, 150 bar = 0,97% e 200 bar = 1,89%) afetaram de modo distinto o rendimento do extrato supercrítico de *H. carinatum*. Apesar do menor rendimento em comparação ao extrato *n*-hexano (6,09%), a extração com CO_2 supercrítico provou ser mais seletiva que a maceração. Nas condições ideais (40°C, 90 bar e 180 minutos), a máxima recuperação de uliginosina B, carifenona A e carifenona B foi, respectivamente, 162,83, 376,33 e 48,79%. O modelo matemático empregado para simular o processo de extração foi apropriadamente correlacionado aos dados experimentais. Considerando as atividades biológicas, todas as espécies investigadas apresentaram um amplo espectro de ação antifúngica, assim como reduziram a migração dos neutrófilos. Os extratos de *H. carinatum*, *H. linoides* e *H. myrianthum* apresentaram os mais baixos valores de concentração inibitória mínima contra *Cryptococcus neoformans* (CIM ≤ 15,6 µg/mL), *Rhodotorula mucilaginosa* (CIM ≤ 62,5 µg/mL), *Candida glabrata* e *C. tropicalis* (CIM = 1,9-250 µg/mL). Para estas plantas, o efeito antiquimiotático variou entre 60 - 100% nas concentrações de 0,31 a 10 µg/mL. Os extratos mais ativos apresentaram elevada concentração de uliginosina B, japonicina A e hiperbrasilol B. Assim, as espécies de *Hypericum* nativas do sul do Brasil apresentam potencial como fonte de novos antifúngicos e anti-inflamatórios.

Palavras-chaves: *Hypericum*, *Brathys*, *Trigynobrathys*, floroglucinol dimérico, benzofenona, benzopirano, flavonoide, ácido fenólico, quimiotaxonomia, extração supercrítica, modelagem matemática, atividade antifúngica, atividade antiquimiotática.

ABSTRACT

Phytochemical and biological study of the *Hypericum* species of the *Brathys* and *Trigynobrathys* sections. The genus *Hypericum* (Guttiferae = Clusiaceae) comprises 494 species placed in 36 taxonomic sections. The representatives of Central and South America belong primarily to sections *Brathys* and *Trigynobrathys*, the two largest sections with 87 and 52 species, respectively. In traditional medicine, these plants are used as antiseptic, diuretic, digestive and as healing agents. Such species have a strong tendency to accumulate dimeric phloroglucinol, besides benzophenones, benzopyrans, flavonoids and phenolic acids, metabolites whose antidepressant, antinociceptive, antimicrobial, antiproliferative and antioxidant activities are described. In view of the therapeutic potential, this work aimed to determine the main phenolic compounds present in flowers of *Hypericum* species native to southern Brazil (*H. campestre*, *H. caprifoliatum*, *H. carinatum*, *H. connatum*, *H. linoides*, *H. myrianthum*, *H. polyanthemum* and *H. salvadorense*) and to Peruvian Páramos (*H. andinum* and *H. laricifolium*); the supercritical CO_2 extraction and analysis of the phenolic compounds of *H. carinatum*; the antifungal and antichemotactic activities of the lipophilic extracts from *H. caprifoliatum*, *H. carinatum*, *H. linoides*, *H. myrianthum* and *H. polyanthemum*. For the experiments, aerial parts in blossom were harvested (2008 - 2010). All collections were authorized by competent agencies of environmental protection. The extraction methods employed were static maceration, ultrasonic bath or supercritical CO_2 (temperature = 40, 50 or 60ºC; pressure = 90, 120, 150 or 200 bar). HPLC analyses were carried out in reverse phase column (C18), isocratic system composed of acetonitrile, water and trifluoroacetic acid, and ultraviolet detection (220, 270 or 254 nm). Peaks were identified by comparison of retention times / co-injection with standards and quantified by calibration curve of the compounds. Antifungal and antichemotactic tests were performed using the broth microdilution assay and neutrophils migration inhibition method. The dimeric phloroglucinol uliginosin B (0.008 - 0.188%) and the flavonoid hyperoside (0.057 - 5.987%) were the main metabolite detected in flowers of the investigated species. *Hypericum caprifoliatum* and *H. andinum* displayed higher yields of these compounds, respectively. Japonicin A

was found in seven species studied at concentrations that varied from 0.003 to 0.087% (*H. myrianthum*). The yield of hyperbrasilol B ranged from 0.006% in *H. laricifolium* to 0.011% in *H. caprifoliatum*. The benzopyrans (HP1 = 0.200%, HP2 = 0.225% and HP3 = 0.327%) and benzophenones (cariphenone A = 0.309% and cariphenone B = 0.062%) occurred exclusively in *H. polyanthemum* and *H. carinatum*. Maximum amounts of chlorogenic acid, isoquercitrin, quercitrin and guaijaverin were observed, respectively, in *H. campestre* (1.458%), *H. andinum* (1.161%), *H. carinatum* (0.231%) and *H. laricifolium* (0.404%). Such results provide a further support for the chemotaxonomic significance of the dimeric phloroglucinols. Temperature (40°C = 3.04%, 50°C = 2.21% and 60°C = 1.05%) and pressure (90 bar = 0.95%, 150 bar = 0.97% and 200 bar = 1.89%) critical did affect differently the yield of supercritical extract of *H. carinatum*. Despite lower yield in comparison to *n*-hexane extract (6.09%), supercritical CO_2 extraction proved to be more selective than maceration. In optimal conditions (40°C, 90 bar and 180 minutes), the maximum amount of uliginosin B, cariphenone A and cariphenone B was, respectively, 162.83, 376.33 and 48.79%. Lastly, the mathematical model used in the process of supercritical extraction was properly correlated to the experimental data. Regarding the biological activities, all investigated species exhibited a broad spectrum of antifungal action as well as reduced neutrophils migration. *Hypericum carinatum*, *H. linoides* and *H. myrianthum* extracts presented the lowest value of minimum inhibitory concentration against *Cryptococcus neoformans* (MIC $\leq$ 15.6 µg/mL), *Rhodotorula mucilaginosa* (MIC $\leq$ 62.5 µg/mL), *Candida glabrata* and *C. tropicalis* (MIC range = 1.9 - 250 µg/mL). For these plants, the antichemotactic effect varied from 60-100% at concentrations of 0.31 to 10 µg/mL. The most active extracts were that presented high amounts of uliginosin B, japonicin A and hyperbrasilol B. Thus, the *Hypericum* species native to Southern Brazil show potential as source of new anti-infectives and anti-inflammatory drugs.

Keywords: *Hypericum*, *Brathys*, *Trigynobrathys*, dimeric phloroglucinol, benzophenone, benzopyran, flavonoid, phenolic acid, chemotaxonomy, supercritical extraction, mathematical modelling, antifungal activity, antichemotactic activity.

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LISTA DE ABREVIATURAS E SIGLAS

CLAE – cromatografia líquida de alta eficiência;

CO_2 – dióxido de carbono;

HP1 – 6-isobutyryl-5,7-dimethoxy-2,2-dimethyl-benzopyran;

HP2 – 7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethyl-benzopyran;

HP3 – 5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl-benzopyran;

ºC - grau Celsius;

CH_3CN – acetonirila;

MeOH – metanol;

HPLC – high-performance liquid chromatography;

TFA – trifluroacetic acid;

HEX –*n*-hexane;

ANOVA – análise de variância;

nm – nanômetros;

t_R – time retention;

$r^2 = R^2$ – coeficiente de correlação linear;

p – nível de significância;

K_1 / K_2 – coeficiente de solubilidade / coeficiente de difusão.

SUMÁRIO

INTRODUÇÃO GERAL

Historicamente, os produtos naturais constituem uma fonte importante de recursos terapêuticos, uma vez que um grande número de fármacos em uso clínico ou são de origem natural ou foram desenvolvidos a partir destes. Na base deste sistema, as plantas figuram como um dos principais alvos dos estudos químicos, farmacológicos e clínicos. Além disso, dos 252 fármacos considerados básicos e essenciais pela Organização Mundial da Saúde (OMS), 11% são exclusivamente de origem vegetal. Constituem exemplos de compostos com aplicações terapêuticas a morfina, alcalóide isolado de *Papaver somniferum* e empregado no tratamento de dores intensas; o glicosídeo digitoxina de *Digitalis sp.*, empregado como cardiotônico; os ginkgolídeos de *Ginkgo biloba*, com propriedades anti-trombóticas e o diterpeno paclitaxel de *Taxus brevifolia*, utilizado como antitumoral (RATES, 2001; BUTLER, 2004; KOEHN e CARTER, 2005*;* GURIB-FAKIM, 2006; BAKER *et al*., 2007; NEWMAN e CRAGG, 2007; GANESAN, 2008; HARVEY, 2008; BARREIRO e BOLZANI, 2009).

A elevada diversidade química e biológica apresentada pelos compostos de origem natural favorece o desenvolvimento de novos fármacos. O potencial das plantas, entretanto, ainda é pouco explorado. Segundo estimativas, o número de espécies vegetais superiores pode chegar a 500.000. Destas, apenas 15 a 17% foram investigadas quanto ao seu potencial medicinal. Sabe-se também que a identificação de produtos com possível utilização econômica ou terapêutica aumenta com a diversidade de espécies. No mundo, metade das plantas superiores ocorre nas florestas tropicais. Países ricos em diversidade vegetal como o Brasil, portanto, apresentam grandes oportunidades na pesquisa e desenvolvimento de novos fármacos (SOEJARTO, 1996; RATES, 2001; GUERRA e NODARI, 2003; KOEHN e CARTER, 2005).

Nos últimos anos, o uso de terapias alternativas e complementares tem aumentado. Algumas práticas passaram a fazer parte inclusive do recurso terapêutico de órgãos oficiais de saúde como o Sistema Único de Saúde (SUS) (BRASIL, 2006). Entre os fatores que justificam o crescente interesse pelas plantas medicinais estão a insatisfação com os resultados da medicina convencional, os efeitos indesejáveis causados pelos medicamentos sintéticos, a falta de acesso aos medicamentos e à medicina institucionalizada, a consciência ecológica e a crença de que o que advém da

natureza é inofensivo (RATES, 2001). No campo científico, o interesse por produtos naturais tem sido justificado pela carência de métodos alternativos ao descobrimento de novos fármacos em áreas como imunossupressão, doenças do metabolismo e infecção (BUTLER, 2004). Além disso, a falta de tratamento efetivo de algumas doenças e a emergência de organismos patogênicos intensificam a necessidade por novos fármacos ou alternativas terapêuticas que combatam tais enfermidades.

O gênero *Hypericum* (Clusiaceae = Guttiferae) possui 494 espécies distribuídas em praticamente todas as regiões do mundo (ROBSON, 2006; CROCKETT and ROBSON, 2011; RUHFEL *et al*., 2011). Popularmente, estas plantas são utilizadas como antissépticos, diuréticos, digestivos e agentes de cicatrização (von POSER *et al*., 2006). Em geral, os principais grupos químicos descritos para o gênero são quinonas policíclicas (hipericina e *pseudo*-hipericina), derivados floroglucinol (hiperforina e *ad*-hiperforina), xantonas (mangiferina e isomangiferina), flavonoides, ácidos fenólicos, taninos e óleos essenciais (KITANOV e NEDIALKOV, 1998; KITANOV, 2001; BERNARDI, 2007; SMELCEROVIC *et al*., 2008). Em relação às atividades farmacológicas destacam-se a atividade antidepressiva (FRANCIS, 2005), antimicrobiana (SCHMITT *et al*., 2001; DALL'AGNOL *et al*., 2003), antitumoral (DONGRE *et al*., 2007), analgésica e anti-inflamatória (PERAZZO *et al*., 2008).

Hypericum perforatum é a espécie mais estudada do ponto de vista químico e farmacológico. Conhecida popularmente como "*erva-de-São-João*", extratos padronizados da planta são empregados no tratamento de depressão leve a moderada. De acordo com estudos farmacológicos *in vitro* e *in vivo*, os compostos hipericina e hiperforina, utilizados como marcadores, constituem os principais responsáveis pela atividade antidepressiva (BILIA *et al*., 2002).

No Brasil, as espécies nativas de *Hypericum* ocorrem principalmente na região Sul, compreendendo cerca de 20 representantes acomodados em duas seções taxonômicas, *Brathys* e *Trigynobrathys* (ROBSON, 1990). As plantas pertencentes a este grupo possuem uma forte tendência de acumular derivados diméricos de floroglucinol (uliginosina B, hiperbrasilol B e japonicina A), além de benzofenonas

(carifenonas A e B), benzopiranos (HP1 = 6-isobutiril-5,7-dimetoxi-2,2-dimetil-benzopiran, HP2 = 7-hidroxi-6-isobutiril-5-metoxi-2,2-dimetil-benzopiran e HP3 = 5-hidroxi-6-isobutiril-7-metoxi-2,2-dimetil-benzopiran), flavonoides (hiperosídeo, quercitrina, isoquercitrina, guaijaverina) e ácidos fenólicos (ácido clorogênico). Tais compostos são considerados os responsáveis pelos efeitos farmacológicos observados onde se destaca a atividade antidepressiva, antinociceptiva, antimicrobiana, antiproliferativa e antioxidante, entre outras (von POSER *et al*., 2006).

A descoberta de novos compostos com utilidade terapêutica e o desenvolvimento de modelos nacionais de saúde são alguns dos fatores que motivam o estudo das plantas. A enorme diversidade biológica dos biomas brasileiros, por sua vez, dada a sua capacidade de gerar conhecimento e inovação tecnológica, constitui uma alternativa promissora nesse processo (BARREIRO e BOLZANI, 2009).

Dados químicos e farmacológicos revelam um grande potencial terapêutico para as espécies do gênero *Hypericum*. O conhecimento das espécies nativas, entretanto, permanece parcialmente explorado. Assim, este trabalho teve como objetivo geral os estudos químico e biológico de espécies de *Hypericum* das seções *Brathys* e *Trigynobrathys*. Os objetivos específicos incluem:

- ✓ Determinação dos principais compostos fenólicos presentes nos extratos *n*-hexano e metanol obtido das flores de espécies de *Hypericum* nativas do Sul do Brasil e Páramos peruanos;

- ✓ Extração de *H. carinatum* com CO_2 supercrítico, modelagem matemática e análise dos compostos fenólicos presentes no extrato;

- ✓ Investigação das atividades antifúngica e antiquimiotática dos extratos lipofílicos de *H. caprifoliatum*, *H. carinatum*, *H. linoides*, *H. myrianthum* e *H. polyanthemum.*

A tese é apresentada e estruturada na forma de capítulos, os quais são na sequência amplamente considerados em uma discussão geral.

CAPÍTULO I

Compostos fenólicos em flores de *Hypericum* da seção *Brathys* e *Trigynobrathys*

INTRODUÇÃO

Quimicamente, fenólicos são compostos que possuem um ou mais anéis aromáticos com um ou mais grupos hidroxila. Distribuídos amplamente no reino vegetal, representam os metabólitos secundários mais abundantes das plantas com mais de 8000 estruturas conhecidas atualmente, as quais variam de moléculas simples a polímeros de alto peso molecular (DAÍ e MUMPER, 2010; IGNAT *et al*., 2011).

Os compostos fenólicos podem ser classificados em diferentes grupos dependendo do número de anéis fenólicos que apresentam e dos elementos estruturais que ligam estes anéis. Entre os principais grupos estão flavonoides, ácidos fenólicos, taninos, cumarinas, quinonas, estilbenos, lignanas, entre outros (IGNAT *et al*., 2011). Biossinteticamente, estes metabólitos são formados por três rotas diferentes: (i) a rota do chiquimato/corismato que produz derivados fenil propanóides; (ii) a rota do acetato/malonato ou policetídeo, a qual produz fenil propanoides de cadeia longa como os flavonoides e algumas quinonas; e (iii) a rota acetato/mevalonato cujos produtos são, por exemplo, os terpenoides aromáticos (BHATTACHARYA *et al*., 2010).

De maneira geral, as plantas produzem uma ampla variedade de substâncias bioativas com importantes aplicações nas áreas da saúde e alimentos. No reino vegetal, os compostos fenólicos atuam na defesa contra organismos patogênicos, parasitas e predadores, na proteção contra a radiação ultavioleta, na atração de polinizadores, como pigmentos e como agentes de sinalização intra e inter-espécies. Particularmente, tais metabólitos são determinantes para a qualidade sensorial e nutricional de frutas, entre outros vegetais, constituindo, nos últimos anos, o foco de muitas pesquisas na área da saúde devido principalmente a sua potente capacidade antioxidante e seus marcantes efeitos na prevenção de enfermidades associadas ao estresse oxidativo tais como câncer, doenças cardiovasculares e neurodegenerativas. Além disso, esses compostos apresentam importantes aplicações industriais, sendo utilizados como corantes e conservantes em alimentos ou na produção de tintas, papel e cosméticos (STAFFORD, 2000; RUIZ e ROMERO, 2001; BHATTACHARYA *et al*., 2010; DAÍ e MUMPER, 2010; IGNAT *et al*., 2011).

Por todas as razões mencionadas acima, a comunidade científica tem dedicado esforços no sentido de caracterizar e quantificar fenólicos de elevado potencial terapêutico. O gênero *Hypericum* compreende mais de 496 espécies acomodadas em 36 táxons amplamente distribuídos no mundo. *Hypericum perforatum* é o membro mais conhecido, dada a sua aplicação terapêutica nos casos de depressão leve a moderada. Os representantes centro-sul americanos pertencem à seção *Brathys* e *Trigynobrathys*. Estudos farmacológicos têm demonstrado para algumas destas plantas atividade antidepressiva, antinociceptiva, inibidora da monoamino oxidase, antimicrobiana e antiproliferativa. Estes efeitos frequentemente estão relacionados à presença de compostos fenólicos. Nesse sentido, uma particularidade das espécies nativas é a forte tendência de acumular derivados de floroglucinol diméricos, além de benzopiranos, benzofenonas, flavonoides, ácidos fenólicos e terpenoides (von POSER *et al*., 2006; CROCKETT e ROBSON, 2011; RUHFEL *et al*., 2011).

Nos sistemas de medicina tradicional e nos produtos beneficiados a partir de *Hypericum perforatum* as flores constituem o principal farmacógeno empregado. Dados da literatura também demonstram que a maior parte do conteúdo fenólico está concentrada nas flores das plantas (AYAN *et al*., 2006; BERNARDI *et al*., 2008; CROCKETT e ROBSON, 2011).

O potencial medicinal e o elevado valor econômico representado por *Hypericum perforatum* à indústria farmacêutica constituem alguns dos fatores que estimulam o estudo fitoquímico de outros membros do gênero. Apesar da elevada diversidade das espécies nativas, estas são relativamente pouco conhecidas, tanto do ponto de vista químico como biológico, justificando, assim, o seu estudo.

O presente capítulo objetivou a análise dos compostos fenólicos acumulados nas flores de espécies de *Hypericum* das seções *Brathys* e *Trigynobrathys*. Os resultados são apresentados e discutidos na forma de um manuscrito científico.

MANUSCRITO 1

FRANCISCO M. C. BARROS, GARI V. C. CCAPATINTA, GABRIELA C. MEIRELES, JÉSSICA M. NUNES, SIMONE T. CARGNIN, SATI SAKAMOTO, SÉRGIO BORDIGNON, CARLA DEL CARPIO, SARA L. CROCKETT, GILSANE L. VON POSER. Determination of phenolic compounds in flowers of *Hypericum* of South Brazil and Peruvian Páramos.

Determination of phenolic compounds in flowers of *Hypericum* species native to South Brazil and Peruvian Páramos

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Abstract

The flowers constitute one of the main sites of accumulation of phenolic compounds in plants of the *Hypericum* genus. In addition to their important pharmacological activities, some metabolites found in species from the section *Brathys* and *Trigynobrathys* appear to have chemotaxonomic significance according to the literature. HPLC analyses were carried out to assess the pattern and accumulation of the dimeric phloroglucinols, benzophenones, benzopyrans, flavonoids and a phenolic acid in flowers of *Hypericum* species native to southern Brazil and Peruvian Páramos. Qualitative and quantitative differences are reported. Uliginosin B and hyperoside were the main components, detected in all species and with maximum concentrations up to 0.188% in *H. caprifoliatum* and 5.987% in *H. andinum*, respectively. The content of japonicin A varied from 0.003 to 0.087% (*H. myrianthum*), while the yield of hyperbrasilol B ranged from 0.006% in *H. laricifolium* to 0.011% in *H. caprifoliatum*. The major compounds in *H. polyanthemum* and *H. carinatum* were the benzopyrans 6-isobutyryl-5,7-dimethoxy-2,2-dimethyl-benzopyran (HP1 = 0.200%), 7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethyl-benzopyran (HP2 = 0.225%) and 5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl-benzopyran (HP3 = 0.327%) and benzophenones cariphenone A (0.309%) and cariphenone B (0.062%), respectively. Maximum amounts of chlorogenic acid, isoquercitrin, quercitrin and guaijaverin were observed, respectively, in *H. campestre* (1.458%), *H. andinum* (1.161%), *H. cariantum* (0.231%) and *H. laricifolium* (0.404%). The results obtained support the taxonomic evidence of the dimeric phloroglucinol derivatives at the section level.

Keywords: *Hypericum*, phloroglucinol derivatives, flavonoids, phenolic acid, chemotaxonomy.

Introduction

The genus *Hypericum* L. (Hypericaceae) is represented by 484 species placed in 36 taxonomic sections on the basis of morphology and biogeography (Crockett and Robson 2011). Ruhfel and co-workers (2011) propose the inclusion of other 10 taxa based on phylogenetic analysis which would total 494 species. The representatives of Central and South America belong primarily to sections *Brathys* and *Trigynobrathys*, the two largest sections with 87 and 52 species, respectively (Crockett and Robson 2011). In the mountain regions of the Peruvian Páramos, 14 species occur, predominantly from section *Brathys* (Crockett et al. 2010), while in southern Brazil, 18 are found, the majority from section *Trigynobrathys* (Robson 1990).

A wide range of pharmacological activities attributed to the extracts of *Hypericum* have led to the isolation and identification of several phenolic compounds. Dimeric phloroglucinols (França et al. 2009), benzopyrans (Ferraz et al. 2001), benzophenones (Bernardi et al. 2005), xanthones (Zhang et al. 2007), flavonoids (Wang et al. 2008), phenolic acids, terpenoids (El-Seedi et al. 2003) and, to a lesser extent, essential oils (Abreu et al. 2004a; Ferraz et al. 2005) are classes of secondary metabolites that have been commonly isolated from *Hypericum* species of sections *Brathys* and *Trigynobrathys*. Compounds of these classes have been shown to possess antibacterial, antifungal, antiviral, antiproliferative, antinociceptive, monoamine oxidase inhibitory and antidepressant activities, among others (von Poser et al. 2006). Structures of the phenolic compounds surveyed are given in Figure 1.

Phenolic compounds play important roles as defensive and signaling in the interactions of the plant with the environment (Gronquist et al. 2001; Boudet et al. 2007). Flowers have been identified as one of the main sites of accumulation of these components in *Hypericum* (Ayan et al. 2006; Bernardi et al. 2008). Some authors also attribute a

chemotaxonomic significance to selected compounds in these classes, especially dimeric phloroglucinols (Ferraz et al. 2002; Nör et al. 2004). Considering the chemical and pharmacological importance of the metabolites, the objective of this study was to assess the pattern and accumulation of selected dimeric phloroglucinols, benzophenones, benzopyrans, flavonoids and a phenolic acid in flowers of *Hypericum* species native to southern Brazil and the Peruvian Páramos. As well as enriching the knowledge about the chemistry of this genus, these data can help in the selection of future targets for biological and phytochemical studies.

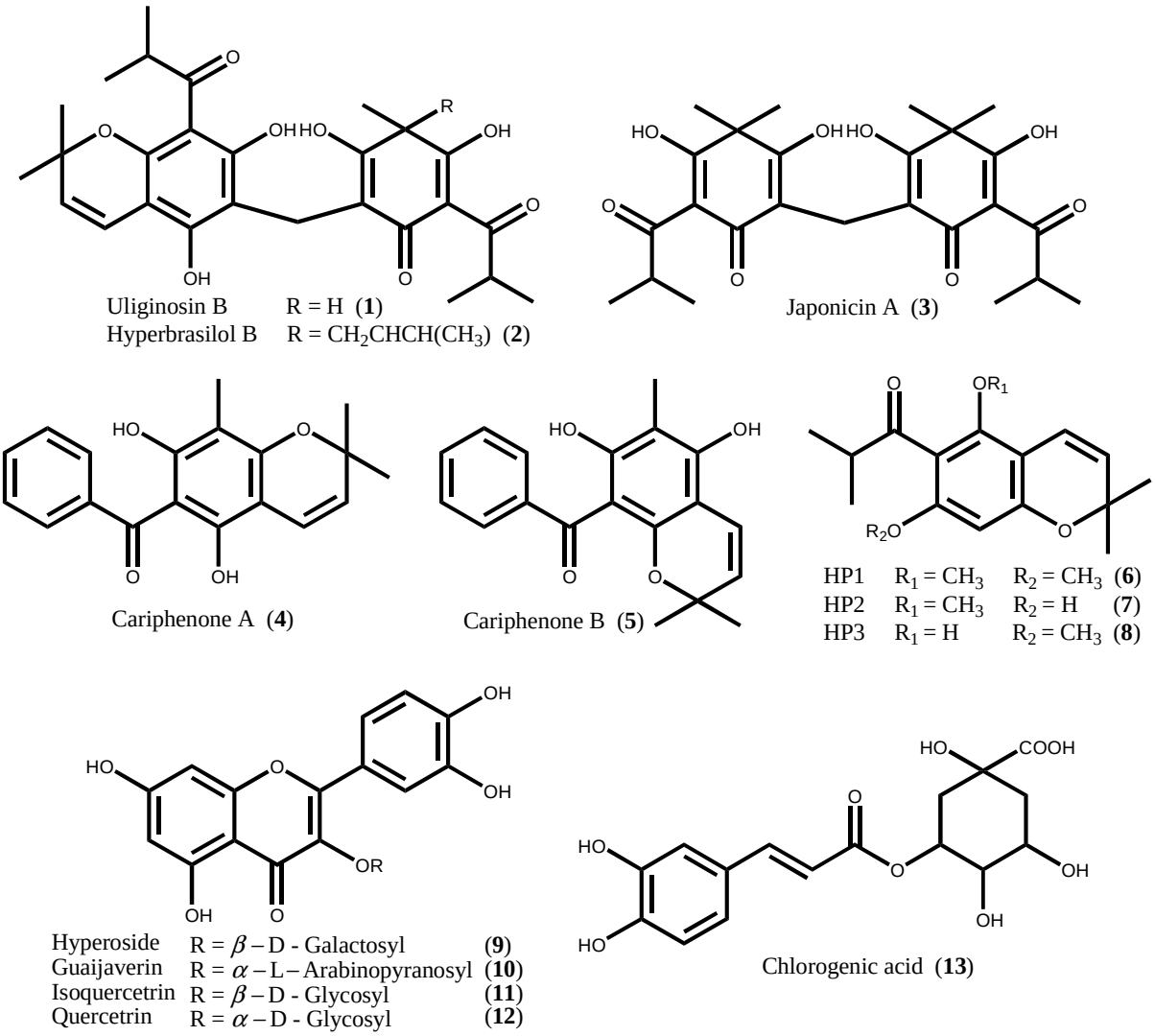

Fig 1 Chemical structure of selected secondary metabolites analyzed in species of *Hypericum* sections *Brathys* and *Trigynobrathys*

Materials and methods

Plant material

Flowers from several individuals of the same species were collected to form the pool used in the experiments (Table 1). Plants were identified by Dr. Sérgio Bordignon (UNILASALLE, RS, Brazil). Voucher specimens are deposited in the herbarium of Universidade Federal do Rio Grande do Sul (ICN). Plant collection was authorized by Conselho de Gestão do Patrimônio Genético (CGEN) and Instituto Brasileiro do Meio Ambiente (IBAMA – 003/2008 P 02000.001717/2008 - 60), besides Dirección General Forestal y de Fauna Silvestre of the Republic of Peru (0147-2010-AG-DGFFS-DGEFFS).

HPLC analysis

Acetonitrile (CH_3CN) and methanol (MeOH) were HPLC grade from Merck (Darmstadt, Germany); trifluroacetic acid (TFA) from Vetec (Rio de Janeiro, Brazil), *n*-hexane (HEX), dichloromethane (DIC) and ethyl acetate (EtOAc) (F. Maia, Cotia, São Paulo, Brazil) were reagent grade. Distilled water (H_2O) was purified by a Milli-Q system.

Uliginosin B (**1**), hyperbrasilol B (**2**), japonicin A (**3**), cariphenone A (**4**), cariphenone B (**5**), 6-isobutyryl-5,7-dimethoxy-2,2-dimethyl-benzopyran (HP1) (**6**), 7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethyl-benzopyran (HP2) (**7**), 5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl-benzopyran (HP3) (**8**), hyperoside (**9**) and guaijaverin (**10**) were isolated and identified from *Hypericum* species as described elsewhere (Ferraz et al. 2002; Nör et al. 2004; Dall'Agnol et al. 2005; Bernardi et al. 2007). Isoquercitrin (**11**), quercitrin (**12**) and chlorogenic acid (**13**) were purchased from Fluka (Steinheim, Germany), Dolisos Laboratories (Paris, France) and MP Biomedicals (Illkirch,

France). The identity and purity of compounds were confirmed by ^{1}H NMR spectroscopy.

Table 1 Collection localities of the *Hypericum* species

Species **Collection loacality (harvest)**	**Voucher number**
H. andinum Gleason.	Ccapatinta et al. 05
Amparaes, Cuzco, Peru (May 2008)	
H. campestre Cham. & Schlecht.	Bordignon et al. 3119
Caçapava do Sul, RS, Brazil (October and December, 2009)	
H. caprifoliatum Cham. & Schlecht.	Bordignon et al. 2287
Porto Alegre, RS, Brazil (October and December, 2009)	
H. carinatum Griseb.	Bordignon & Ferraz 2309
Glorinha, RS, Brazil (October and December, 2009)	
H. connatum Lam.	Bordignon et al. 3076
Capão do Leão, RS, Brazil (October and December, 2009)	
H. laricifolium Juss.	Ccapatinta et al. 07
Cumbemayo, Cajamarca, Peru (November 2008)	
H. linoides A. St.-Hil.	Bordignon et al. 3317
São José dos Ausentes, RS, Brazil (October and December, 2009)	
H. myrianthum Cham. & Schlecht.	Bordignon et al. 3059
Paraíso do Sul, RS, Brazil (October and December, 2009)	
H. polyanthemum Klotzsch ex Reichardt	Bordignon et al. 3118
Caçapava do Sul, RS, Brazil (October and December, 2009)	
H. salvadorense Robinson	Bordignon & von Poser 3452
Glorinha, RS, Brazil (October and December, 2009)	

The dried and powdered flowers of *Hypericum* species (200 mg) were extracted (5 times) successively with 5 mL of HEX and afterwards with 5 mL of MeOH for 20 min in an ultrasonic bath (Ultrasonic, São Paulo, Brazil). The fractions were evaporated to dryness under reduced pressure, dissolved in HPLC grade MeOH and filtered (0.22 mm pore size, Merck) prior to further analysis.

The HPLC system consisted of a Shimadzu liquid chromatography instrument (Shimadzu Corporation, Kyoto, Japan) equipped with a DGU-20A_5 degasser, LC-6AD pumps, SIL-10AD auto sampler, CTO-20AC column oven, SPD-20AV UV/VIS detector and CBM-20A communications module. The chromatographic data were recorded and processed by LC Solution software version 1.24 SP2. For separations, a Waters Nova Pack C18 column (4 µm, 3.9 x 150 mm) and a Waters Nova-Pack C18 60Å guard column (3.9 x 20 mm) were used.

Dimeric phloroglucinols were eluted using an isocratic elution program with 95% CH_3CN, 5% H_2O, 0.01% TFA, monitoring at 220 nm; benzopyrans and benzophenones, with 60% CH_3CN, 40% H_2O, at 270 nm; and chlorogenic acid and flavonoids, with 14% CH_3CN, 86% H_2O, 0.05% TFA, at 254 nm. The analyses were performed at room temperature (25 °C) and at a flow rate of 1 mL.min^{-1}. A volume of 20 µL and three injections were performed for each sample.

Peaks were identified by comparison of their retention times (t_R) and by co-injection with standards (Abreu et al. 2004b). The t_R values were 5.72, 7.29 and 8.85 min. for compounds **3**, **1** and **2**, respectively; 6.17, 8.08, 8.20, 11.52 and 19.41 min. for metabolites **5**, **4**, **6-8**; and 3.32, 20.07, 22.40, 30.67 and 35.24 min for **13**, **9**, **11**, **10** and **12**. The presence of selected metabolites in the extracts was also previously verified by thin layer chromatography using silica gel 60 F_{254} Merck (Darmstadt, Germany) as stationary phase. The mobile phase consisted of HEX:DIC (50:50 v/v) for the compounds **1 to 8** and EtOAc:MeOH:H_2O (100:13.5:10 v/v/v) for **9-13**.

Calibration curves were prepared for selected standards dissolved in MeOH with minimally five data points covering the concentration range of 8.13 – 411.34 µg/mL for chemical **1** (r^2 = 0.9976), 31.05 – 521.08 µg/mL for **4** (r^2 = 0.9999), 16.89 – 400 µg/mL for **6** (r^2 = 0.9999), 25 – 400 µg/mL for **7** (r^2 = 0.9981), 21.11 – 500 µg/mL for

8 (r^2 = 0.9995) and 100 – 2000 μg/mL for **9** (r^2 = 0.9982) and **13** (r^2 = 0.9983). Each curve was obtained by plotting the peak area of the compound at each level versus the concentration of the sample. Japonicin A and hyperbrasilol B were therefore quantified with the uliginosin B calibration curve; cariphenone B, using cariphenone A; and the flavonoids, using hyperoside (Nunes et al. 2010).

Results

In the present study, 10 species of *Hypericum* native to southern Brazil and the Peruvian Páramos were analyzed for the presence and quantity of 13 compounds, several of which are of pharmacological interest. The HPLC analysis allowed a satisfactory separation and detection of the chosen benzophenones, benzopyrans, dimeric phloroglucinols, flavonoids and the phenolic acid. The HEX and MeOH extracts exhibited unique secondary metabolite profiles, although some compounds, such as **1** and **9**, were detected in all samples. The quantitative differences were significant and are shown in tables 2 and 3.

In the HEX extracts, all species contained compound **1** at concentrations ranging from 0.004 to 0.18% (wt/wt). The flowers of *H. caprifoliatum* and *H. polyanthemum* displayed higher yields of **1**. In addition, flowers of seven of the ten species studied contained **3** in concentrations ranging from 0.003 to 0.08%. *Hypericum myrianthum* and *H. campestre* accumulated the highest amounts of **3**. Low amounts of the dimeric phloroglucinol **2** were detected in *H. caprifoliatum* and *H. laricifolium*, while only traces of **2** were observed in *H. connatum*. *Hypericum linoides* also showed a peak with retention time of compound **2**. However, it was found that this peak is resulted of overlap with an unknown compound. The benzophenones **4** and **5** and benzopyrans **6-8** were exclusively found in *H. carinatum* and *H. polyanthemum*, respectively (Table 2).

Table 2 Phenolic compounds present in the *n*-hexane fractions of the *Hypericum* flowers

Species	**%** (weight / dry weight tissue)								
	HEX	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
H. andinum	1.15	0.014	nd	nd	nd	nd	nd	nd	nd
H. campestre	2.10	0.064	nd	0.080	nd	nd	nd	nd	nd
H. caprifoliatum	2.10	0.188	0.011	0.004	nd	nd	nd	nd	nd
H. carinatum	3.30	0.046	nd	nd	0.309	0.062	nd	nd	nd
H. connatum	0.20	0.004	t	nd	nd	nd	nd	nd	nd
H. laricifolium	3.00	0.069	0.006	0.041	nd	nd	nd	nd	nd
H. linoides	3.80	0.020	nd	0.046	nd	nd	nd	nd	nd
H. myrianthum	4.75	0.008	nd	0.087	nd	nd	nd	nd	nd
H. polyanthemum	1.50	0.126	nd	0.003	nd	nd	0.200	0.225	0.327
H. salvadorense	3.20	0.013	nd	0.014	nd	nd	nd	nd	nd

HEX = *n*-hexane extract; **1** = uliginosin B; **2** = hyperbrasilol B; **3** = japonicin A; **4** = cariphenone A; **5** = cariphenone B; **6** = 6-isobutyryl-5,7-dimethoxy-2,2-dimethyl-benzopyran (HP1); **7** = 7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethyl-benzopyran (HP2); **8** = 5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl-benzopyran (HP3); **nd** = not detected; **t** = traces.

Table 3 Phenolic compounds present in the methanolic fractions of the *Hypericum* flowers

Species	**%** (weight / dry weight tissue)					
	MeOH	**9**	**10**	**11**	**12**	**13**
H. andinum	34.35	5.987	0.218	1.161	0.002	t
H. campestre	6.30	0.514	0.116	0.174	0.109	1.458
H. caprifoliatum	50.75	1.553	0.353	0.168	0.120	t
H. carinatum	7.10	0.758	0.228	0.086	0.231	1.014
H. connatum	9.45	0.057	t	0.083	t	0.790
H. laricifolium	30.25	0.715	0.404	1.100	t	t
H. linoides	4.10	0.075	0.082	t	0.187	0.470
H. myrianthum	4.10	1.229	0.142	0.066	t	0.205
H. polyanthemum	8.05	1.063	0.258	0.196	0.190	t
H. salvadorense	8.25	0.377	0.080	0.782	t	1.115

MeOH = methanol extract; **9** = hyperoside; **10** = guaijaverin; **11** = isoquercitrin; **12** = quercitrin; **13** = chlorogenic acid; **t** = traces.

Regarding the MeOH fractions, the flavonoid **9** was the main metabolite detected in concentrations that varied from 0.057% in *H. connatum* to 5.98% in *H. andinum*; **10**, **11**, and **12** varied from trace amounts in several species to 0.40% in *H. laricifolium*, 1.16% in *H. andinum* and 0.23% in *H. carinatum*, respectively. The phenolic acid **13** also varied from trace amounts in several species to a maximum 1.45% in *H. campestre*.

Discussion

Phytochemical profiling studies can be valuable tools in taxonomic analysis of the *Hypericum* genus. HPLC analysis of the HEX extracts allowed the characterization of the different species investigated, supporting the usefulness of the technique as a chemical fingerprinting method. This aspect is particularly important since the limited number of morphological characteristics available to distinguish between some sections and high degree of morphological variability of some specimens create difficulties in the differentiation and identification of individual plants based only on morphological characters. The morphological similarities among *H. caprifoliatum* and *H. connatum* or *H. campestre* and *H. brasiliense* constitute examples (Robson 1990). Until now, however, appropriate methods had not been developed for quality control of *Hypericum* species native to Central-South America.

Dimeric phloroglucinols have been described for the genera *Elaphoglossum*, *Dryopteris*, *Aspidium*, *Myrtus*, *Mallotus*, *Eucalyptus*, *Helichrysum* and *Hypericum* (Singh 2006; Socolsky et al. 2012). In the latter, such compounds have been isolated exclusively from members of sections *Brathys* and *Trigynobrathys* (Table 4). Thus, some authors regard these phenolics as potential chemotaxonomic markers (Ferraz et al. 2002; Nör et al. 2004; von Poser et al. 2006).

Table 4 Dimeric phloroglucinols of *Hypericum* genus – *Brathys* and *Trigynobrathys* sections

Section* / *Species	**Dimeric phloroglucinols**	**References**
Brathys		
H. drummondii	Albaspidins AA and PP	Jayasuryia et al. 1989
	Drummondins A, B, C, D, E and F	Jayasuryia et al. 1991
	Isodrummondin D	
H. gentianoides	Saroaspidin A,	Babka 2009
	Uliginosin A	
	Hyperbrasilol C	
Trigynobrathys		
H. brasiliense	Hyperbrasilol A, B and C	Abreu et al. 2004b
	Isohyperbrasilol B	França et al. 2009
	Isoluliginosin B	Leal et al. 2010
	Japonicin A	Rocha et al. 1995
	Uliginosin A and B	Rocha et al. 1996
H. carinatum	Uliginosin B	Nör et al. 2004
H. connatum	Hyperbrasilol B	Nör et al. 2004
	Japonicin A	Nör et al. 2008
H. caprifoliatum	Hyperbrasilol B	Nör et al. 2004
	Uliginosin B	Nör et al. 2008
H. japonicum	Saroaspidin A, B and C	Hu et al. 2000
	Sarothralen A, B, C and D	Ishiguro et al. 1985
	Sarothralin	Ishiguro et al. 1986
	Sarothralin G	Ishiguro et al. 1987
	Japonicin A	Ishiguro et al. 1990
		Peng et al. 2006
		Singh et al. 2006
H. myrianthum	Japonicin A	Dall'Agnol et al. 2005
	Uliginosin B	Ferraz et al. 2002
		Nör et al. 2008
		Bernardi et al. 2007
H. polyanthemum	Uliginosin B	Nör et al. 2004
H. ternum	Uliginosin B	Bernardi et al. 2007
H. uliginosum	Uliginosin A and B	Parker et al. 1968
		Taylor et al. 1969

Comparing Tables 2 and 4, in eight of ten investigated species, at least one dimeric phloroglucinol has been detected for the first time. In *H. caprifoliatum* and *H. laricifolium*, both phloroglucinols (**1-3**) analyzed were present, as seen in *H. brasiliense*. In accordance with Crockett and Robson (2011), it is interesting to note that compounds such as **1** and **3** have been isolated from species of *Trigynobrathys* growing in different geographic regions, indicating that these compounds may have chemotaxonomic utility at the sectional or subsectional level. Our results provide further support for the chemotaxonomic significance of the dimeric phloroglucinols, although the data cannot be conclusive since the number of species investigated in relation to the total sectional species number is still low. In fact, only 2 and 17% of the representatives of the sections *Brathys* and *Trigynobrathys*, respectively, have thus far been analyzed (Table 4).

The compounds detected in the MeOH fractions (Table 3) are common in many species of *Hypericum* (Su et al. 2008; Nunes et al. 2010). The compounds **9**-**13** are the main polar phenolics reported in *Brathys* and *Trigynobrathys* sections (Ishiguro et al. 1991; Dall'Agnol et al. 2003; El-Seedi et al. 2003; Abreu et al., 2004b; Peng et al., 2006; Bernardi et al. 2007; Zhang et al. 2007; Dourado et al. 2008; Li et al. 2008; Su et al. 2008; Wang et al. 2008; Nunes et al. 2010). Despite of their frequent occurrence, it is not possible to see a clear taxonomic pattern.

The phenolic content varied significantly among the investigated species (Table 2 and 3). The different parts of the plant, development stages and environmental conditions (i.e., location, season, collection) are very common factors of chemical variation. Evaluation of total phenolic compounds in *H. polyanthemum* and *H. ternum*, however, demonstrated that the flowers are the main organ for accumulation (Bernardi et al. 2008; Pinhatti et al. 2010). Additionally, the polar phenolic compounds (phenolic acids and flavonoids) were produced in higher amounts (Li et al. 2008; Su et al. 2008) in accordance with the results presented in table 2 and 3.

The variability of phenolic compounds cited above and the different experimental conditions employed make it difficult to directly compare our results with those of other authors. Nevertheless, the yields of benzopyrans **6-8** are similar to those previously reported by Bernardi et al. (2008). The polar phenolic compounds **9**, **10** and **13** were found in higher concentration in the flowers than in the stems and leafs, as determined by Nunes et al. (2010). Metabolite **12** was obtained in a higher amount in the stems and leaves while the concentration of **11** in flowers was comparable to that found in aerial parts. It is also important to note that the flavonoid content of *H. andinum* and *H. laricifolium*, collected at high altitude regions of Peru, were the highest among all the investigated species. In comparison to the Brazilian species, the Peruvian plants included in this study occur in mountain habitats exceeding 3000 meters above the sea level. Since the flavonoid content of some *Hypericum* species was positively correlated with altitude (Umek et al. 1999; Abreu et al. 2005; Bruni and Sacchetti 2009) this factor could explain such results.

Conclusions

The characterization of selected dimeric phloroglucinols, benzophenones, benzopyrans, flavonoids and chlorogenic acid in *Hypericum* species native to South Brazil and Peruvian Páramos reaffirms the value of this genus as a source of bioactive compounds. The results have provided additional useful evidence supporting the chemotaxonomic significance of dimeric phloroglucinols for the species of the sections *Brathys* and *Trigynobrathys*. Continuing research in our laboratories involves the isolation and structural elucidation of additional bioactive compounds from South American *Hypericum* species.

Acknowledgements

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CAPÍTULO II

Extração de *Hypericum carinatum* Griseb. com CO_2 supercrítico

INTRODUÇÃO

A extração dos compostos de uma matriz constitui uma das primeiras etapas do processo de análise, isolamento e purificação de produtos naturais. Tradicionalmente, os métodos empregados são a extração por Soxhlet, maceração, percolação e destilação. Apesar da facilidade e do pequeno custo operacional, tais técnicas apresentam desvantagens como o baixo rendimento e/ou seletividade e o uso excessivo de solventes orgânicos tóxicos e residuais. O desenvolvimento e o emprego de tecnologias alternativas vêm crescendo nas últimas décadas a exemplo da extração por fluido supercrítico, extração assistida por micro-ondas e com solventes pressurizados (KAUFMANN e CHRISTEN 2002; STICHER, 2007; BIMAKR *et al*., 2009).

Em 1822, o cientista francês Caignard de la Tour descobriu os fenômenos críticos (BERCHE *et al*., 2009). Hannay e Hogarth (1879), por sua vez, foram os primeiros a demonstrar o seu poder de solvatação (HANNAY e HOGARTH, 1879 *apud* LANG e WAI, 2001). Por definição, qualquer substância mantida acima de sua temperatura e pressão críticas constitui um fluido supercrítico, o qual apresenta propriedades intermediárias entre gases e líquidos. A utilização dos fluidos supercríticos na extração de produtos naturais tem como marco o processo de descafeinização dos grãos de café verde em meados de 1970. Desde então, uma ampla variedade de compostos tem sido obtida (MAUL *et al*., 1996; LANG e WAI, 2001; HERRERO *et al*., 2010), sendo o dióxido de carbono (CO_2) o solvente de escolha em mais de 90% dos casos (POURMORTAZAVI e HAJIMIRSADEGHI, 2007).

Idealmente, o processo de extração deve ser exaustivo para os compostos de interesse, além de rápido, simples, de baixo custo e capaz de automação (BENTHIN *et al*., 1999). Entre os fatores que influenciam a extração por fluido supercrítico destacam-se aqueles relacionados à natureza dos solutos (apolar, polar ou iônico), aos parâmetros de extração (pressão, temperatura e tempo) e à natureza da matriz (tamanho das partículas, sítios ativos e conteúdo de água) (CAMEL, 2001). O método tem sido utilizado somente na extração dos compostos bioativos da espécie européia *Hypericum perforatum* (CATCHPOLE *et al*., 2002; GLISIC, *et al*., 2008) e sua

aplicação na extração das espécies de *Hypericum* brasileiras foi descrita apenas para *H. polyanthemum* (CARGNIN *et al*., 2010).

A demanda dos consumidores e a exigência dos órgãos reguladores quanto à qualidade, segurança e eficácia dos produtos naturais denotam o forte impacto econômico que os extratos vegetais geram à indústria, seja ela de alimentos, cosmética ou farmacêutica. Assim, visto que na maioria das vezes o processo extrativo está baseado em abordagens empíricas e/ou em escala laboratorial, a seleção e a otimização do método são consideradas etapas chave para sua industrialização (ZHANG *et al*., 2005; ABBAS *et al*., 2008; KASSING *et al*., 2010).

O presente capítulo teve por objetivo demonstrar a extração seletiva dos derivados de floroglucinol e benzofenonas de *Hypericum carinatum* com CO_2 supercrítico, bem como determinar alguns parâmetros de extração. Os principais resultados são apresentados e discutidos na forma de um manuscrito científico e um resumo expandido apresentado em congresso.

MANUSCRITO 2

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Supercritical extraction of phloroglucinol and benzophenone derivatives from *Hypericum carinatum*: quantification and mathematical modeling

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Running title:

Supercritical extraction of *Hypericum carinatum:* quantification and modeling

Keywords: Supercritical-fluid extraction; Phloroglucinol; Benzophenone; *Hypericum*; Mathematical modeling

Abstract

The aerial parts of *Hypericum carinatum* (Guttiferae) were extracted with supercritical carbon dioxide under constant temperature (40, 50 or 60°C) and gradual pressure increase (90, 120, 150 and 200 bar) aiming at the recovery of enriched fractions containing uliginosin B, cariphenone A and cariphenone B, compounds of pharmaceutical interest. The yields of these substances were determined by high-performance liquid chromatography and compared with those obtained with *n*-hexane maceration. The supercritical-fluid extraction showed higher selectivity than the conventional solvent extraction method. After defining 40°C and 90 bar as the best conditions to obtain the target compounds, a mathematical model was used for the extraction process and a good correlation was achieved with the experimental data.

1 Introduction

Phloroglucinol derivatives are a class of secondary metabolites with wide occurrence in the Guttiferae family [1]. The dimeric phloroglucinol uliginosin B, previously isolated from some *Hypericum* species native to south Brazil (i.e. *H. myrianthum*, *H. polyanthemum* and *H. carinatum*) [2], demonstrated important antinociceptive effect [3]. *H. carinatum* also afforded the benzophenone derivatives cariphenone A and cariphenone B [4], whose chemical class has been highlighted for the cytotoxic and HIV-inhibitory activities [5-7].

Taking into account the pharmacological activities, the low concentration in the vegetal matrix and thermo-oxidative decomposition of phloroglucinol and benzophenone derivatives [8-12], it is important to develop efficient methods to extract such compounds. Conventional methods present few adjustable parameters to control the selectivity of the extraction, employ large amounts of toxic organic solvents, are time-consuming and provide low yields [13-14]. The supercritical-fluid extraction (SFE) has shown promising results in solving such drawbacks [13, 15].

The characteristics of the supercritical carbon dioxide [16-20] suggest its applicability in the extraction of bioactive compounds from *Hypericum* species. Nevertheless, until now it was employed only for *H. perforatum* [8, 21] and *H. polyanthemum* [22]. So, this work investigated the effect of extraction conditions [23] such as temperature, pressure and time regarding the recovery of extracts and concentration of uliginosin B, cariphenone A and cariphenone B. For the condition associated with the higher concentration of these compounds, a mathematical modeling based on mass transfer principles was performed aiming at future scale-up procedure.

The mathematical modeling of extraction must be considered as a fundamental step in the project of an efficient industrial process. Mathematical models are used to simulate processes with no need of experimentation in order to know the extraction process behavior. Therefore, mathematical modeling is a strategy to evaluate the selection of the variable conditions [24]. According to Reverchon and De Marco [25], a model should not be a simple mathematical instrument, but should reflect the physical insight arising from the experimental observations. In this work, the mathematical analysis was performed using differential mass balances for fluid and solid phase in an extraction bed based on the concept of broken and intact cells. The model was used to fit experimental data and simulates two extraction periods, being the former associated to the equilibrium-phase and the latter with the internal diffusion [26].

2 Material and Methods

2.1 Plant material

The aerial parts of *Hypericum carinatum* Griseb. were collected during its flowering stage in Glorinha, Rio Grande do Sul state, Brazil, in December, 2008. The plant material was identified by S. Bordignon (Departamento de Botânica, Universidade Luterana do Brasil). A voucher specimen was deposited in the Herbarium of the Universidade Federal do Rio Grande do Sul (ICN - Bordignon 1520). Plant material was dried at room temperature and powdered (1 mm mesh opening) in a cutting mill.

2.2 Extraction methods

Supercritical extractions were carried out on a pilot-scale automated equipment according to procedures previously described [27]. Powdered plant material (100 g DW) was extracted at constant temperature (40, 50 or 60ºC) while the pressure was successively increased from 90 to 120, 120 to 150 and 150 to 200 bar. The extractions

were performed following the experimental procedure defined by Cargnin et al. [22], i.e., 100 min at 90 bar, 30 min at 120 bar, 30 min at 150 bar and 30 min at 200 bar, using the same plant material. However, instead of only one fraction for each pressure, in this study samples were collected every 10 min in order to evaluate yield and composition of the extracts *versus* extraction time. The supercritical carbon dioxide flow rate was 6.7×10^{-4} $kg.s^{-1}$ (through the extraction vessel) using a flowmeter assay (Sitraus F C Massflo 2100 - Siemens) with accuracy of < 0.1%. Pressure in the extractor was monitored with a digital transducer system, Novus 8800021600, acquired from Novus Produtos Eletrônicos (Brazil) with precision of ±1.0 bar. The temperature controller was connected to thermocouples (PT-100) with accuracy of < 0.5.

In order to compare SFE to the conventional extraction method, powdered aerial parts of *H. carinatum* (10 g DW) were thoroughly extracted by *n*-hexane maceration until constant extract weight. To remove the organic solvent, the extract was evaporated to dryness under reduced pressure.

2.3 HPLC analysis

The *n*-hexane and supercritical CO_2 extracts were treated with acetone and filtered off to remove waxes and insoluble impurities [22]. Then, the enriched fractions were evaporated to dryness, dissolved in HPLC grade methanol, filtered (0.22 µm pore size, Merck) and analyzed by high-performance liquid chromatography.

2.3.1 Benzophenones determination

Cariphenone A analysis was performed using a Waters 600 pump and a Waters 2487 dual λ absorbance detector set to 270 nm. The separation was carried out with an isocratic solvent system (60% CH_3CN, 40% H_2O) through a Waters Nova-Pack C18

column (4 µm, 3.9 x 150 mm) adapted to a Waters Nova-Pack C18 60 A guard column (3.9 x 20 mm) and flow rate of 1 $mL.min^{-1}$. Metabolite determination was assessed by a calibration curve of pure standard isolated from *H. carinatum* and concentrations ranging from 5 to 1400 $\mu g.mL^{-1}$ ($R^2 > 0.999$). The compound identification was made on the basis of the ultraviolet absorption spectra and retention time in comparison with the standard compound. Cariphenone B quantification was performed under the same conditions described above and the content was expressed as milligram of cariphenone A equivalents/100 g plant.

2.3.2 Uliginosin B determination

Uliginosin B yields were determined using the same equipment under isocratic solvent condition (95% CH_3CN, 5% H_2O, 0.01% TFA), flow rate of 1 $mL.min^{-1}$ and detection at 220 nm. Metabolite quantification was assessed by a calibration curve of pure standard and the concentrations ranged from 2 to 800 $\mu g.mL^{-1}$ ($R^2 > 0.999$). The identification was based on the ultraviolet absorption spectra and retention time in comparison with the standard compound.

2.4 Statistical analysis

Mean differences among each extraction condition were tested for significance by ANOVA using a probability value of $P < 0.05$. Tukey's test was used to indicate mean separation among these conditions (SPSS Software, version 10).

2.5 Mathematical modeling

A model for supercritical-fluid extraction based on the concept of broken and intact cells was used to fit experimental data and simulates two extraction periods using the differential mass balances of the solute per unit volume extraction bed. The first period

is guided by phase-equilibrium and the second by internal diffusion into the particles. The mathematical model was formulated by Xavier et al. [26] according to the discussion proposed by Sovová [28]. In this model, the mass of extracted compounds (solute) is assumed to be a *pseudo* component in terms of the mass balance. The solute mass balance in fluid and solid-phase was expressed by two partial differential equations that were analytically solved by Xavier et al. [26]. The extraction curve, written in terms of maximum extract yields, is expressed in two steps; the first one controlled by phase equilibrium and the second, by internal diffusion from particles, having the following expression

$$\frac{M(t)}{M(\infty)} = \frac{K_1\, t}{M(\infty)}. \tag{1}$$

for the first period, where

$$K_1 = \dot{m}\, Y^*, \tag{2}$$

being $\dot{m}$ the solvent flow rate and Y^* is weight fraction for the equilibrium fluid-phase;

and

$$\frac{M(t)}{M(\infty)} = (1 - e^{-K_2\, t}). \tag{3}$$

for the second period, where K_2 is defined as follow

$$K_2 = \frac{k_s a_0}{(1-\varepsilon)}. \tag{4}$$

where k_s is the solid-phase mass transfer, and a_0 is the specific surface area per unit volume of extracted bed.

Summarizing, the extraction curve is linear in function of the time for the first step of the extraction and exponential for the second period [26].

3 Results and Discussion

3.1 Extraction yields

The total extract yields obtained by CO_2 supercritical extraction at 40, 50 and 60 °C and different pressure values are shown in Table 1. The analysis of supercritical extraction of *H. carinatum* showed that the increase in temperature produced a negative effect on the total extracts recovery. This effect is a consequence of the fluid density decrease, which varies proportionally with the pressure [30]. Thus, under constant temperature, the subsequent pressures (120, 150 and 200 bar) tended to afford higher yields. The supercritical fluid density behavior can explain the higher yields obtained at 40ºC and 90 bar since these conditions confer to fluid higher density and, consequently, higher solvation ability.

It is well established that benzopyran, benzophenone and phloroglucinol derivatives present is some *Hypericum* species are soluble in acetone while undesirable waxes and other compounds remain precipitated [22]. As it can be seen in table 1, at 40ºC and 90 bar the yield of the acetone soluble fraction was very high, representing ca. 68% (w/w) of the extract obtained in this condition. In comparison, the extracts acquired at 90 bar - 50 and 60ºC presented only ca. 5% (w/w) and 3% (w/w) of this fraction, respectively. Although the supercritical fluid presents a high solvation power under low temperature (40ºC) and pressure (90 bar), the distinct extraction of acetone soluble and insoluble compounds shows that the properties of the solute are also important to explain the results. The solubility of the low volatile substances, for example, decreases with increasing molecular weight and polarity, while for those highly volatile, the solubility is determined by the vapor pressure, which increases with the temperature [31].

Table 1. Supercritical fluid extraction yields from *H. carinatum* of total extract and fraction obtained after treatment with acetone.

	Yields					
Pressure	**40°C**		**50°C**		**60°C**	
(bar)	% Extract[a]	% Fraction[b]	% Extract[a]	% Fraction[b]	% Extract[a]	% Fraction[b]
90	1.22	0.83	0.59	0.03	0.35	0.01
120	0.25	0.13	0.40	0.20	0.05	0.03
150	0.51	0.27	0.44	0.30	0.29	0.20
200	1.06	0.66	0.79	0.55	0.36	0.30
Total	**3.04**	**1.89**	**2.21**	**1.09**	**1.05**	**0.54**

[a]g of total extract /100 g of plant
[b]g of fraction after treatment with acetone /100 g of plant

In order to compare the extraction efficiency, plant material was also submitted to maceration with *n*-hexane, yielding 6.09% of extract and 4.71% of acetone soluble fraction. The higher yield obtained by the maceration compared to the SFE can be explained by the greater co-extraction of undesirable compounds (i.e. chlorophylls) and consequently lower selectivity of the former method. Similar results are described in literature highlighting the positive effects in the quality of supercritical fluid extracts, such as selectivity, total elimination of solvent residue and compounds stability [17].

3.2 HPLC analysis of phloroglucinol and benzophenone derivatives

The extraction conditions regarding the contents of uliginosin B, cariphenone A and cariphenone B in the *H. carinatum* fractions were determined by HPLC (Fig. 1).

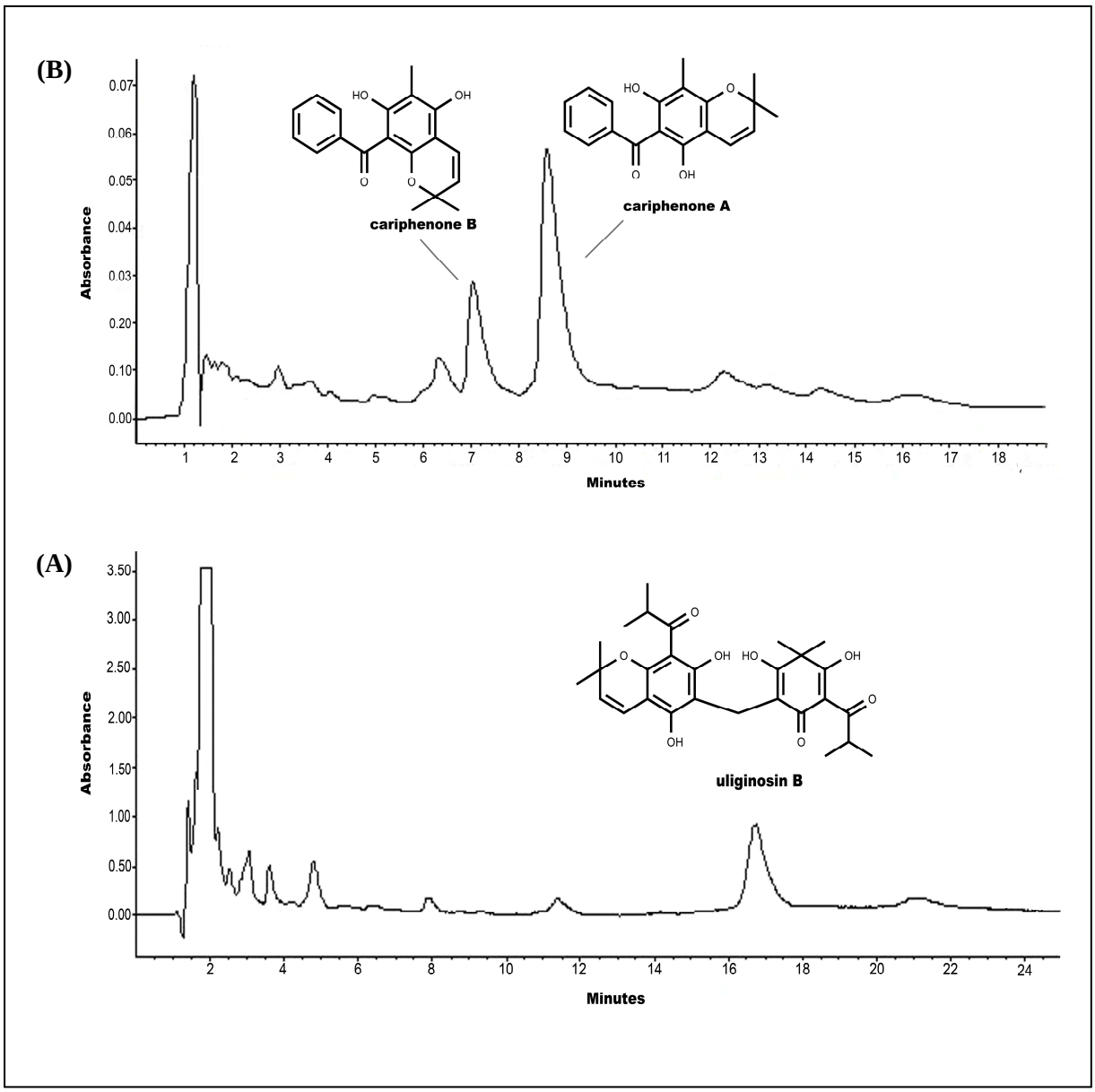

Figure 1. (A) HPLC profile of uliginosin B from the supercritical CO_2 fraction (60 min) obtained at 40°C temperature and 90 bar pressure; (B) HPLC profile of cariphenone A and cariphenone B from the supercritical CO_2 fraction (130 min) obtained at 40°C temperature and 120 bar pressure.

The evaluation of yields and extracts composition in function of the time has been the subject of several studies related to supercritical fluid extraction [32-33]. However, in the studies with *Hypericum* species the influence of time variable was not considered. Aiming to evaluate of the uliginosin B, cariphenone A and cariphenone B yields in relation to the time, the extracts were obtained every 10 min, treated with acetone and analyzed (Tables 2, 3, and 4). In all the experiments, at 90 bar, the end time was defined considering the plant exhaustion (100 min). For other pressures (120, 150, and

200 bar), the extraction time was defined as 30 min, following the same experimental procedure used by Cargnin et al. [22].

The amounts of uliginosin B, cariphenone A and cariphenone B in the acetone soluble fraction of the *n*-hexane extract were respectively: 167.94 ± 3.76, 58.60 ± 1.72, and 26.40 ± 0.65 mg per 100 g of plant. In general, supercritical CO_2 extraction afforded higher amounts of the compounds (Table 2, 3 and 4) than the *n*-hexane maceration, indicating that the supercritical method is more selective. Thereby, significant changes in composition and biological activity of the extracts may occur according to the extraction method selected. Thus, supercritical fluid extraction allows enriching bioactive compounds, increasing the biological activity of the extracts [17, 13, 20].

Observing the results shown in Table 2, it is possible to affirm that the contact time (about 40 min) was necessary to promote the acquisition of the extract. The efficiency of the extraction is also related to the increase of the fluid contact time with the vegetal matrix. Therefore, the magnifying of this time permits a deeper penetration of the solvent in the plant material. Consequently, a higher amount of extract will be spread out characterizing a phenomenon of mass transfer. The intensity of mass transfer is associated to the amount of solvated material and subsequent diffusion through the solid-phase until the interface with the solvent.

In the experiment performed at 40ºC, the pressure of 90 bar afforded higher amounts of uliginosin B and cariphenone A. The highest concentration of cariphenone B was obtained at 200 bar, but this compound was also obtained in considerable quantities at 90 bar (Table 2). At 40ºC, the acetone soluble fraction presented 8.61% - uliginosin B, 5.08% - cariphenone A and 2.58% - cariphenone B. Therefore, the experimental condition 90 bar – 40ºC was the most suitable for the extraction of these compounds.

Table 2. Mass quantities of uliginosin B, cariphenone A, and cariphenone B extracted by CO_2 supercritical fluid from *H. carinatum* at 40°C per 100 g of plant.

Time (min)	Uliginosin B (mg)	Cariphenone A (mg)	Cariphenone B (mg)
		P = 90 bar	
10	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
20	0.23 ± 0.01	0.02 ± 0.00	0.09 ± 0.00
30	3.53 ± 0.09	1.96 ± 0.11	0.09 ± 0.00
40	28.09 ± 1.09	14.09 ± 0.35	0.35 ± 0.00
50	21.19 ± 1.62	9.51 ± 0.18	0.36 ± 0.01
60	17.25 ± 0.14	5.98 ± 0.52	0.29 ± 0.00
70	23.71 ± 2.10	10.67 ± 0.05	0.70 ± 0.00
80	11.85 ± 1.03	7.16 ± 0.39	1.41 ± 0.04
90	15.46 ± 1.42	7.16 ± 0.11	6.12 ± 0.12
100	8.63 ± 0.67	6.10 ± 0.25	4.60 ± 0.34
Σ	**129.97 ± 3.53^{a}**	**62.67 ± 1.47^{a}**	**14.02 ± 0.43^{b}**
		P = 120 bar	
10	11.76 ± 0.90	6.36 ± 0.30	2.40 ± 0.22
20	0.21 ± 0.00	0.36 ± 0.00	0.13 ± 0.00
30	0.14 ± 0.00	0.21 ± 0.00	0.07 ± 0.00
Σ	**12.11 ± 0.90^{b}**	**6.93 ± 0.29^{c}**	**2.60 ± 0.22^{d}**
		P = 150 bar	
10	5.14 ± 0.17	6.69 ± 0.33	1.50 ± 0.12
20	6.49 ± 0.48	5.82 ± 0.38	2.21 ± 0.12
30	2.94 ± 0.27	5.31 ± 0.17	3.01 ± 0.24
Σ	**14.57 ± 0.60^{b}**	**17.82 ± 0.50^{b}**	**6.72 ± 0.05^{c}**
		P = 200 bar	
10	2.06 ± 0.02	1.90 ± 0.07	1.80 ± 0.08
20	1.33 ± 0.02	2.21 ± 0.11	3.66 ± 0.03
30	2.79 ± 0.13	4.43 ± 0.37	19.99 ± 1.18
Σ	**6.18 ± 0.14^{c}**	**8.55 ± 0.51^{c}**	**25.45 ± 1.26^{a}**
Total	**162.83 ± 4.58^{a}**	**95.97 ± 0.82^{a}**	**48.79 ± 0.66^{a}**

^{a,b,c,d} Different letters indicate significant differences among comparable categories ($P < 0.05$).

Table 3. Mass quantities of uliginosin B, cariphenone A, and cariphenone B extracted by CO_2 supercritical fluid from *H. carinatum* at 50°C per 100 g of plant.

Time (min)	Uliginosin B (mg)	Cariphenone A (mg)	Cariphenone B (mg)
P = 90 bar			
10	nd	nd	nd
20	nd	nd	nd
30	nd	nd	nd
40	nd	nd	nd
50	nd	nd	nd
60	nd	nd	nd
70	nd	nd	nd
80	0.01 ± 0.00	nd	nd
90	0.03 ± 0.00	0.01 ± 0.00	nd
100	0.10 ± 0.01	0.04 ± 0.00	0.02 ± 0.00
Σ	**0.15 ± 0.00^{d}**	**0.05 ± 0.00^{d}**	**0.03 ± 0.00^{d}**
P = 120 bar			
10	5.71 ± 0.05	0.87 ± 0.05	0.24 ± 0.01
20	11.47 ± 0.11	4.40 ± 0.04	0.48 ± 0.00
30	10.30 ± 0.44	3.11 ± 0.10	0.56 ± 0.02
Σ	**27.49 ± 0.57^{c}**	**8.37 ± 0.05^{c}**	**1.28 ± 0.01^{c}**
P = 150 bar			
10	16.12 ± 0.44	2.93 ± 0.04	1.10 ± 0.01
20	18.44 ± 0.08	4.80 ± 0.03	1.87 ± 0.03
30	9.31 ± 0.32	2.38 ± 0.05	1.12 ± 0.03
Σ	**43.87 ± 0.16^{a}**	**10.12 ± 0.07^{a}**	**4.10 ± 0.03^{b}**
P = 200 bar			
10	13.23 ± 1.18	3.97 ± 0.12	2.41 ± 0.09
20	12.21 ± 0.35	3.52 ± 0.29	2.34 ± 0.11
30	4.40 ± 0.29	1.86 ± 0.05	2.23 ± 0.08
Σ	**29.84 ± 0.78^{b}**	**9.35 ± 0.41^{b}**	**6.98 ± 0.22^{a}**
Total	**101.35 ± 1.24^{b}**	**27.89 ± 0.35^{c}**	**12.39 ± 0.20^{c}**

^{a,b,c,d} Different letters indicate significant differences among comparable categories ($P < 0.05$); nd = not detected.

Table 4. Mass quantities of uliginosin B, cariphenone A, and cariphenone B extracted by CO_2 supercritical fluid from *H. carinatum* at 60°C per 100 g of plant.

Time (min)	Uliginosin B (mg)	Cariphenone A (mg)	Cariphenone B (mg)
P = 90 bar			
10	0.35 ± 0.02	0.03 ± 0.00	0.06 ± 0.00
20	0.01 ± 0.00	nd	nd
30	nd	nd	nd
40	nd	nd	nd
50	nd	nd	nd
60	nd	nd	nd
70	nd	nd	nd
80	nd	nd	nd
90	nd	nd	nd
100	0.01 ± 0.00	nd	nd
Σ	**0.37 ± 0.02^{c}**	**0.03 ± 0.00^{c}**	**0.06 ± 0.00^{d}**
P = 120 bar			
10	0.14 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
20	0.32 ± 0.01	0.07 ± 0.00	0.08 ± 0.01
30	0.15 ± 0.00	0.06 ± 0.00	0.04 ± 0.00
Σ	**0.61 ± 0.01^{c}**	**0.16 ± 0.00^{c}**	**0.15 ± 0.00^{c}**
P = 150 bar			
10	0.90 ± 0.05	0.13 ± 0.01	0.13 ± 0.01
20	2.04 ± 0.08	0.88 ± 0.02	0.20 ± 0.02
30	17.53 ± 0.35	5.68 ± 0.38	0.81 ± 0.03
Σ	**20.47 ± 0.37^{b}**	**6.69 ± 0.37^{b}**	**1.14 ± 0.01^{b}**
P = 200 bar			
10	16.58 ± 1.24	3.78 ± 0.20	1.45 ± 0.03
20	12.40 ± 0.32	2.59 ± 0.01	1.40 ± 0.04
30	12.63 ± 0.20	3.60 ± 0.01	1.72 ± 0.02
Σ	**41.61 ± 1.36^{a}**	**9.97 ± 0.21^{a}**	**4.57 ± 0.01^{a}**
Total	**63.06 ± 1.22^{c}**	**16.88 ± 0.57^{d}**	**5.96 ± 0.02^{d}**

^{a,b,c,d} Different letters indicate significant differences among comparable categories ($P < 0.05$); nd = not detected.

At 50ºC, the lowest pressure employed in the experiment (90 bar) was not able to extract considerable amounts of the interest compounds due to the supercritical fluid density; thus the proportion of acetone soluble fraction/extract was very small. Consequently, the extracted compounds presented polar characteristics and/or low molecular weight. Increasing pressures result in increasing yields, being 150 bar the ideal pressure to obtain uliginosin B. Cariphenone A and cariphenone B also showed high yields with increasing pressure, being obtained in higher amounts at 150 and 200 bar, respectively. This same profile was observed for the three compounds studied in the experiment performed at 60°C. Therefore, the extraction of such compounds at higher temperatures strongly depends on the CO_2 density.

The extraction of uliginosin B, cariphenone A and cariphenone B in concentrated form would be possible through the use of fractional extraction and/or fractional separation. In this case, the extraction cell and separators are commonly equipped with independent control of temperature and pressure. So, the development of this fractionation requires the improvement of the method since the process of obtaining each compound depends on its differential solubility in the supercritical fluid [13].

Although the solubility of uliginosin B, cariphenone A and cariphenone B in the CO_2 supercritical is not known, it seems to be very similar in view of the chromatographic characteristics of these compounds. Uliginosin B, although presenting several hydroxyl groups, due to the intramolecular hydrogen bonds between the carbonyl and hydroxyl groups, is the less polar among the three compounds. With regard to the benzophenone isomers, cariphenone A is less polar since both hydroxyl groups present in the molecule are in the neighborhood of the carboxyl group favoring the intramolecular bonds. Thus, the approaches of the solubility plus the characteristics of the fluid are coherent with the yields obtained for these compounds.

This work also brings contribution to the medicinal plant research, describing the investigation about benzophenone derivatives extraction from medicinal plant matrix using supercritical fluid. Therefore, aiming at satisfactory results for further isolation and purification of these compounds or enriched fractions for biological investigations, it is clear that the extraction performed at 40ºC and 90 bar must be employed since it proved to be the best experimental condition. Furthermore, the supercritical-fluid method also provides versatility and a notable time economy when compared with *n*-hexane maceration. Besides that, the method permits the use of CO_2 that is potentially an ideal green solvent.

3.3 Mathematical modeling results

The knowledge of thermodynamics parameters as well as the mass transfer rate is an important factor from the industrial point of view to optimization of the supercritical extraction process [34]. Aiming at a future scale-up procedure, the mathematical modeling was performed using extraction mass *versus* time. The mathematical modeling of extraction process can be used for the design of industrial plants with good operational conditions. In this sense, different mathematical models have been proposed to describe the extraction of natural compounds from vegetal matrices by supercritical carbon dioxide [18, 28].

The previous results (Tables 2, 3 and 4) indicate higher yields of the target compounds at 90 bar and 40^{o}C. Therefore another experiment was performed at this condition with the objective of constructing an extraction curve which permitted the mathematical simulation. The experimental data were fitted by the mathematical model previously described. From these data, it was possible to carry out the estimation of the unknown parameters of the model. The results are presented in Fig. 2.

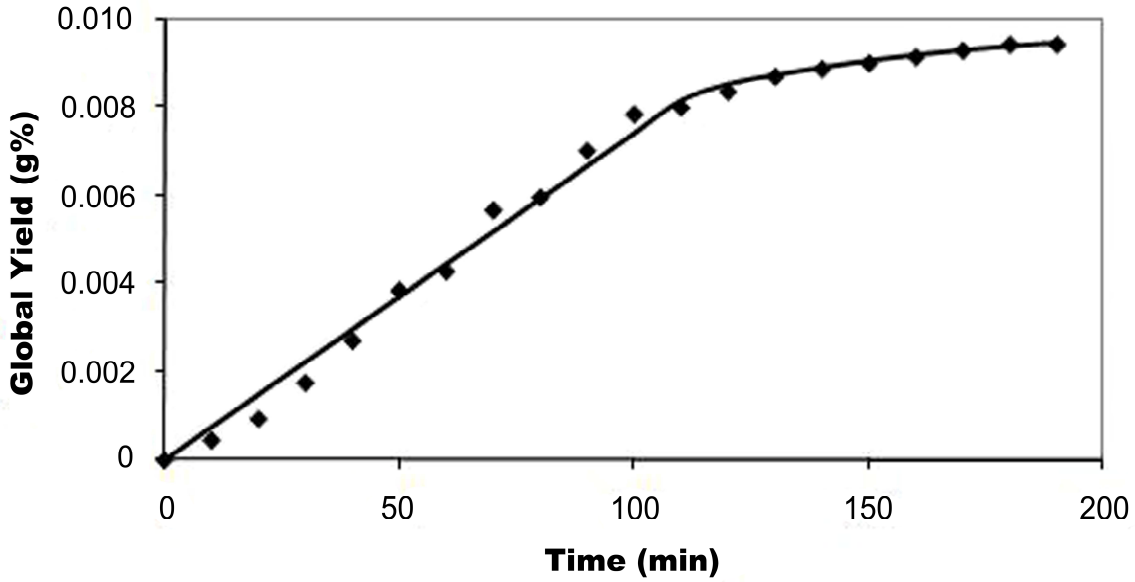

Figure 2. Global yield of the extracts using supercritical CO_2 at 90 bar and 40°C: Experimental data and mathematical fitting (continuous line).

The unknown parameters, K_1 and K_2, were estimated by minimization of the sum of squares of errors between the experimental data and the prediction using the model. The parameter K_1 is related to the extract solubility in the solvent (CO_2) while K_2 is related to the extraction from inner cells in the solid-phase. The estimated parameters for mathematical model were 0.00742 $g.min^{-1}$ and 0.0165 min^{-1} for K_1 and K_2, respectively. The R^2 value was determined as 0.9946. The first step of the curve may be considered a straight line associated to a constant extraction rate while the second step approaches exponentially to a limit value, which was given by the potential supercritical extraction amount of extractable substances. Thus, the results of the mathematical modeling correlated adequately the experimental data at the investigated conditions.

4 Conclusion

This study demonstrated that SFE is a feasible method for selective recovery of phloroglucinol and benzophenone derivatives from *H. carinatum* being 40 °C and 90

bar the best conditions to obtain the target compounds in higher amount and lower time. It was also observed that higher temperatures strongly reduced the yields.

The mathematical model fitted very well the experimental data in which the bioactive compounds were obtained in higher amount. Finally, the model parameters determined could be useful for the scale-up of the *H. carinatum* supercritical extraction process.

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RESULTADOS ADICIONAIS

No manuscrito 2, os procedimentos de extração supercrítica foram obtidos sob temperatura constante e pressão variável em função do tempo. Para determinar indubitavelmente a pressão ótima de extração, um novo experimento foi realizado à temperatura (40ºC) e pressão constantes (90, 150 ou 200 bar) com novo material vegetal, coletado em 2010. Demais condições experimentais seguiram os padrões mencionados. Adicionalmente, os procedimentos de modelagem matemática, realizados previamente em função do extrato, foram aqui obtidos em relação aos compostos investigados (uliginosina B, carifenona A e carifenona B). Os dados são apresentados na sequencia sob forma de um resumo expandido publicado nos anais do *13th European Meeting on Supercritical Fluids*.

RESUMO EXPANDIDO

FRANCISCO M.C. BARROS, FLÁVIA C. SILVA, JÉSSICA M. NUNES, RUBEM M.F. VARGAS, EDUARDO CASSEL, GILSANE L. VON POSER. *Hypericum carinatum*: supercritical fluid extraction, HPLC determination and mathematical modeling. *In*: 13th European Meeting on Supercritical Fluids, The Hague, 2011.

***Hypericum carinatum*: supercritical fluid extraction, HPLC determination and mathematical modeling**

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ABSTRACT

The genus *Hypericum* has been studied worldwide due to its pharmacological and biological effects. Uliginosin B, a dimeric phloroglucinol, and the benzophenones cariphenone A and B, for example, are the major responsible for the anti-proliferative and antimicrobial activities of *Hypericum carinatum*, a native species to southern Brazil. Although presenting therapeutic potential, these compounds are obtained in low yield with conventional extraction methods and can suffer thermal and oxidative decomposition. The use of supercritical carbon dioxide, an environmentally safe solvent, is a promising alternative. Thus, the aim of this study was to determine some extraction conditions in view of future procedures of scale-up. The aerial parts of plant were extracted under constant temperature (40°C) and pressures of 90, 150 and 200 bar. HPLC analysis and mathematical modeling were used to quantify the metabolites and assess the unknown parameters of the extraction, respectively. The better extraction condition to obtain the bioactive compounds was 40ºC and 90 bar. The mathematical model fitted very well the experimental data.

INTRODUCTION

The genus *Hypericum* has been investigated worldwide for its economical, chemical and biological importance. Several species are used in traditional medicine as antiseptic, diuretic, stomachic, wound healing agents and in the treatment of infectious diseases. *Hypericum perforatum* is one of the best-known members especially due to its therapeutic value as an antidepressant drug. The bioactive metabolites commonly described for the genus include naphthodianthrones, flavonoids, phloroglucinol derivatives and xanthones [1-3].

In the southern Brazil, there are about 20 native species. Pharmacological studies have shown that some of them present antidepressant, antinociceptive, MAOI, antiviral, antibacterial, antifungal and anti-proliferative activities. Uliginosin B, a dimeric phloroglucinol, and the benzophenones cariphenone A and B have been pointed out as the major responsible for the anti-proliferative and antibacterial activities of *Hypericum carinatum*, for example [1, 4, 5]. Although presenting therapeutic potential, these compounds are obtained in low yield with conventional extraction methods and can suffer thermal and oxidative decomposition [6, 7].

The use of supercritical fluids is a promising alternative. The supercritical fluids have properties such as high diffusivity, low viscosity and low surface tension conferring attractive characteristics as a solvent for extraction of components from a solid matrix [8]. Carbon dioxide (CO_2) is the most used solvent since it is non-toxic, non-explosive, readily available and of low cost. Furthermore, at supercritical conditions it presents advantages such as selectivity for lipophilic compounds and non degradation of thermolabile substances [9-12], features very appropriate for the extraction of bioactive compounds from *Hypericum* species. Thus, the aim of this study was to assess the effect of extraction conditions (temperature, pressure and time) regarding the recovery of extracts and concentration of uliginosin B, cariphenone A and

cariphenone B. In view of future procedures of scale-up, applications of mathematical modelling were also carried out.

MATERIALS AND METHODS

Plant material

The aerial parts of *Hypericum carinatum* Griseb. were collected during its flowering stage in Glorinha, Rio Grande do Sul, Brazil, in December, 2010. The plant material was identified by S. Bordignon (Departamento de Botânica, Universidade Luterana do Brasil). A voucher specimen was deposited in the Herbarium of the Universidade Federal do Rio Grande do Sul (ICN - Bordignon 1520). Plant material was dried at room temperature and powdered (1 mm mesh opening) in a cutting mill.

Extraction methods

Supercritical extractions were carried out on a pilot-scale automated equipment according to procedures previously described [13]. Powdered plant material (100 g DW) was extracted at temperature of the 40ºC and pressures of 90, 150 and 200 bar. The extractions were performed following the experimental procedure defined by Cargnin et al. [14]. The samples were collected every 10 min in order to evaluate yield and composition of the extracts *versus* extraction time. The supercritical carbon dioxide flow rate was 6.7×10^{-4} $kg.s^{-1}$ (through the extraction vessel) using a flowmeter assay (Sitraus F C Massflo 2100 - Siemens) with accuracy of < 0.1%. Pressure in the extractor was monitored with a digital transducer system, Novus 8800021600, acquired from Novus Produtos Eletrônicos (Brazil) with precision of

±1.0 bar. The temperature controller was connected to thermocouples (PT-100) with accuracy of < 0.5.

HPLC analysis

The supercritical CO_2 extracts were treated with acetone and filtered off to remove waxes and insoluble impurities. Further, the enriched fractions were evaporated to dryness, dissolved in HPLC grade methanol, filtered (0.22 µm pore size, Merck) and analyzed by high-performance liquid chromatography.

Benzophenones determination

Cariphenone A analysis was performed using a Waters 600 pump and a Waters 2487 dual λ absorbance detector set to 270 nm. The separation was carried out with an isocratic solvent system (60% CH_3CN, 40% H_2O) through a Waters Nova-Pack C18 column (4 µm, 3.9 x 150 mm) adapted to a Waters Nova-Pack C18 60 A guard column (3.9 x 20 mm) and flow rate of 1 $mL.min^{-1}$. Metabolite determination was assessed by a calibration curve of pure standard isolated from *H. carinatum* and concentrations ranging from 5 to 1400 $\mu g.mL^{-1}$ ($R^2 > 0.999$). The compound identification was made on the basis of the ultraviolet absorption spectra and retention time in comparison with the standard compound. Cariphenone B quantification was performed under the same conditions described above and the content was expressed as milligram of cariphenone A equivalents/100 g plant.

Uliginosin B determination

Uliginosin B yields were determined using the same equipment under isocratic solvent condition (95% CH_3CN, 5% H_2O, 0.01% TFA), flow rate of 1 $mL.min^{-1}$ and detection at 220 nm. Metabolite quantification was assessed by a calibration curve of pure

standard and the concentrations ranged from 2 to 800 μg.mL^{-1} (R^2 > 0.999). The identification was based on the ultraviolet absorption spectra and retention time in comparison with the standard compound.

Mathematical modeling

A model for supercritical-fluid extraction based on the concept of broken and intact cells was used to fit experimental data and simulates two extraction periods using the differential mass balances of the solute per unit volume extraction bed. The first period is guided by phase-equilibrium and the second by internal diffusion into the particles. The mathematical model was formulated by Xavier et al. [15] according to the discussion proposed by Sovová [16]. In this model, the mass of extracted compounds (solute) is assumed to be a *pseudo* component in terms of the mass balance. The solute mass balance in fluid and solid-phase was expressed by two partial differential equations that were analytically solved by Xavier et al. [15]. The extraction curve, written in terms of maximum extract yields, is expressed in two steps; the first one controlled by phase equilibrium and the second, by internal diffusion from particles, having the following expression

$$\frac{M(t)}{M(\infty)} = \frac{K_1\, t}{M(\infty)}. \qquad (1)$$

for the first period, where

$$K_1 = \dot{m}\, Y^*, \qquad (2)$$

being $\dot{m}$ the solvent flow rate and Y^* is weight fraction for the equilibrium fluid-phase; and

$$\frac{M(t)}{M(\infty)} = (1 - e^{-K_2 t}) . \quad (3)$$

for the second period, where K_2 is defined as follow

$$K_2 = \frac{k_s a_0}{(1-\varepsilon)} . \quad (4)$$

where k_s is the solid-phase mass transfer, and a_0 is the specific surface area per unit volume of extracted bed.

Summarizing, the extraction curve is linear in function of the time for the first step of the extraction and exponential for the second period [15].

RESULTS AND DISCUSSION

The results obtained are summarized in the tables 1, 2, 3 and 4. Uliginosin B, cariphenone A and cariphenone B extraction curves obtained by supercritical CO_2 extraction of *H. carinatum* under temperature (40ºC) and pressure (90, 150 and 200 bar) constants are shown in figures 1, 2 and 3.

Table 1: Total supercritical *H. carinatum* extract yields and respective fractions obtained after treatment with acetone.

Pressure (bar)	Yield 40ºC	
	% Extract[a]	% Fraction[b]
90	0.95	0.91
150	0.97	0.92
200	1.89	0.97

[a]g of total extract /100 g of plant
[b]g of fraction /100 g of plant

In general form, the modeling of the supercritical extraction for the conditions here studied as well as for the total time for the experiments allows to evidence that for 90 bar, the extraction of metabolites of interest is practically controlled for the first stage of the extraction process, in which the higher amounts of these metabolites had been observed. With the increase of the pressure, the second stage of the process becomes present, where the diffusion from internal cells of the vegetal matrix is more predominant in the mass transfer process. The mathematical modeling represented well the experimental data as it can be seen in Figures 1, 2, and 3. The unknown parameters present in the mathematical model were estimated by minimization of the sum of squares of errors between the experimental data and the prediction using the model. The numerical results for these parameters were presented in Table 5.

Especially in pharmaceutical, food and cosmetics industries, the use of supercritical fluids represents an alternative to the conventional methods of extraction since extracts of high quality are achieved. Nevertheless, satisfactory results are dependent on the optimization of process parameters such as pressure, temperature, fluid extraction, modifiers, flow rate, extraction time, among others [7]. According to the results of Table 1, the increased pressure produced higher yields of extract. In addition, in the pressures of 90 and 150 bar the percentage of acetone-soluble fraction represented

about 95% of the total extract. At 200 bar of pressure, however, this value was 51%, indicating an elevated co-extraction of undesirable compounds such as epicuticular waxes. As expected, at constant temperature, the elevation of pressure results higher fluid density and consequently superior power of solvation [8].

Table 2. Mass quantities of uliginosin B, cariphenone A, and cariphenone B in fraction of supercritical extract of *H. carinatum* at 40°C and 90 bar per 100 g of plant.

Time (min)	Uliginosin B (mg)	Cariphenone A (mg)	Cariphenone B (mg)
10	1.30 ± 0.07	15.08 ± 0.51	0.78 ± 0.06
20	1.41 ± 0.10	17.30 ± 0.54	0.84 ± 0.03
30	3.01 ± 0.11	30.59 ± 0.54	1.45 ± 0.02
40	2.48 ± 0.21	37.57 ± 0.90	1.77 ± 0.03
50	6.48 ± 0.48	44.70 ± 2.36	2.10 ± 0.00
60	1.19 ± 0.11	18.78 ± 0.67	0.91 ± 0.03
70	1.75 ± 0.06	28.10 ± 1.32	1.65 ± 0.07
80	1.28 ± 0.05	13.68 ± 1.00	0.71 ± 0.05
90	2.76 ± 0.26	39.21 ± 0.18	1.97 ± 0.02
100	1.96 ± 0.15	32.63 ± 0.16	1.74 ± 0.01
110	0.81 ± 0.00	5.98 ± 0.37	0.39 ± 0.01
120	1.92 ± 0.00	16.11 ± 0.50	0.99 ± 0.03
130	2.48 ± 0.15	28.13 ± 1.03	1.99 ± 0.16
140	0.65 ± 0.04	10.24 ± 0.56	0.76 ± 0.04
150	0.30 ± 0.02	6.61 ± 0.05	0.59 ± 0.01
160	5.10 ± 0.01	12.28 ± 0.01	1.06 ± 0.00
170	5.62 ± 0.06	11.99 ± 0.03	0.99 ± 0.00
180	2.40 ± 0.03	7.32 ± 0.02	0.70 ± 0.00
Total	**42.90 ± 0.66**	**376.33 ± 4.14**	**21.39 ± 0.28**

Table 3. Mass quantities of uliginosin B, cariphenone A, and cariphenone B in fraction of supercritical extract of *H. carinatum* at 40°C and 150 bar per 100 g of plant.

Time (min)	Uliginosin B (mg)	Cariphenone A (mg)	Cariphenone B (mg)
10	1.85 ± 0.15	34.28 ± 0.66	3.11 ± 0.09
20	2.96 ± 0.17	61.81 ± 1.27	6.60 ± 0.22
30	3.13± 0.10	34.99± 1.31	4.98 ± 0.21
40	2.30 ± 0.12	25.27 ± 0.90	3.57 ± 0.22
50	1.16 ± 0.02	11.22 ± 0.43	1.76 ± 0.04
60	1.00 ± 0.05	6.38 ± 0.12	1.19 ± 0.06
70	1.10 ± 0.02	8.10 ± 0.29	1.53 ± 0.05
80	1.47 ± 0.05	14.06 ± 0.81	2.33 ± 0.07
90	0.09 ± 0.00	0.16 ± 0.00	0.08 ± 0.00
100	0.43 ± 0.00	1.12 ± 0.01	0.49 ± 0.00
Total	**15.48 ± 0.06**	**197.40 ± 0.07**	**25.64 ± 0.39**

HPLC analyses were performed aiming to evaluate the recovery of uliginosin B, cariphenone A and B in the fractions of supercritical extract. As it can be observed in the table 2, 3 and 4, the composition of extract varied according to the conditions employed. According to Pourmortazavi and Hajimirsadegh [17], four parameters are extremely helpful in the understanding the solute behavior in supercritical media: (i) the miscibility or pressure in which the solute migrates into the supercritical fluid; (ii) the pressure in which the solute reaches its maximum solubility; (iii) the pressure range of selective fractionation and (iv) a knowledge of the physical properties of the solute. Regarding the conditions employed in this study, the temperature of 40ºC and 90 bar of pressure produced the higher quantity of the three compounds investigated. Moreover, at 40ºC and 150 bar high concentrations of cariphenone B were obtained. It is also important to note that, in comparison to the conventional methods, the time of extraction is reduced to a few hours. Thus, higher selectivity and shorter time are factors that can compensate the costs of investment in the processes of supercritical extraction.

Table 4. Mass quantities of uliginosin B, cariphenone A, and cariphenone B in fraction of supercritical extract of *H. carinatum* at 40°C and 200 bar per 100 g of plant.

Time (min)	Uliginosin B (mg)	Cariphenone A (mg)	Cariphenone B (mg)
10	0.23 ± 0.01	15.65 ± 0.25	1.15 ± 0.05
20	1.45 ± 0.04	69.95 ± 2.20	5.17 ± 0.12
30	2.43± 0.10	85.77 ± 2.07	9.13 ± 0.27
40	1.37 ± 0.02	7.38 ± 0.21	1.98 ± 0.05
50	3.94 ± 0.15	5.19 ± 0.17	3.18± 0.03
60	1.93 ± 0.17	1.10 ± 0.13	1.10 ± 0.03
70	1.92 ± 0.18	0.78 ± 0.03	0.66 ± 0.04
80	0.09 ± 0.00	0.09 ± 0.01	0.05 ± 0.00
90	0.05 ± 0.00	n.d	n.d
100	0.10 ± 0.00	n.d	0.01 ± 0.00
110	0.86 ± 0.04	0.19 ± 0.01	0.23 ± 0.02
120	0.16 ± 0.00	0.09 ± 0.00	0.04 ± 0.00
130	n.d	n.d	n.d
140	n.d	n.d	n.d
Total	**14.53 ± 0.51**	**186.21 ± 1.99**	**22.71 ± 0.32**

Table 5. Numerical values for mathematical parameters

Pressure	Metabolites	K_1	K_2
90 bar	Cariphenone A	2.6588	0.0078
	Cariphenone B	0.1262	-
	Uliginosin B	0.2421	-
150 bar	Cariphenone A	4.4264	0.0418
	Cariphenone B	0.4655	0.0369
	Uliginosin B	0.2542	0.0239
200 bar	Cariphenone A	8.5668	0.1063
	Cariphenone B	0.7444	0.0534
	Uliginosin B	0.2134	0.0230

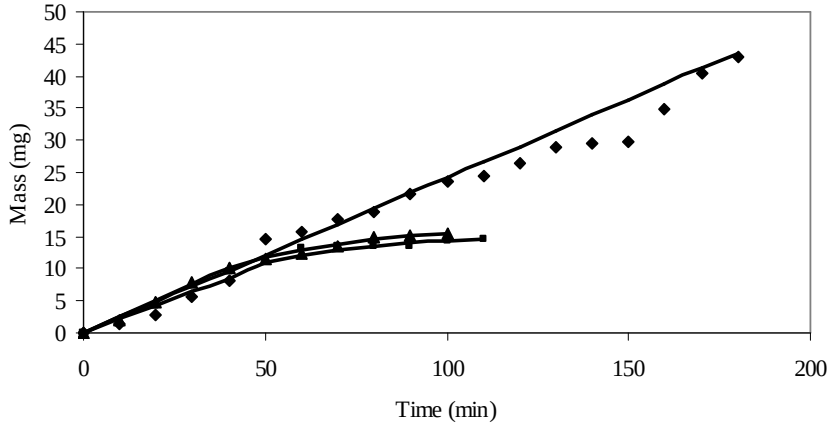

Figure 1. Mass of uliginosin B obtained by supercritical fluid extraction at 40°C and different pressures. The continuous line is for the mathematical fitting and the symbols were used for the experimental data (♦ - 90 bar, ▲- 150 bar, and ■ - 200 bar).

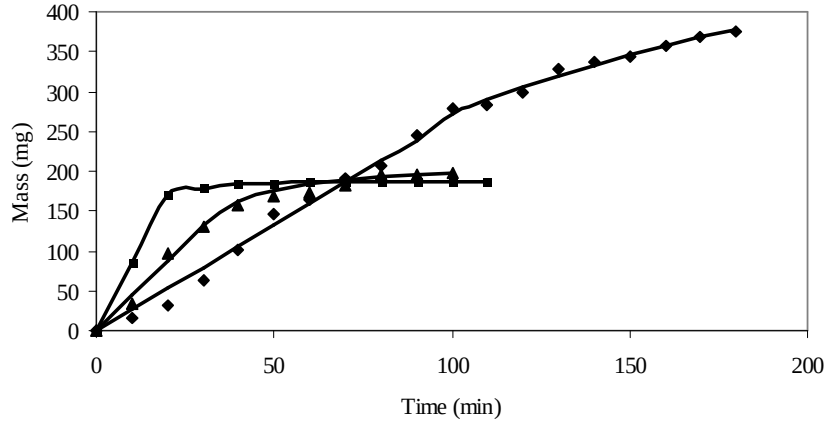

Figure 2. Mass of cariphenone A obtained by supercritical fluid extraction at 40°C and different pressures. The continuous line is for the mathematical fitting and the symbols were used for the experimental data (♦ - 90 bar, ▲- 150 bar, and ■ - 200 bar).

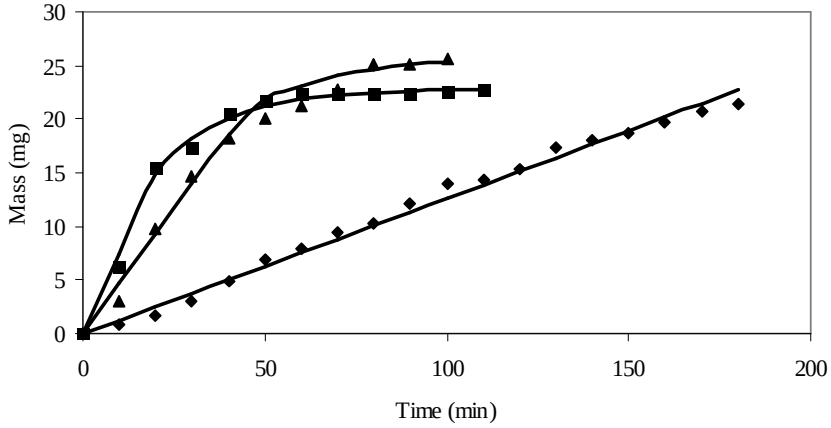

Figure 3. Mass of cariphenone B obtained by supercritical fluid extraction at 40°C and different pressures. The continuous line is for the mathematical fitting and the symbols were used for the experimental data (♦ - 90 bar, ▲- 150 bar, and ■ - 200 bar).

CONCLUSION

The composition of supercritical fluid extract of *H. carinatum* varied in according to temperature and pressure, being 40ºC and 90 bar the better condition for extraction of the bioactive compounds: uliginosin B, cariphenone A and B. Additionally, the condition of 40ºC and 150 bar also represents a great alternative for selective recuperation of cariphenone B. Besides, the time economy is notable especially when compared with the conventional methods of extraction. Depending of the employed condition, it is necessary only 90 to 180 minutes.

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CAPÍTULO III

Atividade antifúngica e antiquimiotática *in vitro* de extratos lipofílicos de *Hypericum* spp.

INTRODUÇÃO

Inegavelmente, os fungos desempenham um importante papel no funcionamento dos ecossistemas. Como decompositores da matéria orgânica, constituem um dos principais responsáveis pela inter-relação dos ciclos de vida na natureza, ao passo que, associados a outros organismos, podem atuar visando à captação de água, sais minerais e nutrientes (HAWKSWORTH, 1991; BLACKWELL, 2011; PAUTASSO, 2012). Muitos destes micro-organismos, em especial *Saccharomyces cerevisiae*, também são empregados na produção de alimentos e bebidas fermentadas (ex.: pão, queijo, iogurte cerveja, vinho), além da fabricação de antibióticos e biofertilizantes (FREY-KLETT, 2011; FAY, 2012).

Segundo algumas estimativas, o número de fungos na natureza pode variar entre 1,5 e 5,1 milhões (BLACKWELL, 2011; HAWKSWORTH, 1991, 2001). Felizmente, poucas espécies são consideradas potencialmente patogênicas para o ser humano, visto que podem causar graves enfermidades e elevadas taxas de mortalidade (HAYNES, 2001; van BURIK e MAGEE, 2001; GARCIA-VIDAL e CARRATALÀ, 2012).

De maneira geral, os fungos patogênicos podem ser divididos em primários e oportunistas. Os patogênicos primários usualmente apresentam reservatório ambiental e infectam indivíduos saudáveis; os oportunistas comumente ocorrem como comensais e aproveitam a debilidade do hospedeiro para causar infecção (HAYNES, 2001; van BURIK e MAGEE, 2001). Em ambos os casos, os mecanismos que levam à doença são complexos (COTTIER e PAVELKA, 2012) e envolvem desde a ausência ou diminuição da imunidade do hospedeiro até uma série de fatores de virulência relacionados ao agente invasor (HAYNES, 2001; van BURIK e MAGEE, 2001).

Na atualidade, as infecções fúngicas mais frequentes são aquelas causadas por espécies de *Candida*, *Cryptococcus*, *Pneumocystis* e *Aspergillus* (GARCIA-VIDAL e CARRATALÀ, 2012). A distribuição desses agentes pode variar em função da geografia, da condição dos pacientes e das unidades de hospitalização (PEMÁN e SALAVERT, 2012). Apesar disso, nas infecções sistêmicas, alguns fatores de risco podem ser identificados. Destacam-se, nesse sentido, a hospitalização por longos

períodos, terapia com antibióticos de amplo espectro, quimioterapia, comorbidades (ex.: neoplasias, HIV, diabetes, cirrose), idade avançada ou prematura, procedimentos médicos invasivos (ex.: transplante de órgãos), entre outros (COLOMBO *et al*., 2006; GARCIA-VIDAL e CARRATALÀ, 2012; PEMÁN e SALAVERT, 2012).

Considerando o tratamento, o diagnóstico precoce e a seleção correta do antifúngico são os fatores de maior impacto no combate ao agente infeccioso. Equinocandinas, anfotericina B e azólicos, por sua vez, constituem os fármacos de primeira escolha nas infecções sistêmicas. O desenvolvimento de novas opções e estratégias terapêuticas, entretanto, continua necessário, tendo em vista os problemas crescentes de resistência aos antifúngicos disponíveis, entre outros fatores (PFALLER, 2012; VANDEPUTTE *et al*., 2012; ZARAGOZA e PÉMAN, 2012).

O uso de terapias combinadas tem sido uma das alternativas empregadas no tratamento de invasões fúngicas graves (RIVAS e CARDONA-CASTRO, 2009; ZARAGOZA e PÉMAN, 2012). Na prática, o emprego da politerapia também visa o tratamento das doenças de base ou sintomas correlacionados. Nesse sentido, alguns estudos têm demonstrado que a modulação ou o controle da resposta inflamatória pode ser um aliado no combate às infecções fúngicas. Este é o caso, por exemplo, de pacientes que apresentam infecção sistêmica recorrente e doença inflamatória crônica como a síndrome inflamatória da reconstituição imune (HOTCHKISS e KARL, 2003; CHAUSSADE *et al*., 2012; COTTIER e PAVELKA, 2012; PERFECT, 2012).

Produtos naturais podem oferecer vantagens na busca de antifúngicos com mecanismos de ação inovadores, tendo em vista sua ampla diversidade química e biológica (LAM, 2007). As espécies de *Hypericum* nativas do sul do Brasil, por sua vez, apresentam potencial como fonte de compostos antimicrobianos (DALL'AGNOL *et al*., 2003; FENNER *et al*., 2005) e anti-inflamatórios (PERAZZO *et al*., 2008). Assim, este capítulo teve como objetivo a investigação da atividade antifúngica e antiquimiotática do extrato lipofílico de *H. carinatum*, *H. caprifoliatum*, *H. linoides*, *H. myrianthum* e *H. polyanthemum*. Os resultados são apresentados e discutidos na forma de um manuscrito científico.

MANUSCRITO 3

FRANCISCO M. C. BARROS, BRUNA PIPPI, BETINA DAUBER, SUELEN C. LUCIANO, ROGER R. DRESCH, MIRIAM APEL, ALEXANDRE FUENTEFRIA, GILSANE L. VON POSER. Antifungal and antichemotactic activities and lipophilic phenolic determination in extracts of *Hypericum* spp. native to South Brazil. **Industrial Crops and Products**, v. 44, p. 294‑299, 2013.

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Antifungal and antichemotactic activities and quantification of phenolic compounds in lipophilic extracts of *Hypericum* spp. native to South Brazil

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Abstract

Plants of the *Hypericum* genus are a notable source of new therapeutic agents including antimicrobial and anti-inflammatory. This study reports the antifungal and antichemotactic activities of the lipophilic extracts of five *Hypericum* species (*H. caprifoliatum* Cham. & Schlecht., *H. carinatum* Griseb., *H. linoides* A. St.-Hil., *H. myrianthum* Cham. & Schlecht. and *H. polyanthemum* Klotzsch ex Reichardt) native to South Brazil. Tests were performed using the broth microdilution assay against 10 species of pathogenic yeasts and neutrophils migration inhibition method, respectively. All samples exhibited a broad spectrum of antifungal action as well as reduced neutrophils migration. *Hypericum carinatum, H. linoides* and *H. myrianthum* Klotzsch ex Reichardt extracts presented the lowest value of minimum inhibitory concentration against *Cryptococcus neoformans* (MIC ≤ 15.6 µg/mL), *Rhodotorula mucilaginosa* (MIC ≤ 62.5 µg/mL), *Candida glabrata* and *C. tropicalis* (MIC range = 1.9 - 250 µg/mL). The antichemotactic effect varied from 60-100% at concentrations of 0.31 to 10.0 µg/mL. The results of analyses by HPLC demonstrated a strong correlation between the phenolic compounds and the antifungal / anti-inflammatory activities. The extracts that presented high amounts of the dimeric phloroglucinol uliginosin B, japonicin A and hyperbrasilol B were the most active. Thus, *Hypericum* extracts show potential as source of new anti-infectives and anti-inflammatory drugs.

Keywords: *Hypericum*, antifungal, antichemotactic, dimeric phloroglucinols, benzophenones, benzopyrans.

1. Introduction

Fungal infections continue to produce high rates of morbidity and mortality especially in severely ill or immunocompromised patients. Species of *Candida* are the most important cause of opportunistic mycoses worldwide. *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* account for 95-97% of all invasive fungal infections caused by yeast of this genus (Pemán and Salavert, 2012). In Brazil, a study of the Brazilian Network Candidemia reported an overall incidence of 2.49 cases per 1000 hospital admissions and mortality rates of 54% (Colombo et al., 2006). Although uncommon, emerging yeasts as *Cryptococcus* spp., *Geotrichum* spp. and *Rhodotorula* spp. are also significant due to their virulence and resistance to the majority of drugs available for treatment (Pemán and Salavert, 2012). Amphotericin B and fluconazole are the most commonly used antifungal agents in clinical practice. Adverse effects, drug interactions, toxicity and increasing resistances, however, are factors that limit the use of these drugs (Colombo et al., 2006; Rajeshkumar and Sundararaman, 2012; Vandeputte et al., 2012; Zaragoza and Pemán, 2012). Considering this scenario, efforts to develop strategies and more effective therapeutic options are needed.

Activation of the immune system is an important step in the host defense against microbial infections. In the early stages of infection, pro-inflammatory mediators stimulate the recruitment of defense cells such as neutrophils and monocytes to combat the infectious agent (Garcia-Vidal and Carratalà, 2012; Kumar and Sharma, 2010). Contradictorily, however, exacerbated inflammatory responses may cause tissue damage and even increase the susceptibility to opportunist pathogens. Some authors have postulated that low-level inflammation promotes fungal colonization and fungal colonization promotes further inflammation in a vicious cycle type (Kumanoto, 2011). Furthermore, it has also been shown that antifungals as amphotericin B can induce signal transduction and inflammatory cytokine release (Sau et al., 2003). Thus, the uncontrolled of the inflammatory process can compromise both the treatment of

infection as the management of other related diseases (Sau et al., 2003; Zelante et al., 2007).

Plants of the *Hypericum* genus (Clusiaceae) are a notable source of potential therapeutic agents. Different extracts and isolated compounds have shown antimicrobial (Gibbons et al., 2002) and anti-inflammatory activities (Ozturk et al., 2002). Besides, several species are used in traditional medicine for the treatment of these disorders (Bussmann et al., 2010; Tunón et al., 1995; van Vuuren and Naido, 2010). Phenolic compounds as phloroglucinols (hyperforin), naphtodiantrones (hypericin) and flavonoids (hyperoside) are the bioactive metabolites commonly described for these plants (Saddiqe et al., 2010).

Dimeric phloroglucinols (uliginosin B, japonicin A, hyperbrasilol B) are considered chemotaxonomic markers of the native *Hypericum* species to Southern Brazil (Ferraz et al, 2002; Nör et al., 2004). Additionally, other metabolites with phloroglucinol pattern (benzophenones and benzopyrans) are also described (von Poser et al., 2006). Some authors suggested that the dimeric derivatives could be responsible for antifungal (Dall'Agnol et al., 2003; Fenner et al., 2005) and anti-inflammatory activities (Perazzo et al., 2008) demonstrated by extracts of some *Hypericum* species. Such studies furnished evidences that could justify the popular use of these plants (Mentz et al., 1997).

The aim of this study was to evaluate the antifungal and antichemotactic activities of lipophilic extracts, rich in compounds with the phloroglucinol substitution pattern, of five *Hypericum* species native to Southern Brazil. In order to correlate the pharmacological results with the main metabolites present in the extracts (Fig. 1), analyses by high performance liquid chromatography (HPLC) were performed.

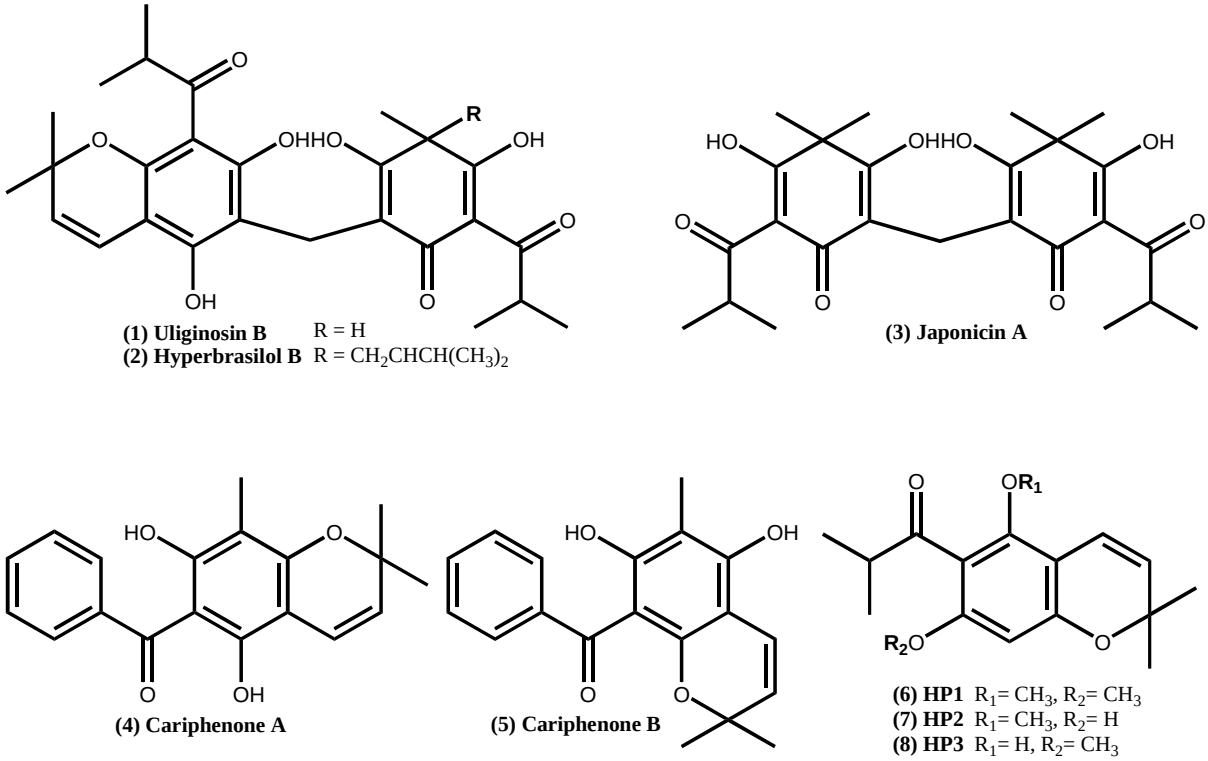

Fig. 1. Dimeric phloroglucinols **(1-3)**, benzophenones **(4 and 5)** and benzopyrans **(6-8)** investigated in *Hypericum* spp. lipophilic extracts.

2. Material and methods

2.1. Plant material

Aerial parts of the plants were collected in the Rio Grande do Sul state, south Brazil, between October and December, 2009 (Table 1). Plants were identified by Dr. Sérgio Bordignon (UNILASALLE, RS, Brazil). Voucher specimens are deposited in the herbarium of Universidade Federal do Rio Grande do Sul (ICN). Plants collection was authorized by IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis) (n◦ 003/2008; Protocol 02000.001717/2008 – 60).

Table 1

Collection localities of the *Hypericum* species.

Species	**Collection locality**	**Voucher number**
H. caprifoliatum Cham. & Schlecht.	Porto Alegre, RS, Brazil	Bordignon et al. 2287
H. carinatum Griseb.	Glorinha, RS, Brazil	Bordignon & Ferraz 2309
H. linoides A. St.-Hil.	São José dos Ausentes, RS, Brazil	Bordignon et al. 3317
H. myrianthum Cham. & Schlecht.	Paraíso do Sul, RS, Brazil	Bordignon et al. 3059
H. polyanthemum Klotzsch ex Reichardt	Caçapava do Sul, RS, Brazil	Bordignon et al., 3118

2.2. Extracts preparation

The dried and powdered plant materials were thoroughly extracted with *n*-hexane at room temperature. The fractions were pooled, evaporated to dryness under reduced pressure and then stored at -20ºC until biological evaluation and HPLC analyses.

2.3. Antifungal activity

A total of 36 strains (clinical isolates and reference strains) of opportunistic yeasts were tested for the antifungal susceptibility: *Candida albicans* (ATCC 28367, ATCC 10231, ATCC 24433, CA 02), *C. dubliniensis* (CD 24A , CD 24P, CD 85P, CO 24P), *C. glabrata* (CG 01, CG 4036, CG 40039, CG 40136), *C. guilliermondii* (CG 40039), *C. krusei* (CK 02, CK 03, CK 04, CK 6258), *C. parapsilosis* (CPA 01, CPA 04, CPA 08, CPA 134), *C. tropicalis* (CT 08, CT 56, CT 750, CT 40098), *Cryptococcus neoformans* (CRY 02, CRY 14, CRY 17), *Geotrichum candidum* (GC 09, GC 18, GC 26, GEO 03) and *Rhodotorula mucilaginosa* (ROH 01, ROH 02, ROH 03, ROH 05).

Screening for antifungal activity was carried out using the broth microdilution method. In order to obtain stock solutions (1000 µg/mL), the *Hypericum* extracts were

solubilized with 0.9% NaCl solution and dimethyl sulphoxide (DMSO). In 96-well microtiter plates, the stock solutions were diluted with Sabouraud dextrose broth (SDB) at 500 µg/mL to the screening test.

Minimal inhibitory concentration (MIC) of the extracts was determined by the broth microdilution method according to M27-A3 documents determined by the Clinical Laboratory and Standards Institute (CLSI, 2008), with RPMI-MOPS (RPMI 1640 medium containing L-glutamine, without sodium bicarbonate - Sigma-Aldrich Co., St Louis, USA - buffered to pH 7.0 with 0.165 mol/L MOPS buffer – Sigma). The concentrations of the extracts ranged from 1.9 to 500 µg/mL and 100 µL-aliquots were inoculated of a flat-bottom 96-well microtiter. The MIC was defined as the lowest concentration of compounds at which the microorganism tested did not demonstrate visible growth. Amphotericin B kindly supplied by Cristália® (Brazil) was used as positive control. The experiments were carried out in triplicate.

The minimal fungicidal concentration (MFC) was determined by sub-culturing volumes of 10 µL from wells without visible grown in Sabouraud dextrose agar (SDA) with Cloranfenicol (Difco, Detroit, USA) and incubated at 35 °C for 48 h. Minimum fungicidal concentration (MFC) was defined as the lowest concentration yielding negative subcultures.

2.4. Antichemotactic assay

Stock solutions (1000 µg/mL) of the *Hypericum* extracts were previously prepared in Hanks buffer and Tween 80 (maximum concentration = 10%). The stock solutions were diluted in rat leukocytes solution to obtain the concentrations of 0.31 to 10.0 µg/mL used in the assay. Chemotaxis evaluation was performed according to the

method described by Suyenaga et al. (2011). The leukocyte/samples was added to the upper wells of a modified Boyden chamber, separated from the chemotactic stimulant (LPS from *Escherichia coli*) present in the lower compartment by an 8.0 μm nitrocellulose filter (Millipore, USA). The leucocytes migration was measured using the microscope. The mean value of migration correspond the micrometer distance from the top of the filter to the farthest plane of focus still containing two cells in five microscopic fields. All experiment was carried out in duplicate.

2.5. HPLC analysis

Acetonitrile (CH_3CN) and methanol (MeOH) were HPLC grade from Merck (Darmstadt, Germany); trifluroacetic acid (TFA) from Vetec (Duque de Caxias, RJ, Brazil) and *n*-hexane (HEX) from F. Maia (Cotia, SP, Brazil) were reagent grade. Distilled water (H_2O) was purified by a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

Uliginosin B (**1**), hyperbrasilol B (**2**), japonicin A (**3**), cariphenone A (**4**), cariphenone B (**5**), 6-isobutyryl-5,7-dimethoxy-2,2-dimethyl-benzopyran (HP1) (**6**), 7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethyl-benzopyran (HP2) (**7**) and 5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl-benzopyran (HP3) (**8**) (Fig. 1) were isolated from *Hypericum* species as described elsewhere (Bernardi, 2007; Dall'Agnol et al., 2005; Ferraz et al., 2002; Nör et al., 2004). The identity and purity of compounds were confirmed by ^{1}H NMR spectroscopy.

The extracts were dissolved in MeOH (HPLC grade) and filtered (0.22 mm pore size, Merck) for further analysis. The HPLC system consisted of a Shimadzu liquid chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a DGU-20A_5 degasser, LC-6AD pumps, SIL-10AD auto sampler, CTO-20AC column oven, SPD-

20AV UV/VIS detector and CBM-20A communications module. The chromatographic data were recorded and processed by LC Solution software version 1.24 SP2. For separations a Waters Nova Pack C18 column (4 µm, 3.9 x 150 mm) and a Waters Nova-Pack C18 60Å guard column (3.9 x 20 mm) were used.

Dimeric phloroglucinols were eluted using an isocratic elution program with 95% CH_3CN, 5% H_2O, 0.01% TFA at 220 nm; benzopyrans and benzophenones, with 60% CH_3CN, 40% H_2O at 270 nm. The analyses were performed at room temperature (25ºC) and at a flow rate of 1 mL/min. A volume of 20 µL and three injections were performed for each samples.

Peaks were identified by comparison of their retention times (t_R) with those of authentic reference standards. Co-injections were carried out for further confirmation of their identification. The t_R values were 5.72, 7.29 and 8.85 min for dimeric phloroglucinols **3**, **1** and **2**; 6.17 and 8.08 for the benzophenones **5** and **4**; and 8.20, 11.52 and 19.41 min for benzopyrans **6**-**8**.

Calibration curves were prepared in MeOH with at least five data points covering the concentration range of 8.13 – 411.34 µg/mL for chemical **1** (r^2 = 0.9976), 31.05 – 521.08 µg/mL for **4** (r^2 = 0.9999), 16.89 – 400 µg/mL for **6** (r^2 = 0.9999), 25 – 400 µg/mL for **7** (r^2 = 0.9981) and 21.11 – 500 µg/mL for **8** (r^2 = 0.9995). Each curve was obtained by plotting the peak area of the compound at each level versus the concentration of the sample. The dimeric phloroglucinols and benzophenones were quantified with uliginosin B and cariphenone A calibration curves, respectively. The yields values were expressed in % (weight compound per weight dry extract).

2.6. Statistical analysis

Control and the treatments were analyzed by ANOVA followed by Tukey's test. Data are expressed as mean ± SEM. Differences were considered statistically significant when $P < 0.05$. Data analyses were performed using the GraphPad Prism 5.0 software.

3. Results

The lipophilic extracts of 5 *Hypericum* species native to South Brazil were investigated against 10 species of emerging yeasts pathogens (Table 2). The extract of *H. carinatum* inhibited the growth of all yeasts tested, being this effect fungicidal in 70% of cases. High percentages of inhibition (60-80%) were also observed to the extracts of *H. myrianthum*, *H. linoides* and *H. caprifoliatum*. Resistances were viewed only against the *Candida* spp. The extract of *H. polyanthemum* proved to be ineffective against all the seven *Candida* species tested, followed by *H. caprifoliatum* (4/7), *H. linoides* (3/7) and *H. myrianthum* (2/7).

The most active extracts were selected for the determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) against different strains of the susceptible yeasts. According to Table 3, *C. neoformans* and *R. mucilaginosa* were the most susceptible yeasts. Regarding these pathogens, the extracts of *H. carinatum*, *H. linoides* and *H. myrianthum* presented MIC values ≤ 15.6 µg/mL and ≤ 62.5 µg/mL, respectively. In addition, *H. myrianthum* extract also displayed the lowest value of MFC (7.8-15.6 µg/mL) against *R. mucilaginosa*. Within the *Candida* genus, the most sensitive species were *C. glabrata* and *C. tropicalis*. In

these cases, MIC values varied in the range of 1.9-250 µg/mL for both the extracts above mentioned.

Table 2

Antifungal screening of *n*-hexane extract of native *Hypericum* species to Southern Brazil.

Yeast	*Hypericum* species[a]				
	H. caprifoliatum	***H. carinatum***	***H. linoides***	***H. myrianthum***	***H. polyanthemum***
***Candida albicans* ATCC 28367**	R	Fungistatic	Fungistatic	R	R
***Candida dubliniensis* CO 24P**	Fungicidal	Fungicidal	R	Fungicidal	R
***Candida glabrata* CG 40136**	R	Fungicidal	Fungistatic	Fungistatic	R
***Candida guilliermondii* CG 40039**	R	Fungistatic	R	Fungistatic	R
***Candida krusei* CK 02**	R	Fungistatic	R	R	R
***Cândida parapsilosis* CPA 08**	Fungicidal	Fungicidal	Fungicidal	Fungicidal	R
***Candida tropicallis* CT 08**	Fungicidal	Fungicidal	Fungicidal	Fungicidal	R
***Cryptococcus neoformans* CRY 17**	Fungicidal	Fungicidal	Fungicidal	Fungicidal	Fungicidal
***Geotrichum candidum* GEO 03**	Fungicidal	Fungicidal	Fungicidal	Fungicidal	Fungistatic
***Rhodotorula mucilaginosa* ROH 03**	Fungicidal	Fungicidal	Fungicidal	Fungicidal	Fungicidal

[a]Tested concentration = 500 µg/mL; R = Resistance.

The previously selected extracts were also evaluated for the chemotactic effect on polymorphonuclear neutrophils in concentrations of 0.31 to 10.0 µg/mL (Table 4). *Hypericum carinatum*, *H. linoides* and *H. myrianthum* extracts showed a similar activity. The effect observed proved to be dose-dependent and over 60% in the lower tested concentration. In preliminary tests (data not shown), the mean chemotactic inhibition of the *H. caprifoliatum* and *H. polyanthemum* extracts was only 43% and 48%, respectively. For these samples, there was no difference between the tested concentrations (5 and 1 µg/mL).

Table 3

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *n*-hexane extract of native *Hypericum* species to Southern Brazil[a].

Yeast	*H. carinatum*			*H. linoides*			*H. myrianthum*		
	MIC	**MIC range**	**MFC**	**MIC**	**MIC range**	**MFC**	**MIC**	**MIC range**	**MFC**
Candida albicans									
CA 02	> 500			> 500					
ATCC 10231	> 500	> 500	> 500	> 500	> 500	> 500	nt	nt	nt
ATCC 24433	> 500			> 500					
Candida dubliniensis									
CD 24A	> 500								> 500
CD 24P	> 500	> 500	> 500	nt	nt	nt	> 500	> 500	> 500
CD 85P	> 500								> 500
Candida glabrata									
CG 01	62.5		> 500	7.8		62.5	31.2		62.5
CG 4036	62.5	62.5 - 250	250	15.6	7.8 - 125	125	15.6	15.6 - 125	125
CG 40039	250		> 500	125		> 500	125		> 500
Candida krusei									
CK 03	> 500								
CK 04	> 500	> 500	> 500	nt	nt	nt	nt	nt	nt
CK 6258	> 500								
Candida parapsilosis									
CPA 01	500		> 500	125		500	250		> 500
CPA 04	500	500	> 500	250	125 - 250	> 500	500	250 - 500	> 500
CPA 134	500		> 500	250		> 500	500		> 500
Candida tropicallis									
CT 56	250		> 500	62.5		125	250		500
CT 750	250	3.9 – 250	> 500	250	31.2 – 250	> 500	250	1.9 – 250	> 500
CT 40098	3.9		31.2	31.2		62.5	1.9		31.2
Cryptococcus neoformans									
CRY 02	3.9		250	1.9		250	7.8		31.2
CRY 14	7.8	3.9 – 15.6	500	1.9	1.9	250	1.9	1.9 – 7.8	250
CRY 17	15.6		500	1.9		250	1.9		1.9
Geotrichum candidum									
GC 09	> 500			500			> 500		
GC 18	> 500	> 500	> 500	500	500	500	> 500	> 500	> 500
GC 26	> 500			500			> 500		
Rhodotorula mucilaginosa									
ROH 01	31.2		500	1.9		250	7.8		15.6
ROH 02	62.5	15.6 - 62.5	125	3.9	1.9 – 7.8	250	7.8	1.9 – 7.8	15.6
ROH 05	15.6		62.5	7.8		250	1.9		7.8

[a]Data antifungal susceptibility in µg/mL; nt = not tested.

Table 4

Effect of *Hypericum* lipophilic extracts on the *in vitro* chemotaxis of polymorphonuclear neutrophils[a].

Sample / Concentration (µg/mL)	Migration (µm)	Migration inhibition (%)
H. carinatum		
10.00	0*	100*
5.00	7.60 ± 1.58*	92.96*
2.50	14.33 ± 3.88*	86.73*
1.25	30.57 ± 8.22*	71.69*
0.63	29.60 ± 6.45*	72.59*
0.31	40.80 ± 7.50*	62.22*
H. linoides		
10.00	0*	100*
5.00	0*	100*
2.50	12.80 ± 3.03*	88.15*
1.25	24.20 ± 8.51*	77.59*
0.63	30.20 ± 4.16*	72.04*
0.31	37.33 ± 9.95*	65.43*
H. myrianthum		
10.00	0*	100*
5.00	12.20 ± 1.14*	88.70*
2.50	21.00 ± 5.98*	80.56*
1.25	31.00 ± 9.39*	71.30*
0.63	18.80 ± 3.03*	82.59*
0.31	29.45 ± 6.64*	72.73*
Positive control (LPS)	108 ± 2.56	0

[a]Chemotaxis is presented as means ± SEM of leukocyte migration. *P<0.05 indicates a significant difference compared to reference chemoattractant (LPS) (ANOVA-Tukey's test).

HPLC analyses were carried out to determine the major phenolic compounds present in the investigated samples. The results are showed in table 5. Uliginosin B was detected in the extract of all species in concentrations ranging from 0.06 to 3.60%. High percentages of this compound were observed in *H. myrianthum* which also presented the higher amount of japonicin A. Additionally, hyperbrasilol B, benzophenones (cariphenone A and B) and benzopyrans (HP1, HP2 and HP3) were detected only in the *H. linoides*, *H. carinatum* and *H. polyanthemum* extracts, respectively.

Table 5

Content (%) of the investigated phenolic compounds in *Hypericum* extracts[a].

Hypericum species	Compounds							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
H. carinatum	1.65 ± 0.008	nd	nd	nd	nd	nd	0.08 ± 0.001	0.58 ± 0.009
H. caprifoliatum	0.06 ± 0.002	0.28 ± 0.002	nd	nd	nd	nd	nd	nd
H. linoides	0.19 ± 0.005	1.44 ± 0.010	1.40 ± 0.010	nd	nd	nd	nd	nd
H. myrianthum	3.60 ± 0.006	7.05 ± 0.033	nd	nd	nd	nd	nd	nd
H. polyanthemum	0.05 ± 0.002	nd	nd	35.70 ± 0.193	42.68 ± 0.027	10.98 ± 0.005	nd	nd

[a]Values are expressed as mean ± standard deviation of three assays; 1 = uliginosin B; **2** = japonicin A; **3** = hyperbrasilol B; **4** = HP1; **5** = HP2; 6 = HP3; **7** = cariphenone A; **8** = cariphenone B; nd = not detected.

4. Discussion

Despite advances in science and technology, the approval of new anti-infectives has declined significantly over the last years (Griffith et al., 2012). Considering the available drugs, some studies also show disparity between the therapeutic areas. The number of antifungal agents for example is about 4 times lower than that of antibacterials (Newman and Cragg, 2007). As a consequence, the pharmacological options against fungal diseases are limited. In this aspect the clinical treatment of systemic infections is reduced to only four classes (fluoropyrimidine analogs, polyenes, azoles and echinocandins) and three molecular targets (inhibition of nucleotides, ergosterol and β-(1-3)-glucan) (Vandeputte et al., 2012; Zaragoza and Pemán, 2012). At least in part, such shortage is due to the eukaryotic nature of the fungal cell and consequent similarity to mammalian metabolism (Newman et al., 2000). Natural products favor the development of new drugs with different targets and mechanisms of action due to their wide structural diversity. In fact, although not representing exclusively the final chemical entity, natural products are a major source of anti-infectives (Newman and Cragg, 2007; Rajeshkumar and Sundararaman, 2012).

Plants are the basis of the systems of traditional medicine. Naturally, many metabolites play a role of defense against pathogenic microorganisms (Rajeshkumar and Sundararaman, 2012). According to the results presented in table 2, the extracts of *H. caprifoliatum*, *H. carinatum*, *H. linoides*, *H. myrianthum* and *H. polyanthemum* exhibited a broad spectrum of action. Similar impressions were also obtained with the lipophilic extracts and fractions of *H. ternum* (Fenner et al., 2005). Considering the MIC (Table 3), it is noteworthy that *H. carinatum*, *H. linoides* and *H. myrianthum* extracts showed higher potency against *C. neoformans* and *R. mucilaginosa.* In this case, the MIC range is comparable to many antifungals clinically used (Pappas et al., 2004). *Cryptococcus* and *Rhodotorula* species can cause infections as meningitis, endocarditis, peritonitis and fungemia with mortality rates in range of 20-40%. Some studies have also shown that the previous administration of broad spectrum antibiotics represents a risk factor for such infections as well as resistance to antifungal agents (Lunardi et al., 2006; Pemán and Salavert, 2012). Thus, the *Hypericum* extracts may represent an alternative for the treatment of these emerging fungal.

According to Ríos and Recio (2005), antimicrobial activity is promising when concentrations below 100 μg/mL for extracts and 10 μg/mL for isolated compounds are achieved. In this range of desirable values, *H. carinatum*, *H. linoides* and *H. myrianthum* extracts demonstrated just inhibit some strains of *C. glabrata* and *C. tropicalis* (Table 3). In general, the MIC range for these yeasts was wide, which could indicate only a limited activity of these extracts. In fact, even at high concentrations, *Candida* species were the only ones to shows resistance to the investigated extracts (Table 2). Such results could be explained by various virulence factors commonly reported for these microorganisms such as adherence, biofilm formation, secretion of hydrolytic enzymes (proteases, phospholipases and haemolysins) and filamentous growth (Silva et al., 2012).

The results of the *in vitro* antichemotactic activity showed that all extracts were able to reduce the migration of neutrophils. Coincidently, the extracts of *H. carinatum*, *H. linoides* and *H. myrianthum* were also the most effective. For these samples, in general 5-10 μg/mL were sufficient to inhibit 100% of the polymorphonuclear migration. Standardized extracts of *H. brasiliense* have demonstrated anti-inflammatory activity in the carrageenin-induced rat paw edema (Perazzo et al., 2008). According to Perazzo *et al.* (2008), the effects observed suggest action in acute processes once the extract did not inhibit the formation of granulomatous tissue. As neutrophils are presented in the acute phase of inflammation, the present results are in agreement with the literature data.

Gradually, mechanisms that promote inflammation and impair antifungal immune response are being discovered. It is known, for example, that *Candida albicans* and *Aspergillus fumigatus* colonization are associated with elevated levels of the pro-inflammatory cytokine (IL-17, IL-23 and Th17) (Kumanoto, 2011; Zelante et al., 2007). Fungal colonization, however, does not necessarily imply infection and disease development. The stability of the host–fungus relationship is maintained by a complex balance of intracellular signals pro- and anti-inflammatory (Cottier and Pavelka, 2012; Romani, 2011). Consequently, control of the inflammatory response may represent a strategy to combat fungal infections (Zelante et al., 2007). In view of this, *H. carinatum*, *H. linoides* and *H. myrianthum* extracts show potential as source of drugs which act in the fungal infections associated with inflammatory disorders.

The findings of this study further demonstrate strong correlation between the antifungal / anti-inflammatory activities and the phenolic content of the *Hypericum* species. Interestingly, the extracts that presented high amounts of dimeric phloroglucinol derivatives were the most active (Tables 2 - 5). Uliginosin B, japonicin A and hyperbrasilol B presented antimicrobial activity (Jayasurya et al., 1989; Jayasurya et al., 1991; Rocha et al., 1995, 1996) and consequently could be the

responsible by effect observed. Some works reports also antimicrobial properties to benzophenones (Baggett et al., 2005) and benzopyrans (Dall'Agnol et al., 2003). The first group of compounds occurring only in *H. carinatum*, plant with high potential fungicide, while the latter group only in *H. polyanthemum*, species ineffective against yeasts of *Candida* genus (Table 2 and 5). Moreover, it is notable that uliginosin B, japonicin A, hyperbrasilol B, cariphenone A and cariphenone B are all poly-hydroxylated molecules, unlike HP1, that does not present OH-group, and HP2 and HP3 that present only one (Fig. 1). Thus, the presence of the –OH group seems to be relevant for the antimicrobial activity.

In conclusion, the *Hypericum* species from South Brazil confirmed their potential as source of new antimicrobial and anti-inflammatory drugs. In general, the lipophilic extracts demonstrated a broad spectrum of antifungal action. *Hypericum carinatum*, *H. linoides* and *H. myrianthum* were highly effective in combating emerging yeasts as *C. neoformans* and *R. mucilaginosa*. Moreover, all the samples investigated were able to reduce the migration of neutrophils revealing the anti-inflammatory potential of the species. The antifungal and anti-inflammatory activities were strongly correlated with the presence of dimeric phloroglucinol derivatives in the *n*-hexane extract. The biological activity of the isolated compounds should be further investigated to confirm the impressions of this study as well as to determine their mechanism of action.

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DISCUSSÃO GERAL

O gênero *Hypericum* tem sido considerado uma unidade natural para muitos taxonomistas, embora existam discussões quanto ao tratamento deste gênero como uma família em separado (Hypericaceae) ou, tradicionalmente, como parte da subfamília Hypericoidae dentro de Guttiferae. Análises filogenéticas suportam a hipótese da existência da família Hypericaceae, a qual compreenderia nove gêneros, entre eles *Hypericum* com mais de 80% da diversidade (CROCKETT e ROBSON, 2011).

A rede filogenética do gênero foi proposta e organizada por Robson (ROBSON, 1977, 2002). Atualmente, *Hypericum* conta com 36 seções taxonômicas delineadas por combinações entre características morfológicas e de distribuição geográfica (CROCKETT e ROBSON, 2011). *Brathys* e *Trigynobrathys* são os táxons predominantemente americanos. O primeiro apresenta dois centros primários de distribuição: Belize - Cuba e as fronteiras entre Venezuela - Colômbia. A partir destes centros, as demais espécies radiam para outros países do próprio continente (Brasil, Argentina, Uruguai, Bolívia, Peru, Chile, Costa Rica, México, Panamá, Estados Unidos e Canadá), assim como para África e Ásia (ROBSON, 1990).

Em que pese o local de origem seja um fator de variabilidade química, os representantes das seções *Brathys* e *Trigynobrathys* mantêm um conjunto de metabólitos secundários comuns (Tabela 2 e 3, manuscrito 1). No caso dos derivados diméricos de floroglucinol, os resultados obtidos reforçam o valor quimiotaxonômico destes compostos. Uliginosina B e japonicina A, por exemplo, foram independentemente isolados de espécies que ocorrem naturalmente em locais muito distintos (Tabela 4, manuscrito 1), demonstrando, assim, que determinadas rotas biosintéticas foram preservadas durante a evolução das espécies e/ou poderiam ocorrer com maior frequência dentro do táxon como um resultado da sua convergência evolutiva (CROCKETT e ROBSON, 2011).

A presença de determinados compostos fenólicos no gênero *Hypericum* também está associada a certas estruturas morfológicas destas plantas. A ocorrência de naftodiantronas como hipericina em *Hypericum perforatum*, por exemplo, está

associada à presença de glândulas escuras, predominantemente nas flores da planta. Tais glândulas ocorrem em cerca de 2/3 das seções (CROCKETT e ROBSON, 2011). As espécies das seções *Brathys* e *Trigynobrathys* não apresentam este tipo glândula, embora sejam dotadas de glândulas pálidas (FERRAZ *et al*., 2002). Estas secretam e acumulam preferencialmente óleos essenciais e derivados de floroglucinol e estão distribuídas, sobretudo, nas folhas, sépalas e pétalas (ABREU *et al*., 2004; ABREU e MAZZAFERA, 2005; AYAN *et al*., 2006; BERNARDI *et al*., 2008; CROCKETT e ROBSON, 2011). Assim, por ser característica distintiva, o acúmulo de determinadas classes fenólicas associadas à presença de glândulas escuras ou pálidas são igualmente importantes do ponto de vista quimiotaxonômico.

Geralmente extratos de plantas contendo fenólicos constituem uma mistura de várias classes solúveis nos solventes empregados. Os mais comuns são metanol, etanol, acetona, água e acetato de etila (NACZK e SHAHIDI, 2004). Os derivados diméricos de floroglucinol, benzopiranos e benzofenonas, contudo, são classicamente obtidos por meio de solventes apolares como éter de petróleo e *n*-hexano (NÖR *et al*., 2004; BERNARDI *et al*., 2005). Embora tais metabólitos apresentem hidroxilas em suas estruturas (Figura 1, manuscrito 1), eles possuem caráter apolar. Esta característica explica-se pelo fato de que muitas das hidroxilas estão envolvidas em ligações intramoleculares, dificultando, assim, a realização de pontes de hidrogênio intermoleculares, fortemente polares.

Os métodos empregados na análise quantitativa dos compostos fenólicos, por sua vez, podem ser classificados em função da determinação do seu conteúdo total ou de um composto / classe específica. No primeiro caso, o método de Folin e suas variações, baseadas na redução do ácido fosfomolibdênico, é o procedimento comumente empregado. Por ser inespecífico, este método detecta todos os grupos de fenólicos encontrados no extrato. Como desvantagem, substâncias redutoras como o ácido ascórbico constituem interferentes; no segundo caso, predominam as técnicas de cromatografia líquida de alta eficiência (CLAE), as quais também são muito aplicadas nos procedimentos de separação e isolamento dos compostos. Nestas situações, os

detectores comumente utilizados são os de luz ultravioleta, visível, arranjo de fotodiodos e de fluorescência (NACZK e SHAHIDI, 2004).

Nas espécies de *Hypericum*, o conteúdo fenólico tem sido avaliado tanto pelo método de Folin-Ciocalteu como por CLAE. Dados ajustados para fins de comparação do extrato *n*-hexano e da fração metanólica das partes aéreas de *H. caprifoliatum*, *H. carinatum*, *H. myrianthum* e *H. polyanthemum* resultaram em variações na faixa de 37 - 86 e 139 - 196 mg equivalentes de quercetina por g de extrato seco, respectivamente (BERNARDI *et al*., 2008). Fritz e colaboradores (2007) obtiveram resultados ligeiramente inferiores para os extratos etanólicos de *H. polyanthemum* e *H. myrianthum*. Em outro estudo, o extrato metanólico das flores de *H. polyanthemum* apresentou variações de 10 a 75 mg/g, dependendo do estágio de desenvolvimento da planta (BERNARDI *et al*., 2008). Diante destes dados são possíveis algumas considerações: primeiro, a menor quantidade de compostos fenólicos nos extratos apolares está coerente com os resultados obtidos (Tabela 2 e 3, manuscrito 1); segundo, considerando que as flores representam, de um modo geral, a menor parte do peso destas plantas (BERNARDI *et al*., 2008), é possível reafirmar que, para os compostos fenólicos investigados, as flores são os principais órgãos de acúmulo.

Em regra, qualquer órgão vegetal possui capacidade de sintetizar e acumular compostos fenólicos. É muito comum, no entanto, que os metabólitos concentrem sua produção em determinados tecidos, a qual pode variar em função de fatores abióticos ou bióticos como os diferentes estágios de desenvolvimento da planta. O maior conteúdo dos benzopiranos HP1 e HP2 em *H. polyanthemum*, por exemplo, tem sido obtido nos botões florais verdes da planta, enquanto HP3 alcança níveis superiores apenas no final do ciclo de floração (BERNARDI *et al*., 2008). Paralelamente, é conhecido que os compostos fenólicos desempenham papéis na interação das plantas com o seu ambiente. Os efeitos alelopáticos atribuídos ao alto conteúdo fenólico em *H. polyanthemum* e *H. myrianthum* poderiam inclusive justificar o padrão de distribuição e adensamento destas plantas (FRITZ *et al*., 2007).

Os compostos fenólicos são frequentemente responsáveis pelas atividades farmacológicas descritas às espécies de *Hypericum*. O derivado dimérico de floroglucinol uliginosina B e o flavonoide hiperosídeo, por exemplo, contribuem para a atividade antidepressiva de *H. caprifoliatum* e *H. polyanthemum* (DAUDT *et al*., 2000; MENDES *et al*., 2002; VIANA *et al*., 2003; VIANA *et al*., 2005; VIANA *et al*., 2006; VIANA *et al*., 2008; HAAS *et al*., 2011). A presença destes metabólitos em outras espécies das seções *Brathys* e *Trigynobrathys* (Tabelas 2 – 4, manuscrito 1), portanto, reforça a importância desse estudo, uma vez que os resultados podem ser úteis na seleção e investigação de novas plantas com equivalente potencial terapêutico.

Em relação aos métodos extrativos, conforme definição descrita por SONAGLIO *et al*., 2003, "*o termo extração significa retirar, da forma mais seletiva e completa possível, as substâncias ou fração ativa contida na droga vegetal, utilizando, para isso, um líquido ou uma mistura de líquidos tecnologicamente apropriados e toxicologicamente seguros.*" Além da adequação tecnológica e da inocuidade fisiológica, a viabilidade econômica também é fator determinante para a escolha do método. Assim, a seleção correta pode determinar quão eficientemente agregamos valor aos recursos biológicos (HANDA, 2008).

As etapas frequentemente envolvidas no processo extrativo incluem a seleção, secagem e moagem do material vegetal; a extração propriamente dita e a filtração; a concentração e a secagem da solução extrativa. O fenômeno extrativo, por sua vez, pode ser descrito como segue: (1) a droga vegetal absorve o solvente promovendo a dilatação da estrutura celular; (2) concomitantemente, os compostos solúveis são dissolvidos pelo solvente; (3) os compostos dissolvidos são transferidos por difusão para a superfície do material vegetal; por fim, (4) os compostos são transportados pelo solvente e removidos do extrator (PEREIRA e MEIRELES, 2010). A padronização de todas as etapas do processo, portanto, contribuem significativamente para a qualidade final do produto.

O emprego de fluidos supercríticos tem ocorrido nas mais diferentes áreas, tais como ambiental, de alimentos, farmacêutica, química e de combustíveis. Embora

incipientes, as aplicações farmacêuticas incluem: extração e análise de fármacos, desenvolvimento de polimorfos, fármacos particulados e sistemas de liberação, co-formulação, purificação de excipientes e esterilização de materiais médico-cirúrgicos, meio para cristalização e reações enzimáticas, formação de complexos e revestimentos, cromatografia e separação de enantiômeros, entre outros (HERRERO, 2010; SEKHON, 2010). Alicerçada em uma abordagem multidisciplinar, a tecnologia dos fluidos supercríticos pode oferecer aplicações exclusivas nos campos da produção, processamento e desenvolvimento de matérias-primas (PEREIRA e MEIRELES, 2010).

Os custos de investimento continuam sendo o maior entrave para aplicação comercial de fluidos supercríticos. Por conta disso, a otimização das condições de extração constitui um dos aspectos de maior relevância para o processo (HANDA, 2008; PEREIRA e MEIRELES, 2010). Neste estudo, foram observadas algumas diferenças entre as condições empregadas. Destaca-se, nesse sentido, o predomínio de uliginosina B e carifenona B no experimento realizado sob temperatura constante e aumento sucessivo de pressão (Tabelas 2-4, manuscrito 2), e de carifenona A quando ambos, temperatura e pressão, foram mantidas constantes (Tabelas 2-4, resumo expandido). De maneira geral, os principais fatores que influenciam o método de extração são o ano, a parte da planta e aspectos edafoclimáticos; a localização dos solutos; a granulometria, a forma, a porosidade e a umidade do material vegetal; o solvente utilizado e sua taxa de fluxo; temperatura e pressão; tipo e percentagem de co-solvente; modo ou perfil de extração, tempo e equipamento empregados (HERRERO, 2010; PEREIRA e MEIRELES, 2010). O sucesso do método, portanto, depende da seleção das condições adequadas de extração, as quais também podem justificar sua viabilidade econômica.

O poder de solvatação dos fluidos supercríticos pode ser manipulado por mudanças nas condições de pressão e temperatura de extração. Este poder de solvatação ajustável constitui uma das grandes vantagens da técnica e é particularmente útil na extração de amostras complexas como drogas vegetais (CASAS, 2010). A extração de *H. carinatum*, por exemplo, mostrou-se mais seletiva

que a maceração com *n*-hexano. O conteúdo de carifenona A e carifenona B, por exemplo, chega próximo do dobro em comparação aquele obtido por maceração com *n*-hexano (Tabela 2, manuscrito 2). A seletividade da extração e/ou solubilidade dos compostos depende do equilíbrio entre a densidade do fluido e as propriedades do soluto, tais como massa molecular, polaridade e pressão de vapor. Assim, a menos que os solutos não tenham qualquer afinidade com a matriz (PEREIRA e MEIRELES, 2010), os dados de solubilidade dos compostos puros não podem ser usados diretamente para predizer a seletividade.

Como regra geral, se outras variáveis são constantes, um aumento na pressão tende a aumentar a densidade do fluido e, por conseguinte, seu poder de solvatação; um aumento da temperatura, por outro lado, tende a reduzir a densidade do fluido. O efeito da temperatura, entretanto, é complexo. Do ponto de vista termodinâmico, a elevação da temperatura também poderia aumentar a pressão de vapor dos solutos, facilitando sua solubilidade no fluido. Adicionalmente, de acordo com as regras cinéticas, o aumento da temperatura pode levar a um movimento térmico mais intenso dos solutos, favorecendo sua dessorção da matriz (CASAS, 2010; PEREIRA e MEIRELES, 2010; SHI, 2010). Assim, os efeitos acima descritos competem entre si durante a extração e são fundamentais para a compreensão do processo.

Tanto a temperatura como a pressão afetaram a composição qualitativa e quantitativa do extrato de *H. carinatum*. Conforme as tabelas 1-4 do manuscrito 2, o aumento da temperatura diminuiu o rendimento total de extrato, bem como a quantidade de uliginosina B, carifenona A e carifenona B. Diante disso, é possível inferir que as moléculas que compõem o extrato apresentam pouca volatilidade e o efeito observado é uma consequência mais pronunciada da diminuição da densidade e/ou do poder de solvatação do fluido. Tais impressões foram confirmadas quando, sob temperatura constante, a pressão foi elevada e os rendimentos dos extratos foram superiores (Tabela 1, resumo expandido). Com exceção da carifenona B, entretanto, o aumento da pressão causou prejuízos à extração seletiva dos compostos (Tabelas 2-4, resumo expandido). Com frequência, o aumento da temperatura e da pressão resulta uma maior extração de compostos pesados, tais como ceras e clorofila (PEREIRA e

MEIRELES, 2010). Assim, a condição ideal para a obtenção dos três compostos em estudo é 40ºC e 90 bar. Além disso, tendo em vista a seletividade, a condição de 40ºC e 150 bar pode representar uma ótima alternativa para a recuperação da carifenona B.

Paralelamente, o tempo de extração influencia tanto a eficiência como os custos operacionais. Um perfil cinético da extração dos compostos bioativos de *H. carinatum* nas diferentes condições empregadas pode ser visto nas figuras 1-3 (resumo expandido). Para a máxima recuperação dos compostos uliginosina B e carifenona A, o tempo de contato entre o fluido e a matriz vegetal foi de pelo menos 180 min a 40ºC e 90 bar; para carifenona B esse tempo reduz e fica em torno de 90 min a 40ºC e 150 bar. Indiscutivelmente, o método supercrítico representa uma grande economia de tempo em comparação à maceração com *n*-hexano. Além disso, as etapas de filtração e concentração do extrato podem ser completamente eliminadas, uma vez que por um simples procedimento de descompressão do fluido o solvente é eliminado. Diante de tudo isso, pode ser considerado um método rápido, eficiente e limpo para a extração de produtos naturais.

Cui e Ang (2002) reportaram que 50ºC e 380 bar representam as melhores condições para a extração dos floroglucinóis hiperforina e *ad*-hiperforina presentes em *H. perforatum*. Outro estudo realizado com a espécie nativa *H. polyanthemum* demonstrou que 40ºC e 90 bar resultaram em altos rendimentos do floroglucinol uliginosina B e do benzopirano HP1 (6-isobutiril-5,7-dimetoxi-2,2-dimetilbenzopirano); para HP2 (7-hidroxi-6-isobutiril-5-metoxi-2,2-dimetil-benzopirano) e HP3 (5-hidroxi-6-isobutiril-7-metoxi-2,2-dimetilbenzopirano), por sua vez, melhores rendimentos foram obtidos a 50ºC e 120 bar (CARGNIN *et al*., 2010). Paralelamente aos resultados obtidos com *H. carinatum*, fica claro que a definição das condições de extração são profundamente dependentes da matriz e, por isso, de difícil generalização.

Simulações matemáticas e computacionais podem ser usadas para predizer e planejar o processo de extração. Basicamente, são ferramentas de engenharia que executam várias tarefas, incluindo cálculos automatizados, balanços de matéria e

energia, estimativas de propriedades físicas, cálculos para concepção de plantas piloto, avaliações econômico-ambientais e otimização de processos (HANDA, 2010). Para nosso conhecimento, este foi o primeiro estudo de modelagem matemática realizado com uma planta do gênero *Hypericum*. Os resultados da simulação para o extrato (Figura 2, manuscrito 2) e para os compostos investigados (Figuras 1-3, resumo expandido) foram condizentes com aqueles obtidos experimentalmente.

Em decorrência disso, visto que as resistências à transferência de massa devem ser evitadas ou minimizadas a fim de aumentar a taxa de extração (PEREIRA e MEIRELES, 2010), dados relativos à solubilidade dos solutos no fluido (K_1) e sua difusão na droga vegetal (K_2) podem ser bastante úteis para a análise do processo extrativo. Nesse sentido, quanto maior o valor de K_1 e menor o de K_2, menores serão as resistências à transferência de massa. O elevado rendimento da carifenona A no extrato obtido a 40ºC e 90 bar de pressão exemplifica muito bem a relação entre as constantes K_1 e K_2 (Tabela 5, resumo expandido). Nesta condição, em que pese a solubilidade da carifenona A seja menor (K_1) em comparação as demais condições, tal efeito é compensado pela menor taxa de difusão do soluto na matriz (K_2). Futuramente, na medida em que novos experimentos forem realizados, os dados relativos a estas constantes poderão ser comparados, facilitando, assim, a otimização e o escalonamento do processo.

De maneira geral, as espécies de *Hypericum* apresentam um amplo espectro antimicrobiano. Para os representantes nativos do Sul do Brasil, destacam-se as atividades antiviral, contra o vírus da imunodeficiência felina (SCHIMITT *et al*., 2001) e herpes (FRITZ *et al*., 2007), antiprotozoária, contra *Trichomonas vaginalis* (CARGNIN *et al*., 2013) e *Leishmania amazonensis* (DAGNINO *et al*., 2012) e antibacteriana, contra *Bacillus subtilis*, *Micrococcus luteus* e *Staphylococcus aureus* (DALL'AGNOL *et al*., 2003; DALL'AGNOL *et al*., 2005; AVANCINI e WIEST, 2008; FRANÇA *et al*., 2009). Em acordo com os dados deste estudo (Tabelas 2 e 3, manuscrito 3), a atividade antifúngica tem sido descrita para *Cryptococcus neoformans*, *Epidermophyton flocosum*, *Saccharomyces cerevisiae* e diferentes espécies de *Aspergillus* sp., *Candida* sp., *Microsporum* sp., e *Trichophyton* sp.

(FENNER *et al*., 2005). Resultados discrepantes foram relatados por DALL'AGNOL e colaboradores (2003). Nesse caso, fatores relacionados ao processo extrativo (ex.: hexano x metanol), ao método do bio-ensaio (ex.: difusão x diluição) e ao perfil de suscetibilidade dos microorganimos poderiam justificar tais diferenças (RÍOS e RECIO, 2005; COS *et al*., 2006).

Ao que tudo indica, diferentes grupos de metabólitos respondem pelas propriedades mencionadas acima. Assim, enquanto taninos e flavonoides (ex.: amentoflavona e luteoforol) constituem os metabólitos bio-ativos para a atividade antiviral (SCHIMITT *et al*., 2001; FRITZ *et al*., 2007), xantonas (ex.: 1,5-di-hidroxixantona, 5-hidroxi-1-metoxixantona e 6-desoxijacareubina) e derivados floroglucinol (ex.: uliginosina B, isoluginosina B, japonicina A, HP1, HP2, HP3) constituem os principais responsáveis pelas atividades anti-*Trichomonas* (CARGNIN *et al*., 2013), leishmanicida (DAGNINO *et al*., 2012), antibacteriana (DALL'AGNOL *et al*., 2003; DALL'AGNOL *et al*., 2005; FENNER *et al*., 2005; FRANÇA *et al*., 2009) e antifúngica (ROCHA *et al*., 1994; FENNER *et al*., 2005), em concordância aos dados correlacionados neste estudo (Tabelas 2, 3 e 5, manuscrito 3).

A maioria dos antifúngicos interfere na biosíntese ou na integridade do ergosterol, o principal esterol da membrana celular dos fungos (RIVAS e CARDONA-CASTRO, 2009). Em que pese o mecanismo de ação dos derivados floroglucinol não seja conhecido, é provável que esses compostos interfiram nas propriedades de membrana dos fungos (SIKKEMA *et al*., 1995; BURT, 2004), uma vez que são lipofílicos. Dados da literatura descrevem, por exemplo, complexas interações entre membranas fosfolipídicas e hiperforina (HOFFMANN *et al*., 2010), o principal derivado floroglucinol de *H. perforatum*, entre outras espécies com potencial antimicrobiano (GIBBONS *et al.*, 2002).

Como evidenciado neste estudo, as hidroxilas também parecem ter um papel relevante na atividade antimicrobiana dos derivados flororoglucinol. Estas impressões corroboram dados da literatura que apontam, por exemplo, ausência de atividade para o benzopirano HP1, molécula que não possui grupos hidroxila, e atividade para HP2,

HP3, uliginosina B e japonicina A, moléculas mono ou poli-hidroxiladas, frente a *S. aureus* (DALL'AGNOL *et al*., 2005). Para Kubo e colaboradores (1995), tais grupos atuariam orientando a ligação dos compostos na fase hidrofílica da membrana fúngica, favorecendo, consequentemente, o alinhamento da cadeia carbonada na fase lipofílica.

Correlações entre a atividade antioxidante inerente aos compostos fenólicos e a atividade antimicrobiana são igualmente descritas (CHUN *et al*., 2005). Os mecanismos, nesse caso, envolveriam perturbações na cadeia de transporte de elétrons, complexação com proteínas, inibição de enzimas e canais iônicos, entre outros (COWAN, 1999; SHETTY e WAHLQVIST, 2004 *apud* CHUN *et al*., 2005). Assim, pelo menos em parte, a atividade antioxidante descrita para a carifenona A (BERNARDI *et al*., 2005) poderia explicar o elevado efeito fungicida demonstrado por *H. carinatum* (Tabela 2, manuscrito 3).

Em muitos casos o papel biológico dos metabólitos secundários está relacionado à defesa da planta contra o ataque de insetos, herbívoros e micro-organimos (COWAN, 1999). Níveis elevados de uliginosina B, HP1, HP2 e HP3 nas partes reprodutivas de *H. polyanthemum* após contato com partes de um fungo autoclavado, *Nomuraea rileyi*, sugere tal função para os derivados floroglucinol (MEIRELLES *et al*., 2012). Estas impressões também foram observadas com relação aos níveis de hiperforina e xantonas em culturas de *H. perforatum* submetidas a estresse biótico com *Colletotrichum gloeosporioides* e *Agrobacterium tumefaciens*, respectivamente (SIRVENT e GIBSON 2002; FRANKLIN *et al*., 2009). Assim como os animais, as plantas desenvolveram habilidades para reconhecer padrões moleculares associados a patógenos (NÜRNBERGER *et al*., 2004) e, consequentemente, ativar a biossíntese dos seus compostos de defesa (FRANKLIN *et al*., 2009). Atualmente, o conhecimento e a manipulação controlada desses mecanismos constitui uma estratégia para obtenção de compostos de interesse terapêutico em *Hypericum* sp. (SIRVENT e GIBSON 2002; FRANKLIN *et al*., 2009; MEIRELLES *et al*., 2012).

A maioria dos antifúngicos usados na clínica apresentam alguma desvantagem em termos de toxicidade, resistência, eficácia, efeito adverso, interação

medicamentosa ou custo. Apenas para exemplificar, os principais fatores que limitam o uso da anfotericina B são a sua nefrotoxicidade e baixa absorção oral (RIVAS e CARDONA-CASTRO, 2009; ZARAGOZA e PEMÁN, 2012). Embora as espécies de *Hypericum* não sejam isentas de toxicidade, tais plantas mostram diferentes perfis de segurança (BETTI *et al*., 2012), indicando que a otimização de parâmetros como dose e vias de administração podem viabilizar o seu uso terapêutico. Ratos tratados pela via intraperitoneal com o extrato éter de petróleo de *H. caprifoliatum* (270 mg/Kg/dia), por exemplo, mostram sinais de toxicidade, enquanto nenhum efeito foi observado para ratos ou camundongos tratados oralmente (50-2000 mg/kg) com o extrato éter de petróleo e ciclo-hexano (VIANA *et al*., 2006). De forma semelhante, nenhum sinal clínico de toxicidade aguda foi observado em camundongos tratados com o extrato ciclo-hexano de *H. polyanthemum* (2000 mg/Kg, v.o), ao passo que doses repetidas (450 e 900 mg/Kg) afetam o ganho de peso e alteram parâmetros bioquímicos e histológicos. Mesmo assim, de acordo com os parâmetros de toxicidade aguda, *H. polyanthemum* foi classificada como segura (BETTI *et al*., 2012). FERRAZ e colaboradores (2009) demonstraram ainda que HP1, HP2 e HP3 (30 mg/Kg, i.p.) não induzem efeitos genotóxicos, embora sinais de mutagênicidade tenham sido observados para HP1. Uliginosina B (90 mg/kg), por sua vez, induz prejuízo motor em camundongos tratados intraperitonealmente, ao passo que, pela via oral, nenhum efeito foi observado. Além disso, ao que tudo indica, tanto os extratos como os compostos isolados apresentam boa biodisponibilidade oral (STOLZ *et al*., 2012).

Considerando a atividade antiquimiotática, é importante destacar que, além das infecções microbianas, muitas outras doenças (ex.: câncer, aterosclerose e Alzheimer) podem ser influenciadas ou até mesmo causadas por desordens da resposta inflamatória (LUSTER, 1998; AKIYAMA *et al*., 2000; COUSSENS e WERB, 2002; LIBBY *et al*., 2002; HOTAMISLIGIL, 2006). Em certos tipos de câncer, por exemplo, dados da literatura apontam que alguns mediadores produzidos pelos neutrófilos poderiam dar início à formação de tumores, bem como favorecer a sua progressão (GREGORY e HOUGHTON, 2011; AMULIC *et al*., 2012). Em contrapartida, é interessante notar que camundongos tratados com o extrato ciclo-hexano de *H.*

polyanthemum (450 – 900 mg/Kg, v.o, 28 dias), planta cujo potencial antitumoral tem sido descrito (FERRAZ *et al*., 2005; GRIVICICH *et al*., 2008), tiveram o número de neutrófilos reduzido (BETTI *et al*., 2012), em acordo com os resultados deste estudo (Tabela 4, manuscrito 3). Deste modo, é possível reafirmar que as espécies de *Hypericum* e seus principais derivados floroglucinol representam alternativas potenciais para o desenvolvimento de fármacos com diferentes alvos terapêuticos.

CONCLUSÕES

- ✓ Uliginosina B foi o principal derivado dimérico de floroglucinol detectado no extrato *n*-hexano das flores de todas as espécies de *Hypericum* das seções *Brathys* e *Tigynobrathys* investigadas. As concentrações do metabólito variaram de 0,008 a 0,188% (p/p);

- ✓ Os benzopiranos HP1, HP2 e HP3 foram determinados exclusivamente em *H. polyanthemum* e em concentrações de 0,200, 0,225 e 0,327% (p/p) no extrato *n*-hexano das flores, respectivamente;

- ✓ As benzofenonas carifenona A e carifenona B foram determinadas exclusivamente em *H. carinatum* e em concentrações de 0,309 e 0,062 % (p/p) no extrato *n*-hexano das flores, respectivamente;

- ✓ Hiperosideo foi o principal flavonóide detectado na fração metanólica de todas as espécies de *Hypericum* das seções *Brathys* e *Tigynobrathys* investigadas. Nas flores, as concentrações do metabólito variaram de 0,057 a 5,987% (p/p);

- ✓ Os resultados reforçam o significado quimiotaxonômico dos derivados diméricos de floroglucinol para as espécies de *Hypericum* das seções *Brathys* e *Tigynobrathys*;

- ✓ As análises por CLAE mostraram-se úteis como método de impressão química, uma vez que permitem diferenciar, a partir dos extratos, as diferentes espécies de *Hypericum* investigadas;

- ✓ Os compostos fenólicos polares foram quantitativamente os principais metabólitos acumulados nas flores das espécies de *Hypericum* das seções *Brathys* e *Tigynobrathys* investigadas;

- ✓ A extração de *Hypericum carinatum* com CO_2 supercrítico mostrou-se mais seletiva que a maceração com *n*-hexano para a recuperação de uliginosina B, carifenona A e carifenona B;

- ✓ Temperatura e pressão afetam de modo distinto o rendimento e a composição dos extratos de *Hypericum carinatum* obtidos com CO_2 supercrítico. Assim, o aumento da temperatura causou efeitos negativos sobre o rendimento do extrato, enquanto o aumento da pressão produziu efeitos positivos;

- ✓ As condições ideais de temperatura, pressão e tempo na extração de *Hypericum carinatum* com CO_2 supercrítico visando à recuperação de uliginosina B, carifenona A e carifenona B foram 40°C, 90 bar e 180 minutos, respectivamente.

- ✓ As máximas quantidades de uliginosina B, carifenona A e carifenona B obtidas na extração de *Hypericum carinatum* com CO_2 supercrítico foram, respectivamente, 162,83 ± 4,58, 376,33 ± 4,14 e 48,79 ± 0,66 mg / 100 g de planta seca;

- ✓ O modelo matemático empregado para simular o processo de extração de *Hypericum carinatum* com CO_2 supercrítico foi apropriadamente correlacionado aos dados experimentais;

- ✓ Os extratos lipofílicos de *H. caprifoliatum*, *H. carinatum*, *H. linoides*, *H. myrianthum* e *H. polyanthemum* exibem um amplo espectro de ação antifúngica;

- ✓ *H. carinatum*, *H. linoides* e *H. myrianthum* mostram maior potência contra *C. neoformans* (Concentração inibitória mínima = 1,9-15,6 μg/mL), *R. mucilaginosa* (Concentração inibitória mínima = 1,9-62,5 μg/mL), *C. glabrata* (Concentração inibitória mínima = 7,8-250 μg/mL) e *C. tropicalis* (Concentração inibitória mínima = 1,9-250 μg/mL);

- ✓ Os extratos lipofílicos de *H. caprifoliatum*, *H. carinatum*, *H. linoides*, *H. myrianthum* e *H. polyanthemum* também apresentam potencial anti-inflamatório, uma vez que inibiram a migração de neutrófilos *in vitro*;

- ✓ Para *H. carinatum*, *H. linoides* e *H. myrianthum* o efeito antiquimiotático foi dose dependente (60-100%) nas concentrações entre 0,31-10 μg/mL;

- ✓ As atividades antifúngica e antiquimiotática foram correlacionadas aos derivados diméricos de phloroglucinol presentes no extrato lipofílico destas plantas.

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