UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Desenvolvimento tecnológico de nanoemulsões contendo extrato padronizado de *Pterocaulon balansae* visando à atividade antifúngica

BRUNA MEDEIROS NEVES

Porto Alegre, 2018

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Tese apresentada por Bruna Medeiros Neves para obtenção do TÍTULO DE DOUTOR em Ciências Farmacêuticas

Orientador: Professor Dr. Helder Ferreira Teixeira
Coorientadora: Professora Drª. Gilsane Lino von Poser

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Profa. Dra. Angela Machado de Campos
Universidade Federal de Santa Catarina

Profa. Dra. Cristiane de Bona da Silva
Universidade Federal de Santa Maria

Profa. Dra. Elfrides Eva Scherman Schapoval
Universidade Federal do Rio Grande do Sul

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APRESENTAÇÃO

A presente tese foi redigida no modelo com encarte de publicações no formato de capítulos seguindo o modelo proposto para elaboração de teses e dissertações recomendado pelo Regimento do Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal do Rio Grande do Sul. Dessa forma, o trabalho encontra-se dividido em:

- Introdução;
- Objetivo geral e objetivos específicos;
- Capítulo I – Artigo de revisão: The genus *Pterocaulon* (Asteraceae) – A review on traditional medicinal uses, chemical constituents and biological properties;
- Capítulo II – Artigo científico: Determination of main coumarins of *Pterocaulon balansae* (Asterareae) by an ultra-fast liquid chromatography method — analytical and bioanalytical assays;
- Capítulo III – Artigo científico: Supercritical CO_2 extraction as a selective method for the obtainment of coumarins from *Pterocaulon balansae* (Asteraceae);
- Capítulo IV – Artigo científico: Topical nanoemulsions as delivery systems for *Pterocaulon balansae* extracts aiming at the treatment of sporotrichosis;
- Discussão geral;
- Conclusões;
- Referências.

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“É o tempo da travessia: e se não ousarmos fazê-la, teremos ficado, para sempre, à margem de nós mesmos.”

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RESUMO

Pterocaulon balansae (Asteraceae) é uma planta utilizada na medicina popular para tratamento de diversas afecções. Recentemente, estudos demonstraram atividade antifúngica de extratos orgânicos de *P. balansae* frente a cepas do fungo *Sporothrix schenckii*. Tal atividade tem sido relacionada à presença majoritária de cumarinas nesses extratos. Neste contexto, o objetivo do presente estudo foi desenvolver nanoemulsões para uso tópico contendo extratos de *P. balansae* visando o tratamento de infecções causadas pelo fungo *S. schenckii*. Em uma primeira etapa foi realizada uma revisão do gênero *Pterocaulon* a fim de compilar dados importantes sobre etnofarmacologia, composição química e atividades biológicas do gênero. Os estudos fitoquímicos mostraram que as espécies de *Pterocaulon* são compostas majoritariamente por cumarinas, apresentando também flavonoides, ácidos fenólicos, poliacetilenos e terpenos. A maior parte das atividades biológicas atribuídas a esse gênero foi associada à presença das cumarinas. Os efeitos biológicos encontrados corroboram os dados etnofarmacológicos que relatam a utilização dessas plantas como anti-inflamatórios, antioxidantes e antifúngicos. Na sequência, a parte experimental deste estudo focou no desenvolvimento e validação de duas técnicas cromatográficas (HPLC e UFLC) para quantificação das cumarinas presentes em extratos de *P. balansae*. Os métodos mostraram-se específicos, lineares, precisos, robutos e exatos. Além disso, o método foi validado para a quantificação das cumarinas em diferentes matrizes analíticas e bioanalíticas visando estudos de desenvolvimento de produtos tópicos e estudos de permeação cutânea. Em uma segunda etapa, foi otimizado um método de extração das cumarinas de *P. balansae* por fluido supercítico utilizando CO_2 como solvente (SFE). Esse extrato (SFE) apresentou um perfil lipofílico, com maiores quantidades de cumarinas com substituição epóxi. Em contraponto, em estudo publicado anteriormente demonstramos a obtenção de um extrato aquoso de *P. balansae* (AE) por maceração a quente, que apresentou um perfil de cumarinas mais hidrofílicas. Observando essas características, optamos por investigar esses dois tipos de extratos (SFE e AE), incorporando-os em nanoemulsões para aplicação tópica. As nanoemulsões foram obtidas por homogeneização à alta pressão conduzindo à obtenção de formulações monodispersas com diâmetro de gotícula e potencial zeta de cerca de 140 nm e -30 mV, respectivamente. Em etapa posterior, foi analisado o perfil de permeação/retenção das cumarinas incorporadas nas nanoemulsões. As cumarinas foram detectadas nas camadas da pele, especialmente quando essa foi lesionada, o que foi confirmado por microscopia confocal. As formulações foram testadas para a atividade antifúngica *in vitro* frente a cepas do fungo *S. schenckii* demonstrando que a incorporação de diferentes extratos de *P. balansae* (AE e SFE) em nanoemulsões conduz à redução da concentração inibitória mínima. O conjunto dos resultados demonstra que nanoemulsões são potenciais carreadores para extratos de *P. balansae* visando sua utilização tópica.

Palavras-chave: Cromatografia líquida de ultraeficiência; cumarinas; nanoemulsões; *Pterocaulon balansae*; *Sporothrix schenckii*.

ABSTRACT

Technological development of nanoemulsions containg standardized extract of *Pterocaulon balansae* aiming at antifungal activity

Pterocaulon balansae (Asteraceae) is a plant used in folk medicine for the treatment of various diseases. Recently, studies demonstrated antifungal activity of organic extracts of *P. balansae* against strains of the fungus *Sporothrix schenckii*. Such activity has been related to the presence of coumarins in these extracts. In this context, the objective of the present study was to develop topical nanoemulsions containing extracts of *P. balansae* for the treatment of infections caused by the *S. schenckii* fungus. In a first step, a review was carried out compiling important data about ethnopharmacology, chemical composition, and biological activities of the genus *Pterocaulon*. Phytochemical studies have shown that *Pterocaulon* species extracts are composed mainly of coumarins, also exhibiting flavonoids, phenolic acids, polyacetylenes, and terpenes. Most of the biological activities attributed to this genus were associated with the presence of coumarins. The biological effects corroborate the ethnopharmacological data that indicate these plants for use as anti-inflammatory, antioxidant and antifungal agents. Afterwards, the experimental part of this study was focused on the development and validation of methods for the quantification of the coumarins contained in *P. balansae* extracts through two chromatographic techniques (HPLC and UFLC). The methods are specific, linear, precise, robust and accurate for quantification of *P. balansae* coumarins. In addition, the method was validated for the quantification of coumarins in different analytical and bioanalytical matrices aiming at development and cutaneous permeation studies. In a second step, an extraction method of coumarins from *P. balansae* by supercritical fluid using CO_2 as solvent (SFE) was optimized. This extract (SFE) presented a lipophilic profile, with higher amounts of coumarins with epoxy substitution. In contrast, in a previously published study we demonstrated the obtaining of an aqueous extract of *P. balansae* (AE) by hot maceration, which had a profile of hydrophilic coumarins. Observing these characteristics, we chose to investigate these two types of extracts (SFE and AE), incorporating in nanoemulsions for topical application. Nanoemulsions were obtained by high-pressure homogenization leading to the production of monodisperse formulations with droplet diameter and zeta potential of about 140 nm and -30 mV, respectively. In a later stage, the permeation/retention profile of coumarins was analyzed when incorporated into the nanoemulsions. Coumarins were detected in the skin layers, especially when it was impaired, which was confirmed by confocal microscopy. The formulations were tested for *in vitro* antifungal activity against *S. schenckii* strains demonstrating that the incorporation of different extracts of *P. balansae* (AE and SFE) into nanoemulsions led to a reduction in the minimum inhibitory concentration. The set of results show that nanoemulsions are potential carriers of *P. balansae* extracts aiming at topical use.

Keywords: Ultra-fast liquid chromatography; coumarins; nanoemulsions; *Pterocaulon balansae*; *Sporothrix schenckii*.

LISTA DE ABREVIATURAS

5MMDC: *5-methoxy-6,7-methylenedioxycoumarin* – 5-metóxi-6,7-metilenodióxicumarina

ACN: *Acetonitrile*

ANOVA: Análise de Variância

BHT: *Butylated hydroxytoluene*

CIM: Concentração inibitória mínima

CFM: Concentração fungicida mínima

CLAE: Cromatografia Líquida de Alta Eficiência

CLUE: Cromatografia Líquida de Ultraeficiência

CT: Cumarinas Totais

DAD: Detector de Arranjo de Diodos

DMSO: *Dimethyl sulfoxide*

DPPH: *2,2-diphenyl-1-picrylhydrazyl*

EMA: *European Medicines Agency*

FDA: *Food and Drug Administration*

FRAP: *Ferric reducing antioxidant power*

HCMV: *Human cytomegalovirus*

HPLC: *High pressure liquid chromatography*

HSV-1-ACVr: *Herpes simplex virus type 1 resistant to acyclovir*

HSV-2-ACVr: *Herpes simplex virus type 2 resistant to acyclovir*

ICH: *International Conference on Harmonisation*

IC_{50}: *Half maximal inhibitory concentration*

LC: *Liquid chromatography*

LIPOID E80®: Lecitina de gema de ovo

LD_{50}: *Lethal dose*

LOD: *Detection limits*

LOQ: *Quantification limits*

MCT/TCM: *Medium chain triglycerides* - triglicerídeos de cadeia média

ME: *Matrix effect*

MIC: Minimum inhibitory concentration

MFC: Minimum fungicidal concentration

MOPS: *Morpholinepropansulfonic acid*

MTT: [(tetra-zolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide)]

NBD-PE: *(N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)-1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine, Triethylammonium Salt*

NBT: *Nitroblue tetrazolium*

NE: *Nanoemulsions* – nanoemulsões

NE_B: *Blank nanoemulsions* – nanoemulsões brancas

NPSH: *Non-protein thiol*

O/W: *Oil in water* – óleo em água

P.E/D: *Porcine epidermis/dermis*/epiderme/derme de pele de orelha suína

PDA: *Photodiode array detection* - detector de arranjo de diodos

PSC: *Porcine stratum corneum layer after tape stripping method*

RF: *Receptor fluid* – fluído receptor

RPMI-1640: *Roswell Park Memorial Institute 1640 broth medium*

RRV: *Ross river vírus*

RSD: *Relative standard deviation*

RSM/MSR: *Response surface methodology* – metodologia de superfície de resposta

SPE: *Supercritical fluid extraction*

TBARS: *Thiobarbituric acid reactive substances*

TC: *Total coumarins*

TRAP: *Total radical trapping antioxidant*

TWEEN 80®: Polissorbato 80

UFLC: *Ultra-Fast Liquid Chromatography*

UPLC-UV-MS: *Ultra Performance Liquid Chromatography – accopled Ultraviolet and mass*

UV: *Ultraviolet* - ultravioleta

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SUMÁRIO

Introdução

A alta incidência de infecções fúngicas invasivas representa uma importante causa de morbidade e mortalidade, estando associada a pelo menos 1,5 milhão de mortes em todo o mundo a cada ano. Os pacientes de grupos especiais, como os gravemente enfermos e imunodeprimidos (ex: politraumatizados, portadores da síndrome da imunodeficiência adquirida ou em tratamento com quimioterápicos), são mais suscetíveis a desenvolver infecções oportunistas por leveduras e fungos filamentosos, o que agrava consideravelmente seu quadro clínico. O tratamento das infecções fúngicas, de modo geral, tem se mostrado limitado devido ao número reduzido de agentes antifúngicos disponíveis e principalmente devido à resistência microbiana adquirida a esses fármacos (BARKER; ROGERS, 2006; CAMPOY; ADRIO, 2017).

Sporothrix schenckii é o agente etiológico da esporotricose, uma micose adquirida pela inoculação de material contaminado do solo, plantas e material orgânico que pode afetar tanto humanos quanto animais causando lesões usualmente limitadas à pele, tecidos subcutâneos e circundantes aos vasos linfáticos. A esporotricose é uma doença predominante em zonas tropicais e temperadas e, recentemente, uma revisão sobre esta doença apresentou o Brasil como o país que tem o maior número de casos relatados em humanos, como também é o país que mais apresenta estudos de pesquisa na área (BARROS; DE ALMEIDA PAES; SCHUBACH, 2011). Especificamente no Estado do Rio Grande do Sul esta é a micose subcutânea mais comum (DA ROSA et al., 2005)

Neste cenário, as plantas medicinais surgem como fonte para novas moléculas, sendo alvo de estudos com extratos, frações e/ou compostos isolados que visam à identificação de potenciais agentes terapêuticos (NEWMAN; CRAGG, 2012, 2016). Nesse contexto uma planta da Família Asteraceae conhecida como "quitoco" (*Pterocaulon balansae)* tem sido bastante estudada, em função de sua alta concentração de cumarinas, compostos aos quais são atribuídas diversas ações farmacológicas (PANATIERI et al., 2017; STEIN et al., 2005, 2006; STOPIGLIA et al., 2011; VIANNA et al., 2012).

Recentemente, um estudo desenvolvido em nosso grupo de pesquisa em parceria com o Laboratório de Fungos Patogênicos (UFRGS), avaliou uma série de extratos metanólicos obtidos a partir de diferentes espécies de *Pterocaulon,* dentre elas *P. balansae,* a fim de obter informações referentes à concentração inibitória mínima (CIM) e à concentração fungicida mínima (CFM) desses extratos frente a cepas do fungo *Sporothrix schenckii*. Todas as cepas testadas apresentaram inibição frente aos diferentes extratos com valores de CIM entre 156 a 1250 µg/mL e CFM entre 312 a 5000 µg/mL. Esses resultados reafirmam a importância de estudos na área da etnofarmacologia como fonte promissora de novos agentes terapêuticos (STOPIGLIA et al., 2011).

Estudos desenvolvidos com a espécie *P. balansae* exploram a extração dos seus compostos ativos por meio de solventes orgânicos (hexano, diclorometano e metanol), empregando métodos de extração em Soxhelt, maceração estática e imersão (PANATIERI et al., 2017; STEIN et al., 2005, 2006; STOPIGLIA et al., 2011; VIANNA et al., 2012). Atualmente, métodos que empregam tecnologias verdes para extração de produtos naturais encontram-se em destaque, pois baseiam-se na descoberta de processos que reduzem o consumo de energia gerando menos resíduos, permitindo o uso de solventes alternativos garantindo a qualidade do produto final (DE MELO; SILVESTRE; SILVA, 2014; JANGHEL et al., 2015; PEREIRA; MEIRELES, 2010). As técnicas e matérias-primas empregadas variam bastante, havendo destaque para os solventes como água, CO_2, líquidos iônicos e métodos como extração por fluido supercrítico, destilação a vapor, destilação por micro-ondas, dentre outros.

Recentemente, nosso grupo de pesquisa demonstrou a obtenção de cumarinas de *P. balansae* utilizando como solvente a água num processo de maceração dinâmica com temperatura (MEDEIROS-NEVES et al., 2015). A viabilidade de uma tecnologia verde para obtenção de um extrato rico em cumarinas se mostrou promissor para obtenção de extratos e/ou frações de *P. balansae*, sendo uma alternativa aos métodos que anteriormente empregavam solventes orgânicos.

Diversos métodos cromatográficos quantitativos podem ser empregados para a análise de teores de ativos em um extrato. Recentemente, descrevemos um método analítico por CLAE a fim de quantificar as cumarinas presentes no extrato aquoso obtido da espécie *P. balansae* (MEDEIROS-NEVES et al., 2015). No entanto, métodos empregando técnicas mais modernas, tais como CLUE também vêm sendo relatados para análise de cumarinas (LI et al., 2014; ZHANG; WEI; YANG, 2017). Tais técnicas são vantajosas, pois suportam altas pressões, possibilitam o uso de colunas cromatográficas com diâmetro interno reduzido (< 2 µm), aumentam a resolução dos picos e diminuem tanto o tempo de corrida cromatográfica como o consumo dos reagentes químicos (MALDANER; JARDIM, 2009).

As nanoemulsões têm sido descritas para incorporação de extratos vegetais de forma a viabilizar sua aplicação, melhorando características como, por exemplo, a maior penetração desses compostos nas camadas da pele. Geralmente constituídas de um núcleo oleoso (de origem natural ou semi-sintética), as nanoemulsões são estabilizadas por uma mistura de fosfolipídeos, que atuam na interface entre óleo e água (JAISWAL; DUDHE; SHARMA, 2015; SINGH et al., 2017).

Com base no supramencionado, a presente tese de doutorado visou o desenvolvimento de uma metodologia bioanalítica para quantificação de cumarinas de *P. balansae* em diferentes materizes biológicas. Além disso, objetiva a otimização de um extrato de *P. balansae* utilizando fluido supercítico, bem como o desenvolvimento de um produto nanoemulsionado contendo extrato aquoso e fluido supercítico de *P. balansae* para o tratamento tópico de infecções fúngicas causadas por *Sporothrix schenckii*.

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VIANNA, D. R. et al. Selective cytotoxicity and apoptosis induction in glioma cell lines by 5-oxygenated-6,7-methylenedioxycoumarins from *Pterocaulon* species. **European Journal of Medicinal Chemistry**, v. 57, p. 268–274, 2012.

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Objetivos

Objetivo geral

Desenvolver nanoemulsões contendo extrato aquoso e extrato supercrítico a partir de *P. balansae*, visando o tratamento tópico da infecção pelo fungo *Sporothrix schenkii*.

O objetivo geral pode ser dividido nos seguintes objetivos específicos:

(i) Validação de métodos analítico e bioanalítico por cromatografia líquida de alta e ultra eficiência para avaliação das cumarinas presentes no extrato aquoso, bem como da cumarina 5MMDC nas formulações e na pele;

(ii) Otimização de um extrato rico em cumarinas a partir das partes aéreas de *P. balansae*, através da extração por fluido supercítico, utilizando CO_2 como solvente;

(iii) Desenvolvimento e caracterização físico-química de nanoemulsões de uso tópico contendo extrato aquoso e por fluido supercrítico de *P. balansae*;

(iv) Avaliação *in vitro* do perfil de permeação cutânea, em células de difusão de Franz, das cumarinas dos extratos aquoso e fluido supercrítico a partir das formulações preparadas;

(v) Avaliação *in vitro* da atividade antifúngica das formulações frente a cepas do fungo *Sporothrix schenkii*.

Capítulo I

Revisão da literatura

1.1. INTRODUÇÃO

O estudo desta tese tem como base a compreensão, pesquisa e desenvolvimento da espécie *P. balansae.* Esta espécie, pertencente à Família Asteraceae, está inserida no gênero *Pterocaulon*, o qual vem sendo investigado pelo nosso grupo de pesquisa nos últimos anos. O interesse em se trabalhar com esse gênero se deu por meio de relatos do uso popular dessas plantas para o tratamento de diversas afecções, em geral utilizadas através do preparo de decoctos e infusões.

A literatura reporta diversas atividades, tais como: digestiva (GOLENIOWSKI et al., 2008), antisséptica (SMITH, 1991), emenagoga (GARLET et al., 2001), antifúngica (SMITH, 1991; AVANCINI et al., 2008), antipirética (RASONAIVO et al., 1992; LANGUEFOSSE et al., 1996), para doenças do fígado (GARLET et al., 2001; GOLENIOWSKI et al., 2008), artrites (ZARDINI, 1984), picadas de serpentes (FILIPOV, 1994), inseticida (ZARDINI, 1984; SMITH, 1991), pesticida (GOLENIOWSKI et al., 2008), e também como aromática (ZARDINI, 1984).

Embora seja de amplo uso popular, esse gênero carece de estudos, com poucos grupos de pesquisa engajados na investigação dessas espécies. Os trabalhos publicados relatam, na grande maioria, a obtenção de extratos e óleos voláteis, a identificação de compostos químicos e atividades biológicas relacionadas.

Neste sentido, para compreender melhor o tema exposto, este capítulo será apresentado na forma de artigo de revisão, redigido nas normas do periódico ao qual foi publicado: *Journal of Etnhopharmacology*.

1.2. ARTIGO

Artigo publicado na revista Journal of Ethnopharmacology:

The genus *Pterocaulon* (Asteraceae) – A review on traditional medicinal uses, chemical constituents and biological properties

The genus *Pterocaulon* (Asteraceae) – A review on traditional medicinal uses, chemical constituents and biological properties

Bruna Medeiros-Neves[a], Helder Ferreira Teixeira[a], Gilsane Lino von Poser[a*]

[a]*Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal do Rio Grande do Sul, Avenida Ipiranga, 2752, Porto Alegre/RS, Brazil.*

Corresponding author: Gilsane Lino von Poser (gilsane@farmacia.ufrgs.br) Address: Av. Ipiranga, 2752, Porto Alegre/RS – Brazil - Fone: 55 51 33085243

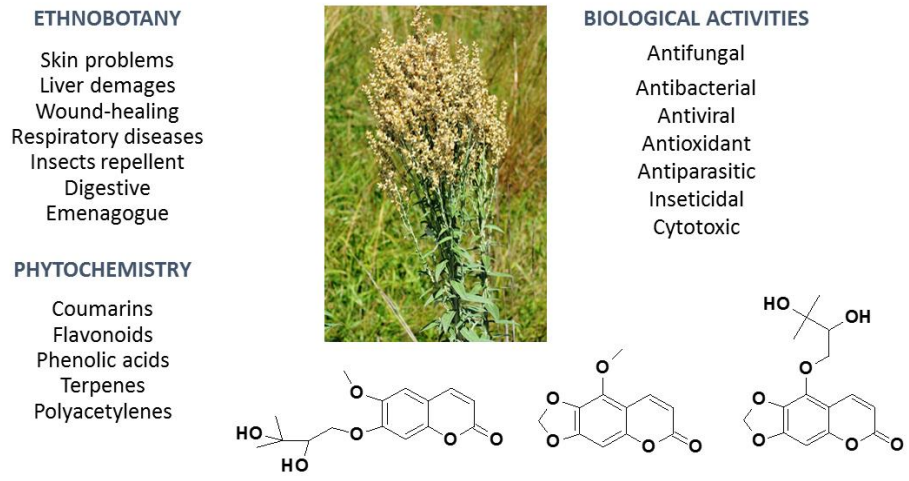
ABSTRACT

Ethnopharmacological relevance: Species of the genus *Pterocaulon* (Asteraceae) are used in different parts of the world for mainly to treat skin and liver diseases, as well as disorders of the respiratory system, among others.
Aim of the study: This review aims to discuss the present state of the art concerning the ethnobotanical uses, secondary metabolites and biological effects of *Pterocaulon* species and their chemical components.
Materials and methods: The available information on the genus *Pterocaulon* was gathered from scientific databases (Web of Science, Pubmed, ScienceDirect, Scopus, ChemSpider, SciFinder ACS Publications, Wiley Online Library). Information was also obtained from local publications, M.Sc. and Ph.D. dissertations. All studies on the ethnobotany, phytochemistry, pharmacology and toxicology of the plants until December 2017 were included in this review.
Results: Approximately 40 coumarins and 30 flavonoids have been isolated from *Pterocaulon* species. Coumarins have been considered the chemotaxonomic markers in the genus and the most active components. Pharmacological studies carried out with extracts and isolated compounds revealed in vitro bioactivities that include antifungal, antiviral, and cytotoxicity. Most of the pharmacological investigations were not correlated with traditional uses of the plants.
Conclusions: Pterocaulon species, a rich source of coumarins, have great ethnomedical potential. Nevertheless, further studies into the pharmacological activities are necessary since none of the purported effects of these plants was fully assessed. In-depth research regarding the toxicity are also required to ensure the safety of these medicinal plants.

Keywords: *Pterocaulon*, Asteraceae, coumarins, flavonoids

Contents

Graphical abtract



1. Introduction

The genus *Pterocaulon* encompasses several species used traditionally for the treatment of skin diseases, hepatic disorders, respiratory illnesses and many other conditions. The plants are usually used as infusions and decoctions. Coumarins are abundant and widely distributed, but the plants also present flavonoids, terpenes and polyacetylenes. Both extracts and compounds isolated from these species have been tested *in vitro* for antiviral, antiparasitic, antifungal and insecticidal activities, among others. In this sense, the aim of this review is to provide, in a comprehensive way, a reference and subsidies for future investigations, and to highlight the role of the *Pterocaulon* species in the ethnobotanical and ethnopharmacological fields as well as their importance as a rich source of coumarins and other natural compounds.

2. Occurrence and botanical description

Synonyms for scientific names of *Pterocaulon* species are reported in Table 1 (The Plant List, 2013). The genus *Pterocaulon* belongs to the family Asteraceae (Compositae), one of the largest among floriferous plants, corresponding to approximately 10% of the Angiosperms. The family comprises about 1700 genera and approximately 25000 species distributed all over the world, except for Antarctica. Asteraceae is a cosmopolitan family, with great concentration of species in subtropical, cold-temperate and temperate regions. The members of the family are customarily distributed in three subfamilies: Barnadesioideae, Cichorioideae and Asteroidae. Some taxonomic revision, dividing the family into a larger number of subfamilies, have been proposed by the Angiosperm Phylogeny Group (APG. Angiosperm Phylogeny Website. Version 14, n.d.) maintaining Asteroidae with more representatives than any other subfamily (approximately 17000 species distributed in 1135 genera). According to some authors, the subfamily is divided into 10 tribes and 57 subtribes. The genus *Pterocaulon*, which

belongs to Asteroidae, is included in the subtribe Plucheinae (Barroso, 1986; Bean, 2011; Bremer, 1994; Funk et al., 1994; Joly, 1991; Lundberg and Bremer, 2003).

The genus *Pterocaulon* Elliot. (1823) comprises 26 species, 12 of which are American (*P. alopecuroides* (Lam.) DC.; *P. angustifolium* DC.; *P. balansae* Chodat; *P. cordobense* Kuntze; *P. lanatum* Kuntze; *P. lorentzii* Malme; *P. polypterum* (DC.) Cabrera; *P. polystachyum* DC.; *P. purpurascens* Malme; *P. pycnostachyum* (Michx.) Elliott.; *P. rugosum* (Vahl) Malme; *P. virgatum* (L.) DC. Among these, only *P. pycnostachyum* do not occur in Brazil. The other 14 species are distributed in other regions of the planet (*P. brachyanthum* A. R. Bean; *P. ciliosum* A. R. Bean; *P. discolor* A. R. Bean; *P. globuliflorus* W. Fitzg; *P. intermedium* (DC.) A. R. Bean; *P. niveum* Cabrera & Ragonese; *P. paradoxum* A. R. Bean; *P. redolens* G. Forst. ex Willd.) Benth. ex Fern. -Vill.; *P. serrulatum* (Montrouz.) Guillaumin; *P. sphacelatum* Benth (Labill.) F. Muell.; *P. sphaeranthoides* (DC.) F. Muell.; *P. tricholobum* A. R. Bean; *P. verbascifolium* (F. Muell. ex Benth.) Benth. & Hook. ex F. Muell. and *P. xenicum* A. R. Bean (Bean, 2011; Heemann et al., 2004; Lima and Matzenbacher, 2008).

The word *Pterocaulon* originates from the Greek "pteros" (wings) and "caulon" (stem) and refers to the winged stem formed from the decurrent leaves, an important morphologic characteristic of the species. The width of the stems is a useful characteristic to distinguish the *Pterocaulon* species. The cauline leaves can be obovate, oblanceolate or elliptical. The upper leaf surface of most species is bullate to a greater or lesser extent. The leaf margins are always dentate to denticulate. Lanate hairs (uniseriate, multicellular) are found in all species, but their density varies considerably. The secondary heads are generally grouped to form a globose or ellipsoidal capitulescence but, in some species, it can be spicate or cylindrical. Capitulescences are mainly sessile but can be consistently pedunculate. The species flourish from May to September and from December to March in the northern and southern hemispheres, respectively (Bean, 2011; Cabrera and Ragonese, 1978; Lima and Matzenbacher, 2008).

Table 1. Scientific names and synonym(s) of reported *Pterocaulon* species (according to The Plant List (2013))

***Pterocaulon* species**	**Synonym(s) and subspecies**
***Pterocaulon alopecuroides* (Lam.) DC.**	*Baccharis erioptera* Benth.; *Chlaenobolus alopecuroides* (Lam.) Cass; *Conyza alopecuroides* Lam.; *Pterocaulon alopecuroides* var. *alopecuroides*; *Pterocaulon alopecuroides* var. *glabrescens* Chodat; *Pterocaulon alopecuroides* var. *polystachyum* DC.; *Pterocaulon alopecuroides* var. *salicifolium* Chodat; *Pterocaulon interruptum* DC.; *Pterocaulon interruptum* var. *interruptum*; *Pterocaulon interruptum* var. *monostachyum* DC.; *Pterocaulon interruptum* var. *polystachyum* DC.; *Pterocaulon latifolium* Kuntze; *Pterocaulon virgatum* f. *alopecuroides* (Lam.) Arechav.; *Pterocaulon virgatum* var. *alopecuroides* (Lam.) Griseb.; *Pterocaulon virgatum* f. *subcorymbosa* Arechav.
***Pterocaulon angustifolium* DC.**	
***Pterocaulon balansae* Chodat**	*Pterocaulon interruptum* auct. non DC.; *Pterocaulon paniculatum* Arechav.; *Pterocaulon polystachyum* var. *tomentosa* Arechav.; *Pterocaulon virgatum* f. *subpaniculata* Arechav.
***Pterocaulon brachyanthum* A. R. Bean**	
***Pterocaulon ciliosum* A. R. Bean**	
***Pterocaulon cordobense* Kuntze**	
***Pterocaulon discolor* A. R. Bean**	
***Pterocaulon globuliflorus* W. Fitzg.**	
***Pterocaulon intermedium* (DC.) A. R. Bean**	
***Pterocaulon lanatum* Kuntze**	*Pterocaulon hassleri* Chodat
***Pterocaulon lorentzii* Malme**	*Pterocaulon malmeanum* Chodat; *Pterocaulon virgatum* f. *angustifolia* Arechav.; *Pterocaulon virgatum* f. *spicata* Arechav.
***Pterocaulon niveum* Cabrera & Ragonese**	
***Pterocaulon paradoxum* A. R. Bean**	
***Pterocaulon polypterum* (DC.) Cabrera**	*Baccharis polyptera* DC.; *Pterocaulon bakeri* Malme; *Vernonia pterocaulon* Baker
***Pterocaulon polystachyum* DC.**	*Pterocaulon polystachyum* var. *polystachyum*
***Pterocaulon purpurascens* Malme**	
***Pterocaulon pycnostachyum* (Michx.) Elliott**	*Chlaenobolus undulatus* Small; *Conyza pycnostachya* Michx.

***Pterocaulon redolens* (G.Forst. ex Willd.) Benth. ex Fern.-Vill.**	*Chlaenobolus spicatus* (Labill.) Cass.; *Conyza redolens* G.Forst. ex Willd.; *Gnaphalium cylindrostachyum* Wall.; *Monenteles redolens* (G.Forst. ex Willd.) Labill.; *Monenteles redolens* (Willd.) DC.; *Monenteles spicatus* Labill.; *Pterocaulon billardierei* F.Muell.; *Pterocaulon cylindrostachyum* C.B.Clarke; *Pterocaulon spicatum* (Labill.) Domin; *Sphaeranthus elongatus* Blanco; *Tessaria redolens* (Willd.) Less.
***Pterocaulon rugosum* (Vahl) Malme**	*Chlaenobolus rugosus* (Vahl) Cass.; *Pterocaulon alopecuroides* var. *mollis* Chodat
***Pterocaulon serrulatum* (Montrouz.) Guillaumin**	*Monenteles glandulosus* F.Muell. ex Benth.; *Monenteles serrulatus* Montrouz.; *Pterocaulon glandulosum* (F.Muell. ex Benth.) Benth. & Hook.f. ex F.Muell.; *Pterocaulon glandulosum* var. *glandulosum*; *Pterocaulon glandulosum* var. *velutineum* Ewart & O.B.Davies; *Pterocaulon serrulatum* var. *serrulatum*
***Pterocaulon sphacelatum* Benth (Labill.) F.Muell.**	*Monenteles sphacelatus* Labill.; *Monenteles tomentosus* Sch.Bip. ex Miq.; *Pterocaulon tomentosus* (Sch.Bip. ex Miq.) Boerl.; *Sphaeranthus erectus* Zoll. & Mor.
***Pterocaulon sphaeranthoides* (DC.) F.Muell.**	
***Pterocaulon spicatum* (Cass.) DC.**	*Pterocaulon spicatum* var. *spicatum*
***Pterocaulon spicatum* var. *brachystachyum* DC.**	*Chlaenobolus rugosa* (Vahl) Cass.
***Pterocaulon tricholobum* A. R. Bean**	
***Pterocaulon verbascifolium* (F.Muell. ex Benth.) Benth. & Hook. ex F.Muell.**	
***Pterocaulon virgatum* (L.) DC.**	*Baccharis virgata* DC.; *Chlaenobolus virgata* (L.) Cass.; *Chlaenobolus virgatus* (L.) Cass.; *Conyza virgata* (L.) L.; *Conyza virgata* var. *virgata*; *Gnaphalium decurrens* Schrank; *Gnaphalium spicatum* Mill.; *Gnaphalium spicatum* f. *spicatum*; *Gnaphalium virgatum* L.; *Pterocaulon pilcomayense* Malme; *Pterocaulon pompilianum* Standl. & L.O.Williams; *Pterocaulon subspicatum* Malme ex Chodat; *Pterocaulon virgatum* f. *subvirgata* (Malme) Arechav.; *Pterocaulon virgatum* f. *virgatum*; Pterocaulon virgatum var. virgatum
***Pterocaulon xenicum* A. R. Bean**	

3. Phytochemistry

Phytochemical studies carried out with *Pterocaulon* species report the presence of a number of chemical constituents, including coumarins, phenolic acids, flavonoids, terpenes, and polyacetylenes. Coumarins are very abundant and considered the main active substances of the genus.

3.1. Coumarins

Coumarins are considered the most active constituents of the *Pterocaulon* species and a total of 41 different compounds have already been isolated from these plants (**Table 2**). To some compounds, trivial names were attributed while many others were reported by the chemical names. The first study reporting the presence of coumarins in the genus dates from 1968, with the isolation of **1** from *P. sphacelatum* (Johns et al., 1968). Subsequently, in 2011, it was verified that the species investigated by Jonh and coworkers was, in fact, *P. intermedium* (Bean, 2011). From *P. sphacelatum*, the compound **5** was isolated (Semple et al., 1999).

The compounds 7-(3-methyl-2-butenyloxy)-5,6-methylenedioxycoumarin, 7-(2',3'-dihydroxy-3-methylbutyloxy)-5,6-methylenedioxycoumarin (the structure of these two compounds was reviewed in a later study (Debenedetti et al.,1997), and it was verified that the positions of the substituents were not adequately assigned) and **13** were found in the aerial parts of *P. balansae* and *P. lanatum.* Other coumarins were isolated from these species: **13**, **18** and **32** from *P. balansae,* and **13**, **15** and **20,** from *P. lanatum* (Magalhães et al., 1981).

Later, the coumarin **25** was found in *P. balansae* (published as *P. interruptum*) (Heemann et al., 2006) and more recently, our research group reported the presence of seven coumarins in the aerial parts of this plant, two of them, **14** and **19**, unpublished for the species, being the latter identified as a new compound (Medeiros-Neves et al., 2015). It is worth mentioning that these seven coumarins were obtained from the

aqueous extract, with the compound **14** being the major components. In the sequence, our research group showed that when using supercritical CO_2, only the coumarins **9**, **18**, **19** and **31** were obtained in a significant amount. The other three compounds appeared as traces (Torres et al., 2017). The chemical composition of both extracts was somewhat divergent from another study using chloroform as solvent, in which coumarin **13** was the most abundant (Barata-Vallejo, 2010).The explanation for this result is that the epoxy-substituted coumarins **18**, **19** and **31** may yield their more polar analogues (**13**, **14** and **25**, respectively) via hydrolysis of the epoxy ring. This conversion is probably due to the solvent and the extraction method used.

The first reports of coumarins from *P. virgatum* are from 1981, with the isolation of the compounds **18** (Bohlmann et al., 1981), **25** and **26** (Debenedetti et al., 1981). In 1994, the isolation of the coumarins **9** and **32** from the aerial parts of *P. virgatum* was reported (Debenedetti et al., 1994a). The latter is one of the compounds isolated from *P. balansae*, cited above, whose structure was not correctly elucidated (Magalhães et al., 1981). *Pterocaulon virgatum* also showed the presence of **3**, **6**, **13**, **27**, **34**, **38** (Debenedetti et al., 1998), **37** (Debenedetti et al., 1999), **10**, **35**, **37**, and **39** (Maes et al., 2006).

The compounds **3**, **9** and **5** were found in *P. serrulatum* (Macleod and Rasmussen, 1999) while *P. purpurascens* afforded **41** and purpurasol (Debenedetti et al., 1992, 1991). Two years later, the same research group (Boeykens et al., 1994) revised the structure of the compound previously published with the name of purpurasol and concluded, based on spectroscopic data, that this compound presents the same structure of the coumarin obtusifol (**40**) previously isolated from *Haplophyllum obtusifolium* (Rutaceae). From the same plant, they also obtained **2** and **36** (Debenedetti et al., 1996).

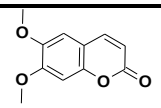
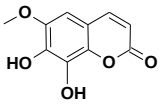
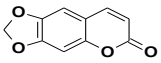
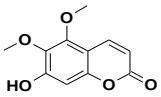
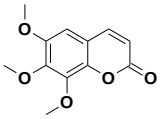
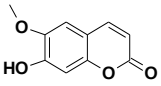
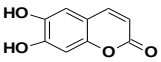
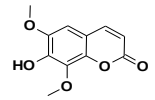
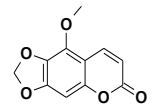
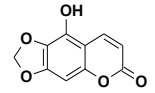
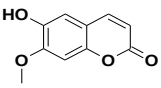
In 1995, the coumarins **13** and **24** were isolated from *P. alopecuroides* (Vilegas et al., 1995). These compounds were previously obtained from *P. balansae, P. lanatum, P. virgatum* and *P. rugosum.* Some authors, analyzing exudates, obtained **9** and **18** from *P. alopecuroides* and **3**, **9**, **16**, and **17** from *P. polystachyum* (Stein et al., 2007). These compounds had already been isolated from *P. polystachyum* together with **6**, **7**, **13**, **12**,

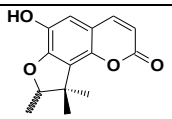
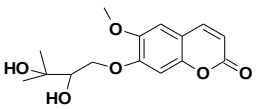
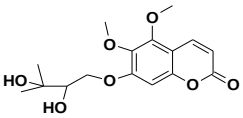
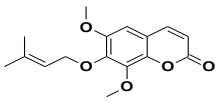
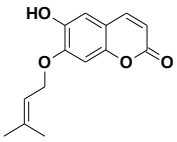
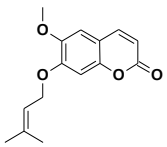
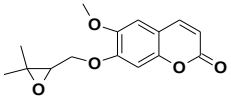
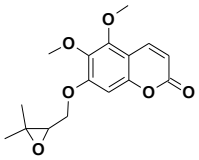
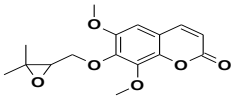
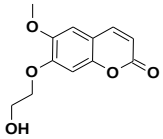
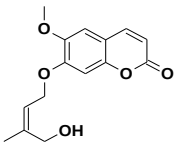
11, **21**, **22**, **25**, **26**, **27**, **28**, **29**, **32**, **33**, **38**, 6-(1,1-dimethyl-2-propenyl)-7-hydroxycoumarin, and 5-hydroxy-6,7-methylenedioxy-8-(3,3-dimethylallyl)coumarin (Palacios et al., 1999; Vera et al., 2001).

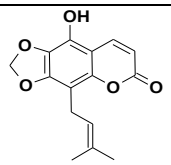
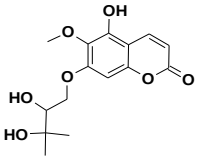
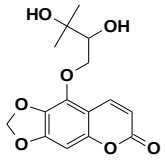
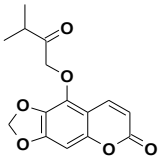
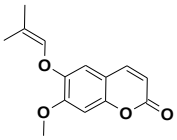
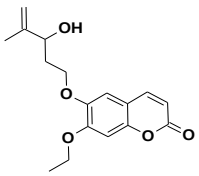
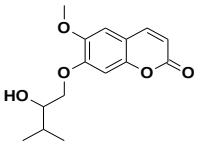
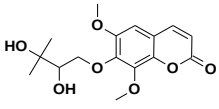
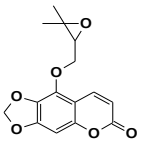
Besides the coumarins above-cited, *P. alopecuroides* afforded the compound **13** and a compound considered by the authors as an artefact (7-(2,2,5,5-tetramethyl-1,3-dioxolan-4-yl)methoxy-6-methoxycoumarin) (Alarcón et al., 2010). The compounds **3**, **4**, **8**, **9**, **15**, **25** and **30** were also isolated from the aerial parts of *P. redolens* (Kanlayavattanakul et al., 2003). In the first report of coumarins from *P. lorentzii*, the compound **25** was characterized by chromatographic methods (Barata-Vallejo, 2010). Later, this coumarin was isolated from the plant as the main compound (Vianna et al., 2012; Miyazaki et al., 2013).

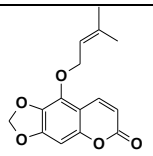
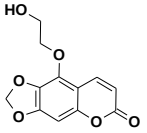
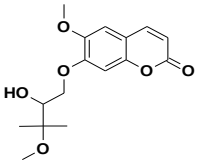
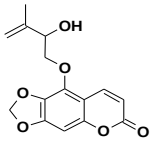
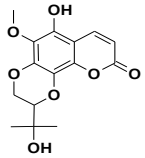
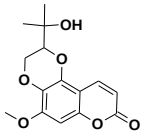
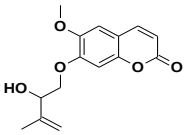
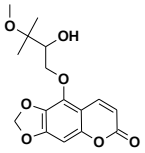
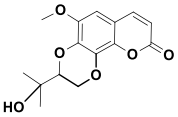
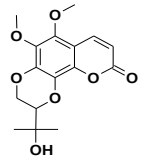
In Asteraceae subtribe Plucheinae, *Pterocaulon* is the only genus known to contain coumarins, and more than 40 different compounds of this class have been found. The compounds are 6,7 dioxygenated (16 coumarins), 5,6,7 trioxygenated coumarins (14 coumarins), 6,7,8 trioxygenated coumarins (eight coumarins) and 5,6,7,8 tetraoxygenated coumarins (three coumarins). Although the species of *Pterocaulon* exhibit other classes of compounds, as it will be shown subsequently, coumarins are characteristic and could be considered chemotaxonomic markers in the genus.

Table 2. Coumarins from *Pterocaulon* species.

	Compound	Structure	Source	Ref
1	6,7-dimethoxycoumarin (Scoparone)		*P. intermedium*	(Johns et al., 1968)
2	7,8-dihydroxy-6-methoxycoumarin (Fraxetin)		*P. purpurascens*	(Debenedetti et al., 1996)
3	6,7-methylenedioxycoumarin (Ayapin)		*P. virgatum*	(Debenedetti et al., 1998)
			P. redolens	(Kanlayavattanakul et al., 2003)
			P. polystachyum	(Palacios et al., 1999; Stein et al., 2007; Vera et al., 2001)
			P. serrulatum	(Macleod and Rasmussen, 1999)
4	5-methoxyscopoletin		*P. redolens*	(Kanlayavattanakul et al., 2003)
5	6,7,8-trimethoxycoumarin		*P. serrulatum*	(Macleod and Rasmussen, 1999)
			P. spachelatum	(Semple et al., 1999)
6	6-methoxy-7-hydroxycoumarin (Scopoletin)		*P. virgatum*	(Debenedetti et al., 1998)
			P. polystachyum	(Palacios et al., 1999)
7	6,7-dihydroxycoumarin (Esculetin)		*P. polystachyum*	(Palacios et al., 1999)
8	6,8-dimethoxy-7-hydroxycoumarin (Isofraxidin)		*P. redolens*	(Kanlayavattanakul et al., 2003)
9	5-methoxy-6,7-methylenedioxycoumarin		*P. virgatum*	(Debenedetti et al., 1994a)
			P. polystachyum	(Palacios et al., 1999; Stein et al., 2007; Vera et al., 2001)
			P. redolens	(Kanlayavattanakul et al., 2003)
			P. alopecuroides	(Stein et al., 2007)
			P. serrulatum	(Macleod and Rasmussen, 1999)
			P. balansae	(Medeiros-Neves et al., 2015)
10	5-hydroxy-6,7-methylenedioxycoumarin		*P. virgatum*	(Maes et al., 2006)
11	7-methoxy-6-hydroxycoumarin (Isoescopoletin)		*P. polystachyum*	(Vera et al., 2001)

12	Demethylnieshoutin		*P. polystachyum*	(Vera et al., 2001)
13	7-(2',3'-dihydroxy-3'-methylbutyloxy)-6-methoxycoumarin (Obtusinin)		*P. balansae*	(Magalhães et al., 1981; Medeiros-Neves et al., 2015)
			P. lanatum	(Magalhães et al., 1981)
			P. alopecuroides	(Vilegas et al., 1995)
			P. virgatum	(Debenedetti et al., 1998)
			P. polystachyum	(Vera et al., 2001)
14	5,6-dimethoxy-7-(3'-methyl-2',3'-dihydroxybutyloxy)coumarin		*P. balansae*	(Medeiros-Neves et al., 2015)
15	6,8-dimethoxy-7-prenyloxycoumarin (Puberulin)		*P. lanatum*	(Magalhães et al., 1981)
			P. redolens	(Kanlayavattanakul et al., 2003)
16	6-hydroxy-7-(3-methylbut-2-enoxy)coumarin (Prenyletin)		*P. polystachyum*	(Palacios et al., 1999; Stein et al., 2007; Vera et al., 2001)
17	Prenyletin methyl eter		*P. virgatum*	(Debenedetti et al., 1998)
			P. polystachyum	(Palacios et al., 1999; Stein et al., 2007; Vera et al., 2001)
18	7-(2',3'-epoxy-3'-methylbutyloxy) 6-methoxycoumarin		*P. balansae*	(Magalhães et al., 1981; Medeiros-Neves et al., 2015)
			P. virgatum	(Bohlmann et al., 1981; Maes et al., 2006)
19	5,6-dimethoxy-7-(2′,3′-epoxy-3′-methylbutyloxy)coumarin		*P. balansae*	(Medeiros-Neves et al., 2015)
20	2',3'-epoxypuberulin		*P. lanatum*	(Magalhães et al., 1981)
21	6-methoxy-7-(2'-hydroxyethoxy)-coumarin		*P. polystachyum*	(Vera et al., 2001)
22	(Haplopinol methyl ether)		*P. polystachyum*	(Vera et al., 2001)

23	5-hydroxy-8-(3',3'-dimethylallyl)-6,7-methylenedioxycoumarin		*P. polystachyum*	(Vera et al., 2001)
24	7-(2',3'-dihydroxy-3'-methylbutyloxy)-5-hydroxy-6-methoxycoumarin		*P. alopecuroides*	(Vilegas et al., 1995)
25	5-(2',3'-dihydroxy-3'-methylbutyloxy)-6,7-methylenedioxycoumarin (Sabandinol)		*P. balansae* *P. lanatum* *P. virgatum* *P. polystachyum* *P. redolens* *P. alopecuroides* *P. lorentzii*	(Heemann et al., 2006; Magalhães et al., 1981; Medeiros-Neves et al., 2015) (Magalhães et al. 1981) (Debenedetti et al., 1981) (Vera et al., 2001) (Kanlayavattanakul et al., 2003) (Miyazaki et al., 2013) (Vianna et al., 2012; Miyazaki et al., 2013)
26	5-(3-methyl-2-butenyloxy)-6,7-methylenedioxycoumarin (Sabandinone)		*P. virgatum* *P. polystachyum*	(Debenedetti et al., 1981) (Palacios et al., 1999)
27	7-(3-methyl-2-butenyloxy)-6-methoxycoumarin		*P. polystachyum* *P. virgatum*	(Palacios et al., 1999) (Debenedetti et al., 1998)
28	6-(2-hydroxy-3-methyl-3-butenyloxy)-7-methoxycoumarin (Isovirgatenol)		*P. polystachyum*	(Vera et al., 2001)
29	6-methoxy-7-(2-hydroxy-3-methylbutyloxy)coumarin (3'-deoxyobtusinin)		*P. polystachyum*	(Macleod and Rasmussen, 1999)
30	2',3'-dihydroxypuberulin		*P. redolens*	(Kanlayavattanakul et al., 2003)
31	5-(2′,3′-epoxy-3′-methylbutyloxy)-6,7-methylenedioxycoumarin		*P. balansae* *P. lanatum*	(Magalhães et al. 1981; Medeiros-Neves et al. 2015) (Magalhães et al. 1981)

32	5-(3'-methyl-2'-butenyloxy)-6,7-methylenedioxycoumarin		*P. balansae* *P. virgatum* *P. polystachyum*	(Magalhães et al. 1981) (Debenedetti et al., 1994a) (Palacios et al., 1999; Vera et al., 2001)
33	5-(2'-hydroxyethoxy)-6,7-methylenedioxycoumarin (5-hydroxyethoxyayapin)		*P. polystachyum*	(Macleod and Rasmussen, 1999; Palacios et al., 1999; Vera et al., 2001)
34	7-(2-hydroxy-3-methoxy-3-methylbutoxy)-6-methoxycoumarin (Virgatol)		*P. virgatum*	(Debenedetti et al., 1998)
35	5-(2-hydroxy-3-methyl-3-butenyloxy)-6,7-methylenedioxycoumarin		*P. virgatum*	(Maes et al., 2006)
36	Purpurasolol		*P. purpurascens*	(Debenedetti et al., 1996)
37	Isopurpurasol		*P. virgatum*	(Maes et al., 2006; Debenedetti et al., 1999)
38	7-(2-hydroxy-3-methylbut-3-enoxy)-6-methoxycoumarin (Virgatenol)		*P. virgatum* *P. polystachyum*	(Debenedetti et al., 1998) (Palacios et al., 1999; Vera et al., 2001)
39	5-(2-hydroxy-3-methoxy-3-methylbutoxy)-6,7-methylenedioxycoumarin		*P. virgatum*	(Maes et al., 2006)
40	Obtusifol		*P. purpurascens*	(Boeykens et al., 1994)
41	Purpurenol		*P. purpurascens*	(Debenedetti et al., 1991; Semple et al., 1999)

3.2. Phenolic acids and flavonoids

Other phenolic compounds cited for *Pterocaulon* species are presented in **Table 3**. Only two studies report the occurrence of phenolic acids in the genus. The compound 3,4 caffeoylquinic acid was isolated as a single compound for the first time from *P. virgatum*, while *P. purpurascens* gave a mixture of caffeoylquinic acids (Martino et al., 1989, 1979).

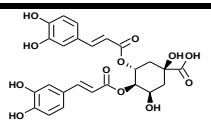
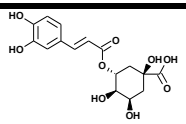
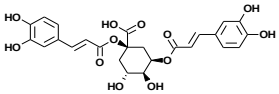
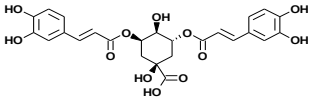
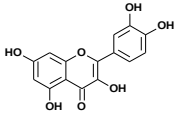
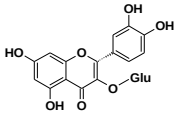
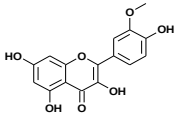
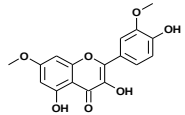
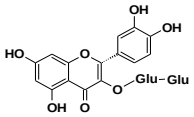
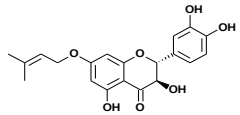
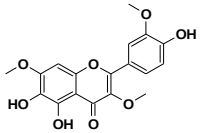
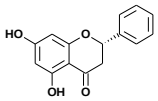
Few studies on flavonoids in *Pterocaulon* species are found in the literature. The compounds **44**, **45**, **46**, **51**, and **52** were isolated from *P. virgatum* (Debenedetti et al., 1983). Further, the same research group described the isolation of **44**, **45**, **55**, **56**, **57** and **62** from *P. purpurascens* (Debenedetti et al., 1987).

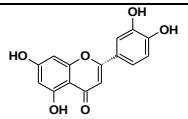
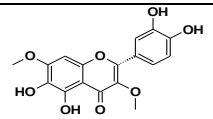
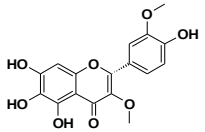
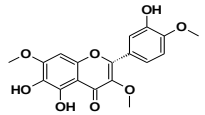
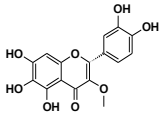
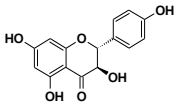
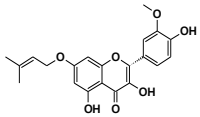
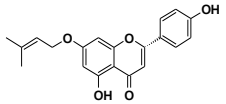
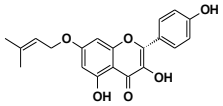
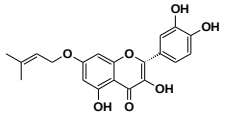
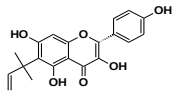
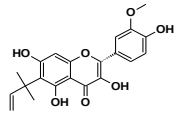
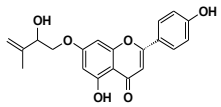
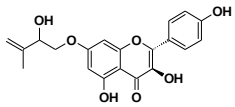
Other species were also investigated for the flavonoids: *P. sphacelatum* afforded **47** (Semple et al., 1999); from the aerial parts of *P. serrulatum* the compound **54** was obtained (Macleod and Rasmussen, 1999); *P. redolens*, native to Thailand, gave **48**, **47** and **55** (Kanlayavattanakul et al., 2003) and from *P. balansae*, the flavonoids **44** and **53** were obtained (Heemann et al., 2006).

In 1995, in addition to the coumarins previously mentioned, the compound **53** was isolated from *P. alopecuroides*. This flavonoid was previously isolated from *P. virgatum* and *P. rugosum* (Vilegas et al., 1995). Subsequently, the compounds **58**, **59**, **60**, **64**, **65** and **66** were isolated from *P. alopecuroides* (Alarcón et al., 2008). From the same plant (*P. alopecuroides*), **61**, **63**, **65** and **67** were obtained (Alarcón et al., 2010).

It is worth mentioning that in the genus *Pterocaulon*, most of the flavonoids isolated until now occur in their aglycone form. It is possible that in these plants the compounds are located in the outer surface of the leaves and flowers together with some coumarins which were demonstrated to be present in *Pterocaulon* exudates (Stein et al., 2007).

Table 3. Phenolic acids and flavonoids from *Pterocaulon* species.

	Compound	Structure	Source	Ref		Compound	Structure	Source	Ref
42	3,4-*O*-dicaffeoylquinic acid		*P. virgatum* *P. purpurascens*	(Martino et al., 1989, 1979) (Martino et al., 1989)	**49**	3- caffeoylquinic acid (chlorogenic)		*P. purpurascens* *P. virgatum*	(Martino et al., 1989)
43	4,5- dicaffeoylquinic acid		*P. purpurascens* *P. virgatum*	(Martino et al., 1989)	**50**	3,5- dicaffeoylquinic acid		*P. purpurascens* *P. virgatum*	(Martino et al., 1989)
44	Quercetin		*P. virgatum* *P. purpurascens* *P. balansae*	(Debenedetti et al., 1983) (Debenedetti et al., 1987) (Heemann et al., 2006)	**51**	Isoquercetin		*P. virgatum*	(Debenedetti et al., 1983)
45	Isorhamnetin		*P. virgatum* *P. purpurascens*	(Debenedetti et al., 1987, 1983)	**52**	Rhamnazin		*P. virgatum*	(Debenedetti et al., 1983)
46	Quercetin-3-diglucoside		*P. virgatum*	(Debenedetti et al., 1983)	**53**	Taxifolin 7-*O*-prenylated		*P. virgatum* *P. alopecuroides* *P. balansae*	(Bohlmann et al. 1981) (Semple et al., 1999) (Heemann et al., 2006)
47	Chrysosplenol C		*P. sphacelatum* *P. redolens*	(Macleod and Rasmussen 1999) (Kanlayavattanakul et al., 2003)	**54**	5,7-dihydroxyflavanone		*P. serrulatum*	(Macleod and Rasmussen, 1999)

48	Luteolin		*P. redolens*	(Kanlayavattanakul et al., 2003)	**55**	Tomentin		*P. redolens* *P. purpurascens*	(Kanlayavattanakul et al. 2003) (Debenedetti et al., 1987)
56	Quercetagetin-3,3‘-dimethyl ether		*P. purpurascens*	(Debenedetti et al., 1987)	**62**	4’-methyltomentin		*P. purpurascens*	(Debenedetti et al., 1987)
57	Quercetagetin-3-methyl ether		*P. purpurascens*	(Debenedetti et al., 1987)	**63**	Dihydrokaempferol		*P. alopecuroides*	(Alarcón et al., 2010)
58	5,4′-Dihydroxy-3′-*O*-methyl-7-(γ,γ-dimethylallyloxy)dihydroflavonol		*P. alopecuroides*	(Alarcón et al., 2008)	**64**	5,4′-Dihydroxy-7-(γ,γ-dimethylallyloxy)flavanone		*P. alopecuroides*	(Alarcón et al., 2008)
59	5,4′-Dihydroxy-7-(γ,γ-dimethylallyloxy)dihydroflavonol		*P. alopecuroides*	(Alarcón et al., 2008)	**65**	5,3′,4′-Trihydroxy-7-(γ,γ-dimethylallyloxy)dihydroflavonol		*P. alopecuroides*	(Alarcón et al., 2010, 2008)
60	5,7,4′-Trihydroxy-6-(α,α-dimethylallyl)dihydroflavonol		*P. alopecuroides*	(Alarcón et al., 2008)	**66**	5,7,4′-Trihydroxy-3′-*O*-methyl-6-(α,α-dimethylallyl) Dihydroflavonol		*P. alopecuroides*	(Alarcón et al., 2008)
61	5,4’-Dihydroxy-7-(2-hydroxy-3-methyl-3-butenyloxy)flavanone		*P. alopecuroides*	(Alarcón et al., 2010)	**67**	5,4-Dihydroxy-7-(2-hydroxy-3-methyl-3-butenyloxy)dihydroflavanol		*P. alopecuroides*	(Alarcón et al., 2010)

3.3. Terpenes

The first species studied regarding the terpenes was *P. virgatum*. From the roots of this plant humulene and thymohydroquinone dimethyl ether were obtained. The aerial parts of the same plant afforded squalene, thymohydroquinone dimethyl ether, taraxasterol and, the corresponding acetate and cetone (Bohlmann et al., 1981). Later, 14-hydroxy-*β*-caryophyllene and 4,5-epoxy-13-hydroxy-*β*-caryophyllene were isolated from *P. serrulatum* (Macleod and Rasmussen, 1999). From *P. balansae*, stigmasterol and 3-*O*-acetyl taraxasterol were identified (Heemann et al., 2006).

The aerial parts of *P. polystachyum* yielded 0.15% of essential oil, which was obtained by hydrodistillation. By GC-MS analysis, 17 compounds were identified: *α*-cubebene, *α*-copaene, *β*-bourbonene, *β*-caryophyllene, *α*-humulene, germacrene D, bicyclogermacrene, *α*-muurolene, *δ*-cadinene, elemol, E-nerolidol, caryophyllene oxide, E-sesquilavandulol, taucadinol, *α*-eudesmol, *α*-cadinol, and E-sesquilavandulyl acetate. The last was the main component, representing 43.8% of the essential oil, followed by E-sesquilavandulol (17.3%) and *β*-caryophyllene (10.0%) (Sauter et al., 2011). Previously, some degraded terpenoids (lololide, 7-epilolyolide, vomifolol and vomifolione) were obtained from this species (Vera et al., 2001).

3.4. Polyacetylenes

Polyacetylenes are a group of compounds more frequently found in species of Asteraceae than in another plant family and they are particularly present in the roots of the plants. This class of compounds was detected in some species of the genus *Pterocaulon*: *P. virgatum* (Bohlmann et al., 1981), *P. alopecuroides, P. balansae, P. lanatum* and *P. rugosum* (Magalhães et al., 1989).

4. Ethnopharmacology and ethnobotany

In a synthesized way, the ethnopharmacological or ethnobotanical data reported for the species of *Pterocaulon* are presented in table 4. In 1984, a study carried out in Argentina, reporting the traditional use of some species of *Pterocaulon* (*P. alopecuroides*, *P. angustifolium*, *P. lorentzii*, *P. polystachyum*, *P. rugosum* and *P. virgatum*) was published (Zardini, 1984). In other studies performed in the same country, *P. purpurascens* (Debenedetti et al., 1992; Filipov, 1994), *P. alopecuroides, P. cordobense* and *P. virgatum* (Debenedetti et al., 1992; Goleniowski et al., 2006), *P. polystachyum* (Debenedetti et al., 1994), *P. pycnostachyum* (Cabrera and Ragonese, 1978; Krag, 1976) were also cited as useful plants. Other authors published studies regarding the popular uses of *Pterocaulon* species in different parts of the world: *P. globuliflorus, P. serrulatum* and *P. sphacelatum* are used in Australia (Smith, 1991); *P. decurrens,* in Madagascar (Rasoanaivo et al., 1992) and *P. alopecuroides,* in Martinica (Longuefosse and Nossin, 1996). In Brazil, the species *P. polystachyum* (Battisti et al., 2013; Garlet and Irgang, 2001), *P. cordobense* (Avancini and Wiest, 2008) and *P. alopecuroides* (Leitão et al., 2009), known as quitoco or quitoco-amarelo, are used for medicinal purposes. Not all the species are cited as useful, but it is necessary to mention that some of them, such as *P. alopecuroides* and *P. balansae* are morphologically very similar being indistinguishable by the people who use them.

As it can be seen in **Table 4**, almost all the ethnopharmacological studies report the use of *Pterocaulon* species in the treatment of skin problems of different etiologies. Although flavonoids and other compounds could be involved in the effects on the skin, the properties popularly attributed to the plants could be due to the presence of coumarins.

As stated before, the coumarins present in the *Pterocaulon* species are soluble in both organic solvents and hot water (Medeiros-Neves et al., 2015; Torres et al., 2017). Therefore, they will be present in the popular preparations, which in most cases are decoctions.

Table 4. Ethnobotanical studies reported for *Pterocaulon* species.

Species	Part of the plant	Preparation	Use	Ref.
P. alopecuroides	L	Decoction	Used for acnes, wounds, insect bites and as antiarthritic agent	(Zardini, 1984)
			Used for the liver	
			Anticephalic, astringent, antitussive, diuretic	
			Oral use in the treatment of chills and fever	(Longuefosse and Nossin, 1996)
		Infusion	Oral use in the treatment of fever	(Longuefosse and Nossin, 1996)
		Dry leaves	Applied in areas with rheumatic pain	(Zardini, 1984)
			Used as aromatic plant insecticidal	
	LS	Decoction	Digestive and against hepatic affections	(Goleniowski et al., 2006)
P. angustifolium	L	Decoction	Used for acnes, wounds, insect bites and as antiarthritic agent	(Zardini, 1984)
			Used for the liver	
P. cordobense	AP	Decoction	Tratament of mycosis	(Avancini and Wiest, 2008)
	LS	Decoction	Against hepatic affections and pesticide	(Goleniowski et al., 2006)
P. decurrens	AP	Decoction	Antipyretic	(Rasoanaivo et al., 1992)
P. globuliflorus	LS	Decoction	Used for relief of colds, influenza and fever	(Smith, 1991)
			Used to treat irritated skin and ringworms (dermatophytosis)	
		Steam	Treatment for colds, influenza and to clear a blocked sinus	
P. lorentzii	L	Decoction	Used for acnes, wounds, insect bites and as antiarthritic agent.	(Zardini, 1984)
			Used for the liver	
			To wash wounds on horses	
P. pycnostachyum	R	Infusion	Emenagogue and abortive	(Krag, 1976)

			Cleaning ulcers	(Cabrera and Ragonese, 1978)
P. polystachyum	L	Decoction	To wash the head in case of insolation	(Zardini, 1984)
			Used for the liver, emenagogue and abortive	(Garlet and Irgang, 2001)
			Heartburn	(Battisti et al., 2013)
	WP	*In natura*	Used as aromatic plant and to ward off fleas and flies	(Zardini, 1984)
			Repellent of fleas and flies and in cases of insolation	(Debenedetti et al., 1994)
P. purpurascens	R	Macerated	Given orally in the treatment of snakebites	(Filipov, 1994)
	AP	Not mentioned	Digestive, insecticide and in the treatment of snakebites	(Debenedetti et al., 1992)
P. rugosum	L	Decoction	Used for acnes, wounds, insect bites and as antiarthritic agent.	(Zardini, 1984)
			Used for the liver	
P. serrulatum	FL	Decoction	Antiseptic for wounds and cuts;	(Smith, 1991)
			For relief from itchy skin and ringworm	
		Steam	Treatment for colds, influenza and to clear a blocked sinus	
		Smoke	Mosquitoes repellent	
P. sphacelatum	FL	Decoction	Used as eye drops to relieve pain and redness of the eyes	(Smith, 1991)
			Antiseptic for infected wounds and cuts	
		Vapours	Cold and influenza relief	
P. virgatum	L	Decoction	Used for acnes, wounds, insect bites and as antiarthritic agent.	(Zardini, 1984)
			Used for the liver	
			Emenagogue and insecticide	
	LS	Decoction	Against hepatic affections and pesticide	(Goleniowski et al., 2006)
	AP	Not mentioned	Digestive, emenagogue, insecticide and in the treatment of snakebites	(Debenedetti et al., 1992)

Aerial parts (AP), Fresh Leaves (FL), Leaves (L), Leaves and Stems (LS), Roots (R), Whole plant (WP)

Several coumarins were investigated for the antibacterial and antifungal activities (Ayine-Tora et al., 2016; Chowdhury et al., 2003; He et al., 2017). Some *Pterocaulon* extracts and isolated coumarins were also studied for these activities (see item 5.1 and 5.2). Besides that, some coumarins are anti-inflammatory (Kirsch et al., 2016) and can have wound healing activity (La et al., 2013). These properties could be related to the purported effects of the plants.

The second most cited use of *Pterocaulon* species is in the treatment of disorders of the liver. Seven among the species that are mentioned for the ethnobotanical importance are used in the therapy of liver damages. Neither the plants nor the coumarins isolated from *Pterocaulon* species were investigated for this activity. Nevertheless, this effect could be attributed to the coumarins since several of them demonstrated hepatoprotective activity, which could be ascribed to their potent antioxidant effects (Atmaca et al., 2011; Chen et al., 2013; Sancheti et al., 2013; Zhang et al., 2013). It is, however, worth commenting that coumarin, present in same edible plants as *Dipteris odorata* (Amazonian vanilla), has been reported as hepatotoxic and carcinogenic in rats, being the toxicity due to the formation of 3,4 epoxide intermediates (Cohen, 1979).

Other ethnomedical use of *Pterocaulon* species is in the treatment of respiratory ailments. Several plants containing coumarins are traditionally used for treating diseases of the respiratory tract. The roots of *Angelica decursiva* (Apiaceae) have been employed in the treatment of cough, bronchitis and upper respiratory tract infections (Lim et al., 2014); decoctions prepared with the leaves of *Mikania glomerata* Spreng. (Asteraceae), rich in coumarin, have similar uses, as anti-inflammatory, anti-asthmatic and bronchodilator agent (Czelusniak et al., 2012). Other plants containing coumarins, such as *Eclipta prostrata* (Asteraceae), *Justicia pectoralis* (Acanthaceae), *Amburana cearensis* and *Pterodon emarginatus* (Leguminosae), are medicinal plants used in the treatment of respiratory tract diseases and demonstrated anti-inflammatory, antinociceptive and bronchodilator activities, justifying their traditional uses (Leal et al., 2000).

Besides that, coumarins are recognized for their anti-inflammatory activity. This effect could be directly related to the antioxidant properties of these compounds (Kirsch et al., 2016). The anti-inflammatory effect could be associated to some of the medicinal uses such as in cases of rheumatic pains, as anti-arthritic agents.

5. Biological activities

5.1. Antifungal activity

An ethnoveterinary study carried out in south Brazil pointed out the usefulness of species of *Pterocaulon* in the treatment of skin diseases popularly diagnosed as mycoses in animals (Avancini and Wiest, 2008). The results of this research have been stimulating most of the investigation on the antifungal activity.

In the first study developed for this purpose, the crude methanolic extract and *n*-hexane, dichlorometane and methanolic fractions of the aerial parts of *P. alopecuroides*, *P. balansae* and *P. polystachyum* were tested against *Candida albicans*, *Candida tropicalis*, *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Microsporum gypseum*, *Trichophyton mentagrophytes* and *Trichophyton rubrum*. The extracts exhibited activity against all the fungi species investigated, showing MIC values of 12.5 to 800 µg/mL. The crude methanolic extract of *P. alopecuroides* exhibited activity against *Cryptococcus neoformans* (MIC = 25 µg/mL). Among the fractions, the *n*-hexane of *P. balansae* presented a broad spectrum of inhibitory action for all fungi tested, including species of *Candida*, showing more pronounced activity against the species of *Trichophyton* (MIC = 12.5 µg/mL). The *n*-hexane and dichloromethane extracts, rich in coumarins, presented the most expressive antifungal activity (Stein et al., 2005). Subsequently, the same research group investigated the activity of the isolated coumarins against the same fungi. The compounds **9**, **3** and **18** did not show activity. Nevertheless, the mixture of **16** (70%) and **17** (30%) demonstrated antifungal activity against *C. neoformans* (MIC = 125 µg/mL) and against the dermatophytes *M. gypseum* (MIC = 31.25 µg/mL), *T.*

rubrum (MIC = 62.5 μg/mL) and *T. mentagrophytes* (MIC = 31.25 μg/mL). The *n*-hexane extracts of the three studied species showed MIC values of 12.5 to 50 μg/mL for the dermatophytes tested (Stein et al., 2006).

The antifungal activity of a methanolic extract of the aerial parts of *P. alopecuroides* was investigated against *Fonsecaea pedrosoi, Fonsecaea compacta, Cladophialophora carrionii, Phialophora verrucosa, Rhinocladiella aquaspersa* and *Exophiala jeanselmei*, agents of chromoblastomycosis, a long-term fungal infection of the skin and subcutaneous tissue. The extract presented MIC values between 625 and 2500 μg/mL. The extract was fungistatic against *Fonsecaea* spp. and fungicide against all other fungi (Daboit et al., 2010). Afterward, the same research group assessed the effect of the crude methanolic extracts of *P. balansae, P. cordobense, P. lanatum, P. lorentzii* and *P. polystachyum* against strains of *Sporothrix schenckii*. All the extracts inhibited the growth of the fungus with MIC values between 156 and 1250 μg/mL and MFC between 312 and 5000 μg/mL. Among the samples tested, *P. polystachyum* extract presented the best results, with MIC ranging from 156 to 312 μg/mL and MFC ranging from 312 to 1250 μg/mL (Stopiglia et al., 2011). Although in both studies the MIC values were high, the authors consider that the plants have potential since the treatment of these mycosis is very difficult. As the experiments were performed with crude extracts, deeper investigations with fractions and isolated compounds are necessary.

In a recent study, the activity of extracts from 89 species native to Argentina was investigated against the fungus *Leucoagaricus gongylophorus*. The hydroethanolic extract (95%) of *P. alopecuroides* showed significant antifungal activity, with MIC of 7.8 μg/mL, being the extract whose MIC most resembled that of the positive control carbendazin (MIC = 0.12 μg/mL) (Napal et al., 2015).

5.2. Antibacterial activity

Although the species are used in the treatment of some diseases caused by bacteria, most of the microorganisms investigated in the few studies available are not related to the ethnomedical uses.

The terpenoids 14-hydroxy-*β*-caryophyllene and 4,5-epoxy-13-hydroxy-*β*-caryophyllene isolated from *P. serrulatum* demonstrated good inhibitory activity against *Bacillus subtillis* and *Escherichia coli*. Coumarins isolated from the same plant were inactive against the microorganisms at the concentrations tested (Macleod and Rasmussen, 1999).

Coumarins and flavonoids isolated from the aerial parts of *P. redolens* were tested on Alamar Blue microplates against *Mycobacterium tuberculosis* strains. Moderate activities were observed for the coumarins **3**, **4**, **8**, **9**, **15**, **25** and the flavonoid **48**, with MIC values of 100, 100, 100, 100, 100, 200 and 100 μg/mL, respectively (Kanlayavattanakul et al., 2003).

In 2008, the activity of decoction and hydroalcoholic extracts of *P. cordobense* was evaluated against *Staphylococcus aureus* and *Salmonella choreraesuis.* Both extracts were active against *S. aureus* (Avancini and Wiest, 2008). In other study, the ethanolic extract of *P. sphacelatum*, Australian medicinal plant used in the treatment of respiratory infections, was investigated against strains of *Mycobacterium fortuitum* and *Mycobacterium smegmatis.* The extract was active against the later, with MIC value of 60 μg/mL (Meilak and Palombo, 2008). In the same year, the antimicrobial activity of flavonoids from the aerial parts of *P. alopecuroides* was evaluated against *Bacillus cereus*, *Bacillus subtilis*, *Salmonella typhimurium* and *Proteus mirabilis.* The compounds were not active against the Gram-negative bacteria. The compounds **64** and **66** were active against *Bacillus cereus* and *Bacillus subtilis* (MIC < 25 μg/mL), whereas **60** e **65** showed moderate activity against the same bacteria, with MIC values of 200 μg/mL and 50 μg/mL, respectively (Alarcón et al., 2008).

5.3. Antiviral activity

Few reports on antiviral activity of extracts and compounds obtained from *Pterocaulon* species were found. The studies were not motivated by ethnomedical uses since none of the virus assayed are associated to the diseases popularly treated with these plants.

Different plant species used in the traditional medicine of the Australian Aboriginal people, among them *P. sphacelatum*, were investigated for the antiviral activity. The ethanol extract of this plant was tested against one DNA virus (human cytomegalovirus) and two RNA viruses (Ross River virus and poliovirus type 1). The extract presented activity only against poliovirus, inhibiting poliovirus-induced CPE by more than 75% in the crystal violet assay at a non-cytotoxic concentration (6-52 µg/mL) (Semple et al., 1998).

In sequence, the same researchers investigated the antiviral activity-guided fractionation of the ethanol extract of *P. sphacelatum* using an inhibition of poliovirus-induced cytopathic effect assay. The flavonoid chrysosplenol C (**47**) isolated from this extract was active against poliovirus with an EC_{50} of 0.27 µg/mL (0.75 µM), and a maximum non-toxic concentration to actively growing BGM cells of 3.91 µg/mL (10.86 µM) (Semple et al., 1999).

The total ethanol extract from *P. alopecuroides* inhibited the *Herpes simplex* virus type 2 resistant to acyclovir (HSV-2-ACVr) with a percentage of inhibition higher than 70%. The compound **13**, isolated from this extract, showed a viral inhibitory rate almost two times higher than that of the extract, with a percentage of inhibition of 94.2%. The isolated compound was also active against HSV-1-ACVr (84.1%) (Silveira et al., 2009).

5.4. Antioxidant activity

The antioxidant activity of *Pterocaulon* extracts and isolated compounds was not investigated deeply. Most of the studies are preliminary and were not based on *in vivo* models. However, *in vivo* experiments are expected to demonstrate antioxidant activity once coumarins, their major components, are recognized as potent antioxidant agents

(Torres et al., 2014). The most relevant biological activities that have been described for coumarins such as anti-inflammatory, antiproliferative and neuroprotective effects can be, at least in part, connected with inhibition of oxidative stress, by avoiding the production of ROS or inhibiting the consumption of endogenous antioxidants (Bubols et al., 2013).

The infusion of *P. polystachyum* in the concentration of 60 µg/mL demonstrated dose-response relationship, exhibiting chain-breaking antioxidant activity in Fe^{2+}/ascorbate-induced lipid peroxidation of rat liver microsomal fractions (IC_{50} = 13.5 µg/mL), and scavenged peroxyl radicals in an aqueous assay system (Paya et al., 1996).

The ethanol extract of *P. alopecuroides* evaluated by DPPH assay, presented CE_{50} value of 19.06 µg/mL. The extract was partitioned, and the fractions were also evaluated showing CE_{50} values of 140.41 µg/mL (dichloromethane), 18.81 µg/mL (ethyl acetate) and 17.71 µg/mL (*n*-butanol). The IC_{50} value of the standard (*Ginkgo biloba*) was 49.79 µg/mL (Silveira et al., 2009).

Through different assays (DPPH; TRAP) the methanolic fraction of *P. alopecuroides* and *P. balansae* showed good capacity to capture the radicals. In addition, in the tests (TBA-RS; NPSH) with brain and liver tissue of rats, the extracts demonstrated a clear capacity to prevent the initiation of free radical-mediated chain reactions (Ferreira, 2009).

Still in the same year, a screening carried out with a series of plants from the Province of Córdoba, Argentina, demonstrated the antioxidant capacity of *P. cordobense* (704.4 ± 8.8 µmol of Fe(II)/g) by the FRAP assay and the antioxidative effect (IC_{50} = 301.1 ± 10.2 µg/mL) by the DPPH radical scavenging activity (Borneo et al., 2009).

In a recent study, the antioxidant potential of alcoholic extracts and hexane, chloroform, and ethyl acetate fractions of *P. lorentzii* and *P. alopecuroides* and the coumarin **25**, isolated from both species, was investigated. All the extracts, fractions and isolated coumarin were evaluated using the spectrophotometric method of phosphomolybdenum, DPPH assay and thiobarbituric acid reactive substances test

(TBARS). In the phosphomolybdenum test, the ethyl acetate fraction of both plants was the most active. In the DPPH assay, the acetate fractions of both plants were also the most active. In the TBARS test, the crude extracts of the two species showed significant results in comparison to BHT, the positive control (Miyazaki et al., 2013).

5.5. Antiparasitic activity

Although ethnomedical studies do not highlight the use of *Pterocaulon* species in the treatment of parasitic diseases, a study with the species *P. interruptum* (syn. *P. balansae*) was carried out. The results showed high *in vitro* antiparasitic activity, inhibiting the hatching of trichostrongilidae eggs from sheep. In 48 h the ethanolic extract inhibited 100% of the embryonic development. Tests *in vivo* demonstrated a 47% reduction in the number of eggs eliminated in the fecal material (Furtado, 2006).

Considering the antifungal effect already described for the species *P. polystachyum*, and that some antifungal drugs are also antiprotozoal agents, the activity of this plant against strains of *Acanthamoeba castellanii* was investigated. The greatest activity was observed in the treatment with the hexane fraction, which lysed approximately 66% and 70% of the trophozoites in 48 and 72 h, respectively, preventing encystment (Ródio et al., 2008).

Afterwards, the same research group evaluated the amebicidal activity of the essential oil of *P. polystachyum* against *Acanthamoeba polyphaga*. The oil obtained from this species, mentioned in item 3.3, was solubilized in 1% Tween 80® and water and tested at the concentrations of 20, 10, 5, 2.5 and 1.25 mg/mL. In 24 h, the oil was able to kill 60%, 71.6% and 81.1% of trophozoites at concentrations of 1.25, 2.5 and 5 mg/mL, respectively. In 48 h, the oil had the capacity to kill 46%, 46.3% and 80.7% of trophozoites at concentrations of 1.25 2.5 and 5 mg/mL, respectively. Based on the results obtained, the authors concluded that the essential oil of *P. polystachyum* was active in a dose-dependent manner (Sauter et al., 2011).

The *n*-hexane extract of *P. balansae* incorporated into a nanoemulsion was assessed against *Acanthamoeba castellanii* showing a behavior dose-dependent and incubation time-dependent. A reduction of 95% of trophozoite viability was detected after 24 h of incubation with the nanoemulsion containing 1.25 mg/mL of coumarins, an effect similar to that obtained with chlorhexidine. After analyzing the results, the authors suggest that the formulation developed has the potential to be a novel strategy for the treatment of ocular keratitis caused by *Acanthamoeba* (Panatieri et al., 2017).

5.6. Insecticidal activity

Pterocaulon polystachyum and *P. purpurascens*, plants used in Argentina as insecticides, were investigated against the larva of the mosquito *Aedes aegypti*, vector of several viruses. The dichloromethane extract of *P. polystachyum* showed activity with LC_{50} ~ 150 μg/mL, whereas *P. purpurascens* showed no insecticidal activity (LC_{50}> 500 μg/mL) (Ciccia et al., 2000).

Five coumarins (**3**, **9, 16**, **17** and **21**), isolated from *P. polystachyum*, were tested against *Spodoptera frugiperda* (Lepidoptera: Noctuidae) larvae. The addition of 200 μg of the coumarins per g of diet induced 100% of phagodepression. Compounds **3** and **16**, and the equimolar mixture of the both exhibited the strongest phagodepression. Doses of 50 μg/g of **3** and **16** incorporated in the larval diet caused 80 and 50% of pupal mortality. Equimolar binary mixtures of coumarins were phagodepressors against *S. frugiperda*, indicating that the mixture of these compounds is more active against the larvae (Vera et al., 2006).

5.7. Cytotoxic activity

Cytotoxicity studies carried out with *Pterocaulon* species are relatively recent and are not related to the traditional uses of the plants. In the first study, published in 2000, the authors observed the cytotoxic activity of the dichloromethane extract of *P.*

polystachyum, by the ability of this sample to inhibit in 30% the development of crown gall tumors. Subsequently, a DNA assay (DNA-MG assay) indicated that the cytotoxicity could be related to the interaction of the compounds present in this extract with the DNA (Mongelli et al., 2000)

In another study, different extracts of *P. polystachyum* (petroleum ether, dichloromethane, ethyl acetate, methanol and an infusion) and the coumarins **9** and **32** were evaluated for the proliferation and differentiation of human promonocytic U-937 cells by the trypan blue exclusion test. The petroleum ether extract was the only one that reduced cell proliferation ($p < 0.01$) and induced cell differentiation. The coumarins **9** and **32**, isolated from this extract, inhibited the growth of human leukemia U-937 cells in a time and concentration-dependent manner, with IC_{50} values of 2.2 µM and 3.5 µM, respectively. In addition, these coumarins were also able to induce CD88 functionality and NBT reduction, markers of monocytic cell differentiation (Riveiro et al., 2004).

Afterwards, the cytotoxic and mutagenic potential of infusions prepared with leaves of the same plant were investigated using the *in vivo* onion (*Allium cepa*) root-tip cell test. Infusions were tested at the concentration commonly used in folk medicine (2.5 mg/mL) and at twice (5 mg/mL) and four times (10 mg/mL) this concentration. The greatest inhibition of onion root-tip cell division and decreased mitotic index was obtained with the infusion prepared in the concentration of 10 mg/mL (Knoll et al., 2006). Two studies demonstrated the cytotoxic effect of the extracts of *P. polystachyum* which could be related to a possible interaction with DNA (Knoll et al., 2006; Mongelli et al., 2000). The cytotoxicity of this species was also observed through the reduction of the proliferation and induction of the cell differentiation and the effects was attributed to the coumarins present in the extracts (Riveiro et al., 2004).

Pterocaulon redolens and *P. alopecuroides* also exhibited cytotoxic activity. Compounds isolated from the ethanol extract of *P. redolens* were assessed by the sulforhodamine B (SRB)-assay in human tumor cell lines of breast cancer (BC) and human small cell lung cancer (NCI-H187). Among them, the flavonoid **47** showed moderate cytotoxic activity against both cell lines with IC_{50} values of 5.52 and 9.25

µg/mL, respectively (Kanlayavattanakul et al., 2003). More recently, the cytotoxicity of the ethanol extract of *P. alopecuroides* (100 µg/mL) was evaluated against RBL-2H3 cells. The extract presented 96.26 % of cytotoxic effect, being more active than terfenadine (positive control = 66.43 %) (Silveira et al., 2009).

The promising results described above motivated the investigation of isolated coumarins for the antileukemia activity. The compounds **25** and **39** were able to inhibit U-937 cells growth and induce cell differentiation after 48 h of treatment, with IC_{50} values of 191.9 µM and 25.3 µM, respectively (Riveiro et al., 2009). More recently, coumarin **9** demonstrated significant cytotoxicity against human (U138-MG) and rat glioma cells (C6) with IC_{50} values of 31.6 µM and 34.6 µM, respectively (Vianna et al., 2012).

6. Toxicology

Regarding the toxicity of the extracts and compounds isolated from these plants, only very few studies addressing this issue were found. In all of them different extract and species were investigated, being difficult to compare the results. The first toxicity study carried out with *Pterocaulon* species evaluated the oral acute toxicity in mice of the dried aqueous extracts of *P. polystachyum* and *P. virgatum*, testing a dose of about 1000 mg/kg, 10 times higher than those used in folk medicine. The animals did not show significant alterations in the consumption of water or food during the study period (15 days). No behavioral change, signs of toxicity, death or macroscopic changes in organs vital were observed (Gorzalczany et al., 1999).

More recently, a study with the hexane extract of the same species was carried out. The acute and subacute toxicity on mice was investigated after oral administration of the doses of 100, 200 and 400 mg/kg. The extract caused modifications in biochemical parameters and morphological alterations in tissues. Nevertheless, there was no mortality and no visible signs of lethality were seen in mice. The results also revealed genotoxicity in kidney tissue in comet assay (concentration of 100 and 400 mg/kg) but no mutagenicity was detected by the micronucleus test (Regner et al., 2011).

In 2006, the acute and subacute toxicity of the hydroalcoholic extract of *Pterocaulon interruptum* (syn. *P. balansae*) was studied on mice. The results, analyzed after intraperitoneal and oral administration, allowed estimating the LC_{50} of the extract between 500 and 1000 mg/kg (Furtado, 2006).

The ethanol extract of *P. lorentzii* and *P. alopecuroides*, as well as the fractions hexane, chloroform, ethyl acetate and the isolated coumarin **25** were tested by the brine shrimp lethality bioassay. No toxicity was detected since all the samples exhibited LC_{50} values higher than 1,000 μg/mL. In the hemolysis evaluation, the ethanol extract and the fractions did not cause hemolysis, as shown by both methods used (Dilution method and Blood agar plates method) (Miyazaki et al., 2013).

7. Concluding remarks

This review presents the results of the investigations on *Pterocaulon species* that have been conducted so far. Among the 26 species distributed throughout the world, 13 were reported as medicinally useful, 12 were the subject of research on the chemical composition and only 11 were examined regarding some biological aspects. The data collected in the available literature turn evident that the plants of the genus *Pterocaulon* have great pharmacological potential. At the same time, these species are still scantily explored and many gaps in the knowledge exist. Thus, there is a clear need for more in-depth research into the pharmacological activities since none of the purported effects of the plants was fully studied. In fact, it was observed that most of the pharmacological investigations were not correlated with traditional uses of the plants. It is noteworthy to highlight that one of the most cited traditional claim of these plants, i.e. in the treatment of liver disorders was not pharmacologically evaluated. The only study related to the traditional use that is somewhat developed is about the antifungal activity. But even so, the studies were performed with organic extracts. Thus, to validate the popular use of the plants, the experiments should be repeated with aqueous extracts, decoctions and infusions, which is the form used by the population.

Further researches must also be focused on furthering the knowledge of new technologies for the extraction and isolation of the compounds in the necessary amount to deeply investigate the pharmacological activities as well as their mechanisms of action. Finally, a fundamental issue has still to be addressed. Detailed studies of the different species, concerning the toxicity, including genotoxicity, are required to ensure the safety of these medicinal plants.

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Author's contribution

All authors developed the concept for the study; B. Medeiros-Neves conducted the literature survey and drafted the paper. H.F. Teixeira and G.L. von Poser supervised the work and revised the manuscript.

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Capítulo II

Desenvolvimento e validação de metodologia analítica para quantificação de cumarinas de Pterocaulon balansae *em amostras analíticas e bioanalíticas*

2.1. INTRODUÇÃO

A maioria dos estudos relacionados a *Pterocaulon* e seus principais componentes está focada na obtenção de extratos/frações, isolamento dos compostos, identificação por técnicas espectroscópicas e determinação da atividade biológica. Existe, neste cenário, uma lacuna com relação a métodos cromatográficos capazes de quantificar os compostos a partir de extratos da planta.

Diferentes técnicas são empregadas para a quantificação dos compostos ativos presentes em uma planta medicinal, como por exemplo as metodologias por cromatografia líquida (FEKETE et al., 2014). Neste contexto, são amplamente descritas as técnicas por CLAE e CLUE. Embora a técnica por CLAE seja mais amplamente difundida, a tendência atual tem sido a utilização de métodos denominados de ultraeficientes. Tais métodos são capazes de suportar altas pressões, possibilitando o uso de colunas cromatográficas com tamanhos de partícula menores, melhorando a resolução dos picos, diminuindo o tempo de análise e por consequência, resultando na redução do consumo de solvente (MALDNER & JARDIM, 2009)

Visando o desenvolvimento de metodologias confiáveis, rápidas e eficientes para as análises qualitativa e quantitativa dos produtos, a realização de uma etapa denominada "validação de método analítico e/ou bioanalítico" é recomendada pelos guias e compêndios mundiais (SHABIR, 2003; NOVÁKOVÁ & VLCKOVÁ, 2009). Essa validação assegura que a metodologia desenvolvida seja precisa, específica, reprodutível e robusta, garantindo confiabilidade durante a rotina de uso.

O presente capítulo descreve o desenvolvimento e validação de métodos por CLAE e CLUE, com o objetivo de quantificar as cumarinas presentes no extrato aquoso de *P. balansae*, bem como nas nanoemulsões contendo o extrato aquoso e a cumarina 5MMDC, e o perfil de permeação destas cumarinas na pele de orelha suína. O capítulo está escrito no formato de manuscrito.

3.2. ARTIGO

Manuscrito em preparação

Determination of main coumarins of *Pterocaulon balansae* (Asterareae) by an ultra-fast liquid chromatography method — analytical and bioanalytical assays

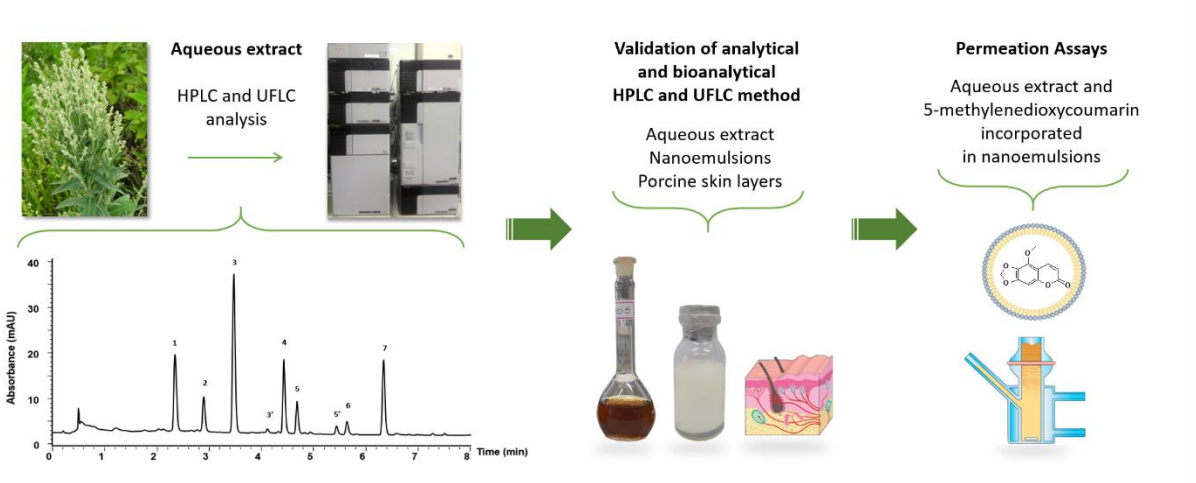
Determination of coumarins from *Pterocaulon balansae* (Asteraceae) by an ultra-fast liquid chromatography method — analytical and bioanalytical assays

Bruna Medeiros-Neves[a], Marina Cardoso Nemitz[a], Nathalya Tesch Brazil[a], Martin Steppe[a], Gilsane Lino von Poser[a], and Helder Ferreira Teixeira[a *]

[a] Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal do Rio Grande do Sul, Avenida Ipiranga, 2752, Porto Alegre/RS, Brazil.

*Corresponding author: Helder Ferreira Teixeira (helder.teixeira@ufrgs.br) Address: Av. Ipiranga, 2752, Porto Alegre/RS – Brazil - Fone: 55 51 33085231

Graphical Abstract



Abstract

Coumarins from *Pterocaulon balansae* have received increasing attention due to some interesting findings regarding their biological activities, especially for the treatment of skin disorders. This study aimed to develop and validate chromatographic methods (HPLC and UFLC) for the analytical and bioanalytical determination of coumarins of *P. balansae* in extracts, topical formulations, as well as in porcine skin after *in vitro* permeation/retention studies. Chromatographic separation was performed on a Phenomenex-C_{18} RP column for HPLC-UV and Shim-pack XR ODS column for UFLC-DAD. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B) using gradient elution, flow rate and column oven was HPLC (1 mL min^{-1}; 30 ºC) and UFLC (0.55 mL min^{-1}; 55 ºC) for 45 min and 8 min, respectively. The linearity curve used as standard 5-methoxy-6,7-methylenedioxycoumarin (5MMDC) in a concentration range from 0.1 to 7.5 μg mL^{-1}. The accuracy was evaluated at three levels: 0.5, 2.5, and 5.0 μg mL^{-1} for 5MMDC. The mean, standard deviation, RSD and 95% confidence interval for all concentration levels were determined for the three days. These methods showed to be specific, precise, accurate, and linear (0.1 to 7.5 μg mL^{-1}) for determination of 5MMDC in all samples. The matrix effect was determined for complex matrices, and low effect was observed during the analysis. The method's robustness was confirmed using the Plackett-Burman experimental design. The methodologies proposed proved to be linear, precise, and accurate to estimate coumarins from aqueous extract of *P. balansae*, without significant differences between both methods (HPLC and UFLC). However, UFLC analysis time was shortened to about one-fourth the time and consumes significantly less eluent, being more eco-friendly in comparison with HPLC.

Keywords

Pterocaulon balansae; UFLC/HPLC; coumarin assay, permeation/retention; 5-methoxy-6,7-methylenedioxycoumarin

1. Introduction

Various species of *Pterocaulon* Ell. genus have been used in the traditional medicine for the treatment of several diseases. A well-documented literature described different activities of *Pterocaulon* extracts, especially for the treatment of skin disorders (FILIPOV, 1994; GARLET; IRGANG, 2001; GOLENIOWSKI et al., 2006; LONGUEFOSSE; NOSSIN, 1996; RASOANAIVO et al., 1992; SMITH, 1991; ZARDINI, 1984). In last years, some reports have described the antifungal activity of extracts obtained from different *Pterocaulon* species (AVANCINI; WIEST, 2008; STEIN et al., 2005, 2006; STOPIGLIA et al., 2011). STEIN and co-workers evaluated antifungal activity of extracts of *P. balansae*, *P. polystachyum* and *P. alopecuroides* demonstrating a pronounced activity against the dermatophytes *Trichophyton rubrum*, *T. mentagrophytes* and *Microsporum gypseum* (STEIN et al., 2006). More recently, methanol extracts obtained from different species of *Pterocaulon* were evaluated against clinical isolates of the fungus *Sporothrix schenckii* (STOPIGLIA et al., 2011).

The main biological activities of *Pterocaulon* Ell. genus have been related with the coumarins content in the extracts and/or preparations (MEDEIROS-NEVES; TEIXEIRA; VON POSER, 2018). Our research group validated an HPLC/UV method to estimate coumarins from aqueous, *n*-hexane, and supercritical fluid *P. balansae* extracts (MEDEIROS-NEVES et al., 2015; PANATIERI et al., 2017; TORRES et al., 2017). The proposed method led us to determine accurately seven *Pterocaulon* coumarins. However, the quantification of coumarins in crude extracts of *P. balansae* proved to be a difficult task. In fact, the system requires a long period of analysis (almost 40 min) to achieve the separation of these coumarins with a satisfactory resolution. Different chromatographic techniques can be currently used to improve analysis of plant extracts aiming the determination of active compounds in products and/or biological samples (HÖGNER et al., 2013; LI et al., 2016; SILVA et al., 2011). In this way, the use of ultraefficient methods comes to enhance liquid chromatography techniques because withstand high pressures, enable the use of chromatographic columns with a reduced internal diameter (<3 µM), increasing the resolution of peaks, and also

decreasing both the chromatographic run and chemical reagents consumption (BUCAR; WUBE; SCHMID, 2013; FEKETE et al., 2014; GAIKWAD et al., 2010; WAKSMUNDZKA-HAJNOS; SHERMA, 2011).

Therefore, the first aim of this study was to develop an ultra-fast liquid chromatography (UFLC) method to determine the main coumarins of *P. balanseae* aqueous extract. Initial UFLC chromatographic conditions were set by using the Method Transfer Program (Proeminence, Shimadzu Corporation) based on our previous report using HPLC (MEDEIROS-NEVES et al., 2015). After that, both HPLC and UFLC methods were validated and compared to estimate coumarins content in topical nanoemulsions and porcine ear skin samples from *in vitro* permeation/retention studies.

2. Material and Methods

2.1. Chemical and Materials

Ethanol and methanol were purchased from Nuclear (Brazil), and acetonitrile and formic acid were acquired from Tedia (HPLC grade, USA). Egg-lecithin (LipoidE-80®) and medium chain triglycerides (MCT) were purchased from Lipoid GmbH (Ludwigshafen, Germany). Ultrapure water was obtained from a Milli-Q® Plus apparatus by Millipore (Billerica, USA).

2.2. Plant Material

Aerial parts of *Pterocaulon balansae* Chodat. were collected in Canoas, RS, Brazil, in February 2013. The species was identified by Sérgio A. L. Bordignon (La Salle University Center - Canoas, UNILASALLE, Brazil). Voucher specimen was deposited in the herbarium of the Universidade Federal do Rio Grande do Sul (ICN 157762). Plant collection was authorized by Ministry of the Environment - MMA, Chico Mendes Institute for Biodiversity Conservation - ICMBio. System Authorization and Information on Biodiversity – SISBIO (number 38017-1).

2.3. Apparatus and Chromatographic conditions

2.3.1. HPLC analysis

The HPLC method was performed on a Shimadzu LC-20AT system (Kyoto, Japan), equipped with an UV detector controlled by LC-solution Multi-PDA software (Kyoto, Japan). Phenomenex-C_{18} Synergi column (150 mm × 4.6 mm, 4 µm) coupled to a refillable pre-column filled with C_{18} silica was used in the analysis. The mobile phase consisted of a gradient of (A) formic acid 0.1% and (B) acetonitrile, filtered and degassed. The gradient elution was 0-17% B (0-0.01 min), 17-20% B (0.01-10 min), 20% B (10-15 min), 20-25% B (15-20 min), 25-27% B (20-22 min), 27-30% B (22-25 min), 30-35% B (25-30 min), 35% B (30-35 min), 35-17% B (35-40 min). LC system operated at flow rate of 1 mL min^{-1} for 45 min at 30 °C with the injection volume of 20 µL and the wavelength was adjusted to 327 nm.

2.3.2. UFLC analysis

The system used was Shimadzu Prominence UFLC System (Shimadzu, Japan) equipped with a diode array detector (SPD-M20A). The output signal was monitored and processed using Shimadzu LC-solution Multi-PDA software (Kyoto, Japan). Chromatographic separation was performed on a Shim-pack XR ODS column 100 x 2.0 mm i.d.; particle size, 2.2 µm guarded by an in-line pre-column C_{18} SecurityGuard™ ULTRA (Phenomenex,USA).

The mobile phase consisted of (A) formic acid 0.1% (v/v) and (B) acetonitrile. The gradient elution was 0-17% B (0-0.09 min), 17-25% B (0.09-1.45 min), 25% B (1.45-2.20 min), 25-32% B (2.20-2.90 min), 32-37% B (2.90-4.77 min), 37-39% B (4.77-4.90 min), 39-40% B (4.90-5.60 min), 40% B (5.60-7.20 min), 40-22% B (7.20-7.80 min), 22-17% B (7.80-8.00 min). The column was re-equilibrated with 17% B for 2 min before the next analysis. The flow rate was set in 0.55 mL min^{-1} up to 8 min, the wavelength was adjusted to 327 nm, injection volume 5 µL, and the analysis was carried out at 55 ^{o}C.

2.4. Solutions

2.4.1. Stock and reference solutions

The 5MMDC, used as a chemical marker, was obtained according to the methodology previously described by Medeiros et al. (MEDEIROS-NEVES et al., 2015) A stock solution of 5MMDC was solubilized in dimethyl sulfoxide (DMSO) in calibrated volumetric flask, adjusted to the concentration of 40 µg mL^{-1} with acetonitrile (ACN), sonicated for 30 min and used for analytical validation. The reference solutions were prepared by the stock solution dilution with ACN:H_2O (1:1 v/v).

2.4.2. Matrices solutions

2.4.2.1. Aqueous extract

The aqueous extract (AE) was prepared with the dried aerial parts of *P. balansae* and water 2% (w/v) at 60 °C in the multipoint stirrer with water bath (Dist-DI920) for 4h07min. These extraction conditions were set from a previous optimization study. In order to quantify the coumarins, an aliquot of this extract was diluted 10x in ACN:H_2O (1:1 v/v), filtered by membrane of 0.22 µm, and analyzed in HPLC and UFLC.

2.4.2.2. Nanoemulsions

Nanoemulsion (NE_B) was obtained by spontaneous emulsification procedure. The oil phase of the formulation consists of medium chain triglycerides 16.0% (w/w), egg lecithin 4.0% (w/w) and aqueous phase of polysorbate 80 1.0% (w/w) and water up to 100%. An adequate aliquot was diluted in ACN:H_2O (1:1 v/v), filtered by membrane of 0.22 µm, and analyzed.

2.4.2.3. Porcine skin layers

Full thickness skin was removed from the back of the porcine ear. After removal of subcutaneous fat and reducing the size of the hair, the skin was cut into round pieces, and the separation of the stratum corneum was carried out by *tape stripping* procedure. The first stripped tape was discarded, while the following 14 tapes were placed in test tubes and used for the stratum corneum analysis (P.SC). The remaining layer

(epidermis/dermis) was reduced to tiny pieces and placed in different test tubes. To extract the coumarins from skin layers, 2 mL of methanol were added, and the samples were kept in an ultrasound bath for 45 minutes, resulting in the sample named porcine epidermis/dermis skin layers (P.E/D).

2.4.2.4. Receptor fluid for permeation studies

The receptor fluid (RF) for the permeation/retentions studies was a mixture of the 40% ethanol in phosphate buffer pH 7.4. An adequate aliquot was filtered by membrane of 0.22 µm and analyzed.

2.5. *Validation*

The developed HPLC and UFLC methods were validated according to the official guidelines. For nanoemulsions it was used the ICH specifications (ICH, 2005), while for aqueous extract and biological matrix (skin layers) it was used the FDA and EMA recommendations (EUROPEAN MEDICINES AGENCY, 2012; FOOD AND DRUG ADMINISTRATION, 2013). The results were analyzed by Student's t test and analysis of variance (ANOVA) using a significance level of $\alpha = 0.05$. The parameters specificity, linearity, limit of detection, limit of quantification, accuracy, precision and robustness were determined.

2.5.1. Specificity

The interference of the matrix composition was determined by injecting samples containing only matrices, and matrices spiked with the aqueous extract (AE) and the standard solution of 5MMDC at a concentration of 5.0 µg mL^{-1}. The coumarins present in different matrices were identified based on their UV spectra between 200 and 400 nm and their retention times, confirmed by the Mass, UV and NMR data of our previous report (MEDEIROS-NEVES et al., 2015).

2.5.2. Linearity, precision and accuracy

Calibration standards of 5MMDC at concentrations of 0.1, 0.5, 1.0, 2.5, 5.0, 7.5 µg mL^{-1} in ACN:H_2O (1:1 v/v) were prepared. The standard calibration curve was constructed using peak area versus known concentrations of 5MMDC. The linear regression line was used to determine the linearity and concentration of the samples. The linearity of 5MMDC was determined using six sets of the calibration standards. The detection and quantification limits (LOD and LOQ, respectively) were calculated from the calibration curve, using the values of standard deviation of the intercept (σ) and of the slope (S) (LOD 3.3 σ/S and LOQ 10 σ/S). To determine the accuracy, the aqueous extract was prepared and spiked with known amounts of analyte, at the three concentrations of the 5MMDC solution: low, medium and high, corresponding to 0.5; 2.5 and 5.0 µg mL^{-1}, respectively, performing five determinations for each concentration. The results represent the mean recovery (%) for three independent samples. The repeatability of the method was determined by analysis of five determinations of 5MMDC in three points of the analytical curves, during the same day under the same experimental conditions. The intermediate precision values were obtained by assaying freshly prepared solutions as 5MMDC analytical curve on three different days. The results were expressed in relative standard deviation (RSD %).

2.5.3. Robustness

The robustness in each matrix was investigated by the Plackett-Burman design. The factors: column oven temperature, flow rate, initial organic composition and formic acid concentration, were analyzed in low levels (-1) and high levels (+1) to both methods, according the **Table 1**. The four factors selected were tested with eight experiments designed in accordance with Heyden et al. (VANDER HEYDEN et al., 2001).

Table 1. Factors and levels used in evaluating the robustness of methods.

Factors	HPLC			UFLC		
	Nominal	-1	+1	Nominal	-1	+1
T (ºC)	30	29	31	55	54	56
Flow rate (mL min^{-1})	1.0	0.9	1.1	0.55	0.54	0.56

Formic acid (%)	0.1	0.08	0.12	0.1	0.08	0.12
ACN (%)	17	16	18	17	16	18

2.5.4. Assessment of the matrix effect

The slopes obtained in standard curves of 5MMDC standard diluted in the mobile phase were compared with the slopes obtained in standard curves of 5MMDC-spiked in each matrix. The studied matrices were AE, SFE, NE_B, P.E/D, P.SC and RF. Three standard curves were obtained, in three consecutive days, by plotting the peak area versus the concentration of the 5MMDC standard (0.1, 0.5, 1.0, 2.5, 5.0, 7.5 µg mL^{-1}) in acetonitrile 50% (v/v) and in the matrices solutions. Five replicates were analyzed for each concentration level. The matrix effect was calculated based on the ratio of the peak area in the presence of matrix to the peak area in absence of matrix, following the equation: $ME\% = 100 \times [1 - (S_m/S_s)]$, where S_m=slopes of the standard curves of the 5MMDC and S_s=slopes of the standard curves of the 5MMDC in the matrix (EUROPEAN MEDICINES AGENCY, 2012).

2.5.5. Recovery of coumarins after extraction from porcine skin

To evaluate the recovery of coumarins, before the extraction procedure of porcine skin layers, the matrices were spiked with stock solution of 5MMDC in theoretical concentrations of 0.1, 0.5, 2.5 and 5.0 µg mL^{-1}. After that, 2 mL of methanol was added in each matrix test tube, and the samples were maintained in an ultrasound bath for 45 minutes, filtered through a 0.22 µm membrane and analyzed by HPLC and UFLC.

2.5.6. Coumarins stability in matrices

The stability of matrices spiked with 5MMDC was determined after 48 hours storage at room temperature. The stability of these solutions was studied by conducting analysis and observing any change in the chromatographic pattern, compared with freshly prepared solutions.

2.5.7. System suitability

For determination of system suitability, the chromatographic parameters, such as, time retention, theoretical plates (*N*), resolution (Rs) and tailing factor (T) were calculated. The number of theoretical plates (*N*) is used to describe the quality of chromatographic column, where the greater the value of *N* (> 2000), better the quality of the column. The separation between two compounds measured by the resolution (Rs), where Rs > 1.5 represents good separation, and Rs ≥ 2.0 a desirable target for methods development. The tailing factor is a measure of peak tailing, considering good values T < 1.5 (SNYDER; GLAJCH; KIRKLAND, 1997).

2.6. Method application

In permeation studies, 5MMDC loaded-nanoemulsions (NE_{5MMDC}) and aqueous extract loaded-nanoemulsion (NE_{AE}) in the concentrations of 0.50 mg mL^{-1} (w/w) of 5MMDC were used. The nanoemulsions were obtained by means of spontaneous emulsification procedure. The oil phase of the formulation consists of medium chain triglycerides 16.0% (w/w), egg lecithin 4.0% (w/w). The aqueous phase was polysorbate 80 1.0% (w/w) and water up to 100%. In the preparation of $NE_{5MMDC,}$ the 5MMDC was previously solubilized in oil and acetone under constant agitation and after, mixture of egg lecithin and ethanol was added. In the preparation of NE_{AE}, the aqueous extract was used in the aqueous phase, replacing water. The positive control used was a dispersion of 5MMDC in propyleneglycol. To determine the 5MMDC incorporated into NE_{5MMDC} and NE_{AE}, an adequate aliquot was diluted in acetonitrile 50% (v/v), filtered by membrane of 0.22 µm, and analyzed.

The permeation/retention of 5MMDC from nanoemulsions NE_{5MMDC} and NE_{AE} was evaluated using Franz type diffusion cells. The porcine ears skin used was devoid of hair, and was stored at -20 °C. The Franz type diffusion cells have internal area of 1.77 cm^2 and a volume of 10 mL acceptor phase interface. The circular sections of porcine skin, prepared as described in the section 2.4.2.3, were previously dipped in phosphate buffer pH 7.4 solution for 15 minutes. After hydratation, the skin was placed on top of

the cell. These skins were used as the interface between the donor and acceptor medium through the Franz cell. The acceptor medium was phosphate buffer:ethanol 60:40 (v/v). The cells were maintained in thermostatic bath at 32 ± 1.0 °C and stirred at 480 rpm. In the donor compartment about 500 µL of formulations (NE_{5MMDC} and NE_{AE}) were placed maintaining the sink conditions. The 5MMDC concentration was determined in the skin and in the acceptor phase after 8 hours of application of the sample in the donor phase. The amount of coumarin retained in the skin was determined after extraction of 5MMDC with 2 mL of methanol and ultrasound bath for 45 min. The 5MMDC was quantified by HPLC and UFLC and the results expressed in µg of 5MMDC per area of skin.

3. Results and discussion

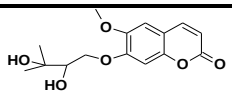
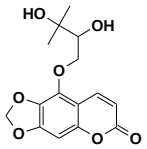
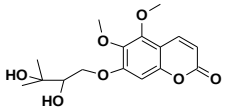
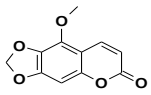
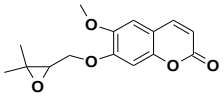
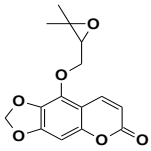
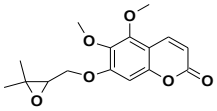
3.1. UFLC method optimization: chromatographic conditions

As stated before, our research group has developed an HPLC/UV methodology for the quantification of coumarins in aqueous extract of *P. balansae*. The coumarins content was estimated based on the linearity curve of the isolated 5-methoxy-6,7-methylenedioxy coumarin (5MMDC). 5MMDC was chosen as a chemical marker of *P. balansae* extract due to some advantages over other coumarins found in the extract. The compound is easily isolated, is present in different *Pterocaulon* species and demonstrated biological activities in different experimental models (LI et al., 2008; MEDEIROS-NEVES et al., 2015; STEIN et al., 2007, 2006; VIANNA et al., 2012). LC system operated at flow rate of 1 mL min^{-1} for 45 min at 30 °C with the injection volume of 20 µL and the wavelength was 327 nm. The column used in the analysis was Phenomenex-C_{18} Synergi (150 mm × 4.6 mm, 4 µm) coupled to a refillable pre-column filled with C_{18} silica. The mobile phase was of a gradient of (A) acetic acid 2% and (B) acetonitrile. The gradient elution was described in 2.2.1 (MEDEIROS-NEVES et al., 2015). Figure 1 shows a typical HPLC/UV chromatogram and **Table 2** the chemical structure of the coumarins detected in the *P. balansae* aqueous extract.

Based on the conditions mentioned above, the method was adapted by changing the acid acetic to formic acid because of its compatibility in various liquid chromatographic

techniques (HPLC, UFLC, UPLC) coupled with UV detector and Mass. In fact, some acids (acetic and trifluoroacetic) have restrictions on the use in techniques that have coupled mass detector. The program used to transfer the HPLC methods to UFLC, MethodTransferProgram – Proeminence UFLC is available from Shimadzu. The conditions shown by the HPLC chromatogram (**Figure 1**) provided an initial condition (Method B1) for UFLC, shown in **Figure 2**. However, the transfer method from an HPLC conventional column (~ 5 µm) to a UFLC column presenting a particle diameter of 2-3 µm must consider some other analytical modifications such as adjustment of the temperature of the column oven. High oven temperatures provide a reduction in viscosity of the mobile phase, allowing the system to maintain a reasonable pressure, and result in more rapid analysis. Thus, a first adaptation of the method in the column oven temperature and the injection volume was required.

Table 2. Structure of the coumarins presents in aqueous extract of *Pterocaulon balansae*.

	Compound	**Structure**
1	6-methoxy-7-(3'-methyl-2',3'-dihydroxybutyloxy)coumarin	
2	5-(2',3'-dihydroxy-3'-methylbutyloxy)-6,7-methylenedioxycoumarin	
3	5,6-dimethoxy-7-(3- methyl-2', 3'-dihydroxybutyloxy)coumarin	
4	5-methoxy-6,7-methylenedioxycoumarin (5MMDC)	
5	6-methoxy-7-(2',3'-epoxy-3-methylbutyloxy)coumarin	
6	5-(2',3'-epoxy-3'-methylbutyloxy)-6,7-methylenedioxycoumarin	
7	5,6-dimethoxy-7-(2',3'-epoxy-3-methylbutyloxy)coumarin	

The initial method, called Method B1 consists of flow rate of 0.45 mL min^{-1} using a gradient of 0-17% B (0-0.01 min), 17-20% B (0.01-2.80 min), 20% B (2.80-4.20 min), 20-25% B (4.20-5.60 min), 25-27% B (5.60-6.30 min), 27-30% B (6.30-7.00 min), 30-35% B (7.00 -8.40 min), 35% B (8.40-9.80 min), 35-17% B (9.80-11.20 min) with the oven temperature 55 °C, injection volume of 5µL and analysis time of 12.6 min. As can be seen in **Figure 2,** by the Method B1, it was not possible to notice all peaks in the aqueous extract matrix, probably due to the low flow rate of the mobile phase and the % organic phase in the elution gradient. The study was continued by making minor changes in the flow rate and the column oven temperature, resulting in the Method B2, which was the flow rate of 0.55 mL min^{-1}, with a minor change at the beginning of the gradient, which was increased to 0-17 % B (0-0.05 min), maintaining the same conditions of oven temperature, injection volume and analysis time of Method B1.

As can be seen in **Figure 2**, the Method B2 was not still in accordance with the recommendations for the chromatographic parameters analyzed: number of theoretical plates (> 2000), tailing factor (<1.5) and especially the factor resolution between peaks (> 1.5), while peak 3 ' to the peak 4 showed a Rs <1.5 and the peak 5' at peak 6, having a Rs <1. From this point some modifications of the mobile phase and elution gradient were necessary setting the oven temperature conditions, flow rate and injection volume of Method B2, aiming to improve chromatographic parameters and reducing the analysis time.

The method B3 (**Figure 2**) showed the best results of optimization techniques, laying down the conditions gradient elution was 0-17% B (0-0.09 min), 17-25% B (0.09-1.45 min), 25% B (1.45-2.20 min), 25-32% B (2.20-2.90 min), 32-37% B (2.90-4.77 min), 37-39% B (4.77-4.90 min), 39-40% B (4.90-5.60 min), 40% B (5.60-7.20 min), 40-22% B (7.20-7.80 min), 22-17% B (7.80-8.00 min). The flow rate was 0.55 mL min^{-1} up to 8 min, the wavelength was adjusted to 327 nm, injection volume 5 µL, and the analysis was carried out of 55 ºC.

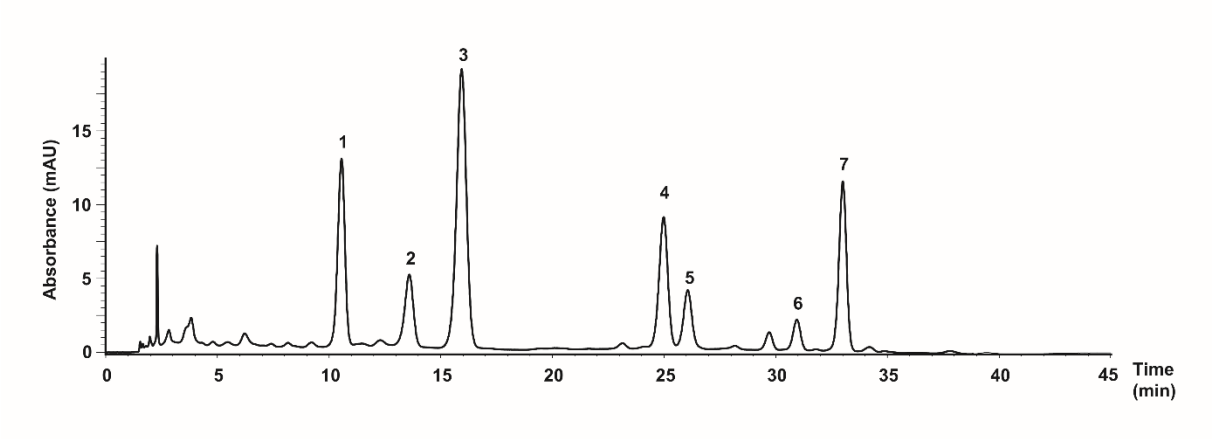


Figure 1. Chromatographic profile of the HPLC method: 0-17% B (0-0.01 min), 1 mL min^{-1} for 45 min at 30 °C with the injection volume of 20 µL. The chemical name and structure of the *Pterocaulon balansae* coumarins 1-7 are presented in **Table 2**.

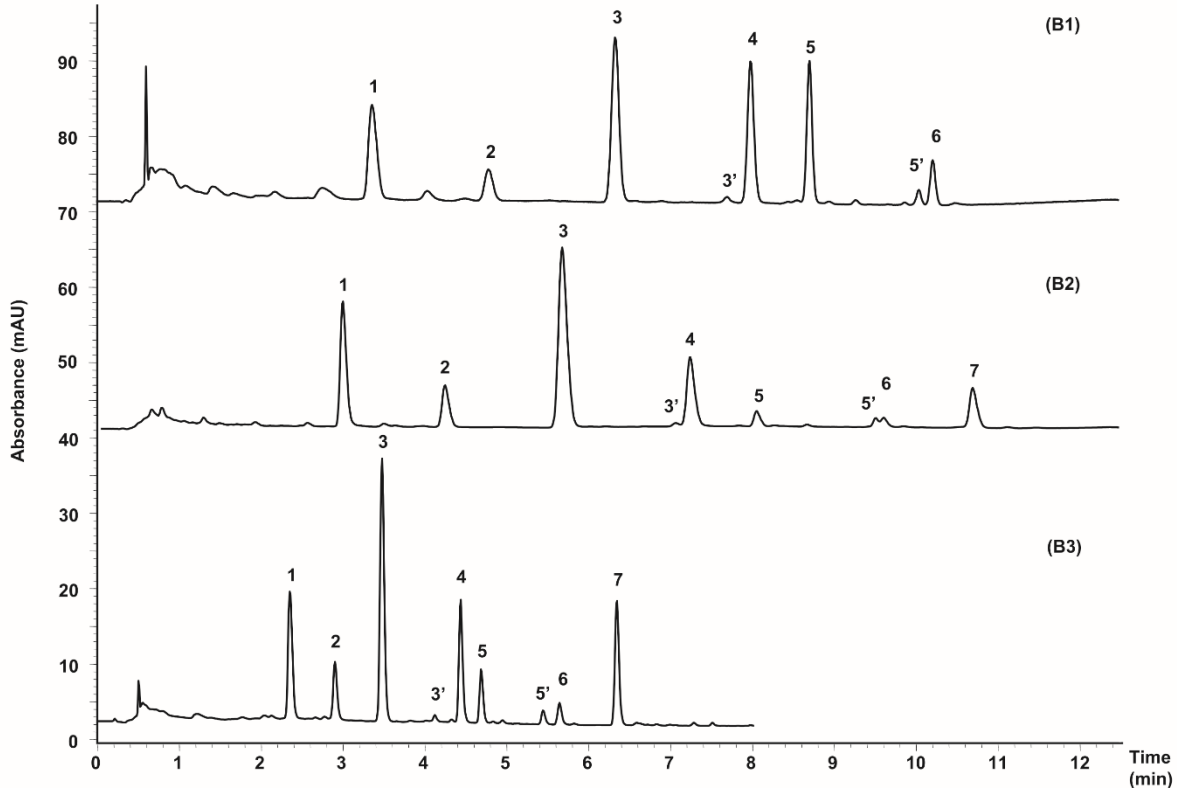


Figure 2. Chromatographic profile of the development of methods, where **B1.** UFLC Method: 0.45 mL min^{-1}, initial elution gradient of 0-17% B, oven temperature 55 °C, injection volume of 5µL and analysis time of 12.6 min; **B2.** UFLC Method: 0.55 mL min^{-1}, was increased to 0-17 % B (0-0.05 min), **B3.** UFLC Method: 0.55 mL min^{-1} up to 8 min, injection volume 5 µL, and the analysis was carried out 55 ºC. The peaks 3' and 5' correspond to coumarins, with maximum UV absorption at 244/336 and 245/ respectively. However, these compounds are in low amount in the aqueous extract for a complete structural elucidation.

3.2. Method validation

3.2.1. *Specificity*

The retention times for coumarin peaks in HPLC were 10.55, 13.59, 15.92, 24.97, 26.04, 30.94 and 32.99 for 1, 2, 3, 4, 5, 6 and 7, respectively. The retention times for UFLC were 2.34, 2.89, 3.46, 4.43, 4.68, 5.63 and 6.33 and characteristic λ_{max} (maximum UV absorption) at 229/293/343, 239/327, 228/327, 240/325, 236/292/341, 243/324 and 235/325 nm for 1, 2, 3, 4, 5, 6 and 7, respectively. There was no matrix interference, showing that the peaks of coumarins were free from any substance that may be co-eluted, demonstrating that the proposed method is specific for the simultaneous analysis of coumarins of *P. balansae* in all matrices analyzed.

3.2.2. Linearity, precision and accuracy

The linearity of these methods, shown in **Table 3**, was assessed by means of the concentration of 5MMDC *versus* the corresponding mean peak area. The standard calibration curve exhibited an excellent linearity and a good determination coefficient for all the standard compounds over the given range of 0.1–7.5 µg/mL of 5MMDC. The sensitivity of these methods was evaluated by determining the limits of detection (LOD) and quantification (LOQ) calculated by standard curves, as presented in **Table 3**.

The precision and accuracy of post-extraction spiked-matrices were evaluated at four levels: 5MMDC stock solution was tested at concentrations of 0.1 µg mL^{-1}, 0.5 µg mL^{-1}, 2.5 µg mL^{-1} and 5.0 µg mL^{-1} for each sample. The repeatability of the method for 5MMDC was performed by comparing the relative standard deviation (% R.S.D.) of nine determinations per concentration for three consecutive days. For the intermediate precision were compared standard deviations of the determinations of each concentration of the standard curves for three consecutive days. The results showed % R.S.D less than 2% for experiments on the same day or three different days, indicating that both methods are precise, according to the official codes (ICH, 2005). The results, shown in the **Table 3,** were evaluated by means of an ANOVA single factor.

The accuracy results for 5MMDC in all matrices were within the 99.31 to 102.04 % range for the HPLC, and the 102 to 106 % range for UFLC. Despite the complexity of the different matrices, the UFLC method can be considered precise and accurate according to official guidelines.

3.2.3. Robustness

The robustness of an analytical procedure was a measure of its ability to remain unchanged for small, deliberate variations in method conditions, representing the reliability under normal conditions of use. A multivariate approach using design of experiments is often recommended in robustness testing since a number of different factors can be analyzed concurrently with a reduced number of experiments. As shown in **Figure 3**, in both methods no significant factors were revealed for all analyses as the calculated t-values were lower than the t-critical values ($\alpha = 0.05$). Thus, there were no significant changes in the assay results in terms of the percentage of 5MMDC contents with the changes made in the experimental conditions, thereby demonstrating the proposed method's robustness.

3.2.4. Assessment of the matrix effect

An important issue in the method development is the possible occurrence of matrix effect. For this reason, during the development of an analytical and bioanalytical method, it is essential to consider the effect of all matrices involved, which can be easily detected by comparing the response obtained from a standard solution with that from a spiked pre-treated sample. When the response range is between -20% < ME% <20%, the matrix effects is deemed low; when it is between -50% < ME% <-20% or 20%> ME% > 50%, it is considered medium, and when it is between ME% < -50% or ME% > 50%, it is considered high (NIESSEN; MANINI; ANDREOLI, 2006; WATANABE et al., 2014). The matrix effects for 5MMDC present in the AE, SFE, NE_B, P. E/D skin layer, P. SC layer after tape stripping process and RF were expressed by ME% and are presented in **Table 3**. The data indicate that samples exhibited low matrix effects in HPLC (ME% > -11.39) and UFLC (ME% > -11.96).

3.2.5. Coumarin stability in matrices

The evaluation of the stability showed that the concentration of 5MMDC in different matrices remained constant after storage at 25 ºC for 48 h. Amounts of 5MMDC in all matrices after this period varied from 99.45% to 101.99%.

Table 3. Linearity data of the standard and the matrix effect for each matrix studied by the methods for HPLC and UFLC.

		Standard	**AE**	**NE_B**	**P. E/D**	**P. SC**	**RF**
HPLC	Equation	y = 52077x + 478.22	y = 46753x + 279.21	y = 47487x + 312.44	y = 54073x + 521.73	y = 49406 + 489.28	y = 52533 + 489.28
	R^2	0.999	0.998	0.997	0.999	0.997	0.997
	LOD	0.030	0.021	0.034	0.052	0.022	0.022
	LOQ	0.092	0.095	0.088	0.091	0.093	0.093
	ME (%)	-	-11.39	-9.67	3.69	-5.41	0.87
UFLC	Equation	y = 13142x + 120.20	y = 11137x + 131.40	y = 11973x + 420.40	y = 13611x + 120.45	y = 12249x + 131.40	y = 13245x + 340.40
	R^2	0.999	0.998	0.998	0.998	0.996	0.995
	LOD	0.030	0.022	0.025	0.034	0.031	0.023
	LOQ	0.091	0.094	0.092	0.093	0.090	0.091
	ME (%)	-	-11.96	-9.97	3.44	-5.57	0.78

AE: aqueous extract of *P. balansae*; NE_B: blank nanoemulsion; P. E/D: porcine epidermis/dermis; P. SC: porcine stratum corneum after *tape stripping* process; RF: receptor fluid; R^2: determination coefficient; LOD: limit of detection; LOQ: limit of quantification; ME: matrix effect.

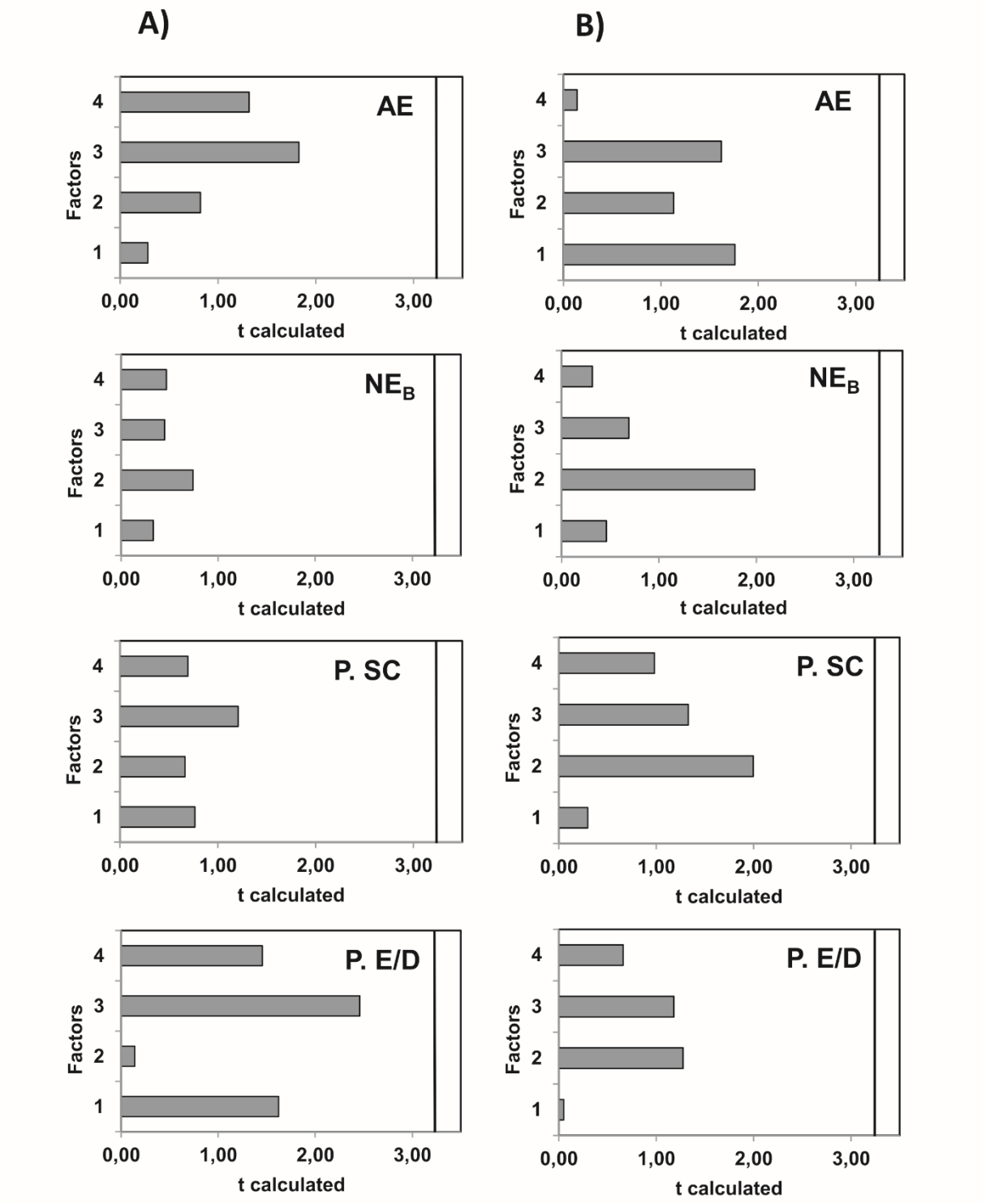


Figure 3. Bar charts representing the t-calculated for quantitative determination (assay) of the investigated factors (1, 2, 3, and 4) in Plackett-Burman experimental design and their t-critical, represented by the vertical line. Column **A** represents the HPLC method and **B** the UFLC method, where, 1: column oven temperature, 2: initial flow rate, 3: AF concentration, 4: initial organic composition, AE: aqueous extract of *P. balansae*; NE_B: blank nanoemulsion; P. E/D: porcine epidermis/dermis; P. SC: porcine stratum corneum after *tape stripping* process.

3.2.6. Coumarin extraction form skin layers

The recovery data for 5MMDC quantification after extraction of previously spiked-matrices are shown in **Table 4**, and was within FDA recommendations for bioanalytical method validation. Taken together, the recovery yields are satisfactory and demonstrated that 45 min was sufficient for the complete 5MMDC extraction from matrices, and no matrix components interfered during the procedure. Furthermore, 5MMDC recovery showed adequate precision in all assessed matrices (RSD < 8.83%).

Table 4. Recovery data of the 5MMDC in biological matrices by the methods for HPLC and UFLC.

Method	Nominal (μg mL¹)	Recovery (%) (RSD)	
		P. E/D skin layer	P. SC skin layer
HPLC	0.1	86.86 (3.43)	88.98 (5.12)
	0.5	90.48 (3.57)	91.63 (2.36)
	2.5	85.90 (2.52)	92.11 (6.45)
	5.0	89.14 (2.20)	84.10 (7.31)
UFLC	0.1	85.28 (4.51)	89.39 (6.19)
	0.5	88.78 (4.51)	87.63 (6.36)
	2.5	85.68 (3.95)	85.38 (7.55)
	5.0	85.04 (2.42)	85.27 (8.83)

P. E/D: porcine epidermis/dermis; P.SC: porcine stratum corneum after tape stripping process; RSD = relative standard deviation.

Table 5. Parameters of system suitability of the HPLC and UFLC methods for determination of 5MMDC in aqueous extract of *P. balansae*.

Parameters	**Method**	**1**	**2**	**3**	**3'**	**4**	**5**	**5'**	**6**	**7**	**Reference values**
Theoretical plates (*N*)	**A1**	4966	5845	6025	22754	18592	22713	42267	40136	44128	*N* > 2000
	B3	6967	12650	16349	38491	32751	39623	45354	43036	55950	
Resolution (R_s)	**A1**	-	4.64	3.05	10.03	2.75	1.51	5.76	2.07	3.30	R_s > 1.5
	B3	-	5.12	5.44	6.73	3.45	2.62	7.71	1.89	6.48	
Tailing factor (T)	**A1**	0.92	0.90	0.95	0.93	0.96	0.99	0.93	0.97	0.95	T < 1.5
	B3	1.40	1.38	1.30	1.29	1.38	1.36	1.36	1.35	1.31	
Retention time (min)	**A1**	10.55	13.59	15.92	23.11	24.97	26.04	29.70	30.94	32.99	-
	B3	2.34	2.89	3.46	4.11	4.43	4.68	5.43	5.63	6.33	

A1 = HPLC and **B3** = UFLC, n=6.

3.2.7. System suitability

The suitability of the system was verified by standard substances routine analysis carried out on the experimental conditions. The values of the parameters and variability (RSD%) of 5MMDC for each method were, HPLC: retention time 24.97 (0.04); theoretical plates 18592 (0.94); tailing factor 0.96 (0.10); resolution between pic 3' and 5MMDC 2.75 (0.61); resolution between 5MMDC and pic 5 1.51 (0.44), UFLC: retention time 4.43 (0.08); theoretical plates 32751 (1.59); tailing factor 1.38 (0.79); resolution between pic 3' and 5MMDC 3.45 (1.33); resolution between 5MMDC and pic 5 2.62 (0.42). The parameters (**Table 5**) indicate that the system is suitable for the analysis.

3.3. Method application

In vitro permeation studies were carried out in Franz-type diffusion cells to compare the effects of 5MMDC permeation/retention profile from nanoemulsions containing this coumarin or in the *P. balansae* extract. After 8 h, the retention of 5MMDC from NE_{AE} and NE_{5MMDC} was significantly different ($P < 0.05$) from the control with propyleneglycol, however, without statistical differences among formulations. The amount of 5MMDC retained on skin was approximately 3 μg/cm² for both formulations in both methods, as shown in **Table 6**.

The increase of the skin retention of bioactive compounds from nanoemulsions has been described in the literature and is probably due to the higher interaction of nanostructures with the stratum corneum improving the permeation/retention through the skin (DE VARGAS et al., 2012). The permeation of 5MMDC from formulations was also significantly different ($p < 0.05$) from the control with propyleneglycol. However, the amount of 5MMDC permeated through the skin from NE_{AE} was significantly lower ($p < 0.05$) (0.27 ± 0.03 μg/cm²) in comparison with the formulation containing the isolated coumarin (0.46 ± 0.06 μg/cm²). Taken together, these preliminary results suggest that the amount of 5MMDC retained in the skin was not influenced by the presence of the other components of *P. balansae*; however, the amount of 5MMDC permeated was

lowered in the presence of these compounds. In the **Table 6**, we can see that HPLC and UFLC methods were able to quantify the 5MMDC in nanoemulsions NE_{AE} and NE_{5MMDC} in amount of approximately 0.46 mg mL^{-1} in both formulations. The values of 5MMDC were not significantly different ($P > 0.05$) between methods, demonstrating interchangeability of the methods.

Table 6. Determination of the 5MMDC in real samples.

Application of methods		Mean of six replicates (RSD)	
		HPLC	UFLC
Samples			
NE_{5MMDC} (mg mL^{-1})		0.45 (1.51) [f]	0.46 (1.67) [f]
NE_{AE} (mg mL^{-1})		0.46 (1.45) [g]	0.47 (1.34) [g]
Skin retention			
Total skin (µg $cm^{2\ -1}$)	NE_{5MMDC}	2.84 (17.46)[a b]	2.82 (16.49)[a b]
	NE_{AE}	3.02 (15.86)[a b]	3.00 (18.24)[a b]
RF (µg $cm^{2\ -1}$)	NE_{5MMDC}	0.46 (12.94) [c e]	0.43 (11.76) [c e]
	NE_{AE}	0.27 (9.82) [d e]	0.28 (14.82) [d e]

[a] NE_{AE} and NE_{5MMDC} were no significantly different ($P < 0.05$)
[b] NE_{AE} and NE_{5MMDC} were significantly different ($P < 0.05$) from the control with propyleneglycol
[c and d] NE_{AE} and NE_{5MMDC} were significantly different ($P < 0.05$)
[e] NE_{AE} and NE_{5MMDC} were significantly different ($P < 0.05$) from the control with propyleneglycol
[f] NE_{5MMDC} in HPLC and UFLC method were no significantly different ($P < 0.05$)
[g] NE_{AE} in HPLC and UFLC method were no significantly different ($P < 0.05$)
RF: receptor fluid

4. Conclusions

This study showed the development and validation of LC methods for the determination of coumarins from aqueous extracts of *P. balanseae* in analytical and bioanalytical samples. The determination of coumarins was based on analytical validation carried out for 5-methoxy-6,7-methylenedioxy coumarin (5MMDC), a phytochemical marker extracted from this medicinal plant. Both HPLC and UPLC proved to be linear, precise, accurate and robust to estimate coumarins in different matrices without significant differences between the methods developed. Thus, the methods seem to be analytically

interchangeable. However, UPLC method was faster (almost 4-fold) and consumes less eluent being more eco-friendly in comparison with traditional HPLC.

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Capítulo III

Emprego da green chemistry *para obtenção de extrato rico em cumarinas através da extração por SC-CO_2*

3.1. INTRODUÇÃO

O terceiro capítulo desta tese foi realizado em colaboração com o Dr. Fernando Torres, orientado da Prof. Gilsane von Poser, que no âmbito da sua tese de doutorado estudou cumarinas isoladas de *Pterocaulon* e cumarinas sintéticas.

A parceria deste trabalho surgiu no sentido de explorar técnicas alternativas para obtenção de cumarinas de *P. balansae* que seguissem as diretrizes das tecnologias verdes. Até o momento, o que existia reportado na literatura para a espécie *P. balansae* eram métodos de extração convencionais (maceração estática, Soxhelt) empregando solventes orgânicos (hexano, metanol, diclorometano) para a obtenção de extratos e frações de *P. balansae* ricos em cumarinas.

A ideia de trabalhar com extratos "mais limpos" surgiu durante a minha dissertação de mestrado, quando desenvolvemos um método de maceração a quente sob agitação utilizando água como solvente, com a finalidade de obter um extrato rico em cumarinas a partir das partes aéreas de *P. balansae*. Este trabalho, publicado em 2015 na revista *Molecules*, despertou o interesse em explorar novas metodologias para obtenção de extratos com menor geração de resíduos na natureza.

Atualmente, as discussões sobre o emprego de tecnologias verdes vêm tomando força e os pesquisadores têm voltado seus projetos para o desenvolvimento de novas técnicas para obtenção de compostos ativos a partir das fontes naturais. Essas iniciativas buscam a otimização de processos por meio da inovação, utilização de energias renováveis e emprego de solventes alternativos (água, CO_2, agro solventes), resultando num menor impacto no meio ambiente (CHEMAT; VIAN; CRAVOTTO, 2012; CUE; ZHANG, 2009; TOBISZEWSKI; NAMIEŚNIK, 2017).

A partir do contexto apresentado, esta parte do trabalho realizado em parceria teve por objetivo a extração de cumarinas das partes aéreas de *P. balansae* por SC-CO_2, otimizando as condições de extração para a maior recuperação dos compostos. O capítulo está escrito no formato de artigo seguindo as normas da revista em que foi publicado: *Journal of CO_2 Utilization.*

1.3. ARTIGO

Publicado na revista Journal of CO_2 Utilization

Supercritical CO_2extraction as a selective method for the obtainment of coumarins from *Pterocaulon balansae* (Asteraceae)

Supercritical CO_2 extraction as a selective method for the obtainment of coumarins from *Pterocaulon balansae* (Asteraceae)

Fernando Cidade Torres[a], Bruna Medeiros-Neves[a], Helder FerreiraTeixeira[a], Daniel Kawanoa[a,b], Vera Lucia Eifler-Lima[a], Eduardo Cassel[c], Rubem Mário Figueiró Vargas[c], Gilsane Lino von Poser[a]

[a] Graduate Program in Pharmaceutical Sciences, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

[b] Pharmaceutical Sciences Faculty, State University of Campinas, Campinas, SP, Brazil. [c] Graduate Program of Matherials Engineering and Technology, Pontifical Catholic University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

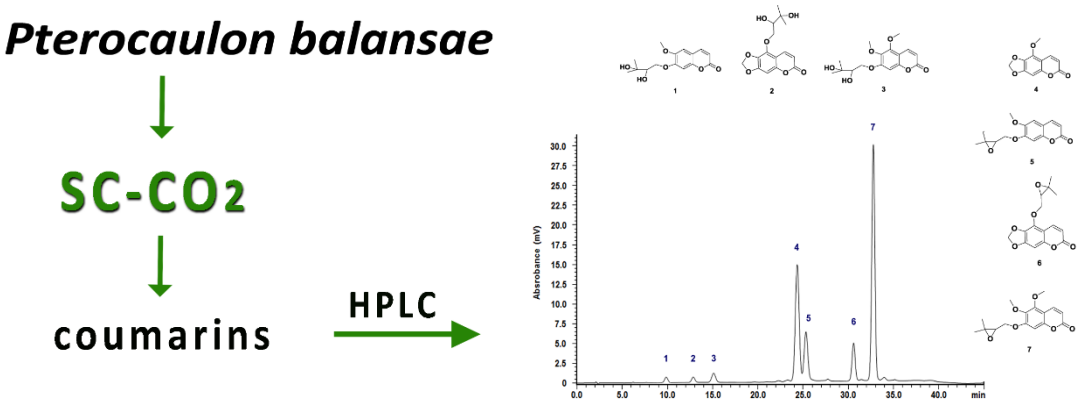
Corresponding author. Tel.: +55 51 33085289; fax: +55 51 33085437. E-mail address: gilsane@farmacia.ufrgs.br (G.L. von Poser).

Abstract

Aiming to recover fractions enriched with coumarins, the aerial parts of *Pterocaulon balansae* Chodat (Asteraceae) were subjected to supercritical fluid extraction (SFE) using CO2 as solvent under constant temperature (40 ^{o}C) and gradual pressure increments (90, 120, 150 and 200 bar). The yields of the coumarins in the extracts were calculated using high-performance liquid chromatography analyses, which allowed the identification of seven coumarins previously reported by our research group. Coumarin 7-((3,3- dimethyloxiran-2-yl)methoxy)-5,6-dimethoxy-2*H*-chromen-2-one was identified as the major component of all extracts but we also identified considerable amounts of epoxy- substituted coumarins. The mild conditions provided by the supercritical CO_2 extraction (SC-CO_2 extraction) may considerably reduce the ring-opening of the epoxy groups in the coumarins from the aerial parts of *P. balansae,* when compared to the conventional extraction procedures.

Keywords: *Pterocaulon balansae*; coumarins; supercritical fluid extraction; carbon dioxide; epoxy-coumarins.

Graphical abstract



Highlights

- *Pterocaulon balansae* was subjected to CO_2 supercritical fluid extraction.

- Coumarins were efficiently recovered and quantified by HPLC.

- The method proved to be effective to obtain coumarins containing epoxy group.

1. Introduction

Supercritical fluid extraction using carbon dioxide as solvent (SC-CO_2 extraction) can be an useful tool to increase both the yields and the selectivity profile in the extraction of organic compounds from plants [1,2]. SC-CO_2 extraction has been successfully used by our research group for the extraction of phenolic compounds from *Hypericum* species [3- 5]. Given the importance of coumarins for the cosmetic and pharmaceutical industries and the increasing demand for green chemistry, several studies have highlighted the value of SC-CO_2 extraction for the isolation of coumarins [6-11]. Nonetheless, no study has been performed concerning the use of this tool in the extraction of coumarins from *Pterocaulon* species. This species shows a distinct chemical profile, mainly characterized by 6,7-dioxygenated, 5,6,7-trioxygenated and 6,7-methylenedioxy coumarins [12,13] for which we have a particular interest due to their activities against cancer cells and pathogenic fungi [14,15]. Additionally, plants from the genus *Pterocaulon* (Asteraceae) are used in veterinary medicine in the South region of Brazil to treat animal skin diseases, popularly known as “mycoses” [16].

In a previous study, our research group developed a method to characterize coumarins from *Pterocaulon balansae* by UPLC-UV-MS, highlighting the existence of seven coumarins (Fig. 1) in the aqueous extract [17]. Coumarins **1**, **2**, **4**, **5** and **6** were previously described for *P. balansae* [18,19] and some studies also reported the quantification of these compounds in this species [17,20].

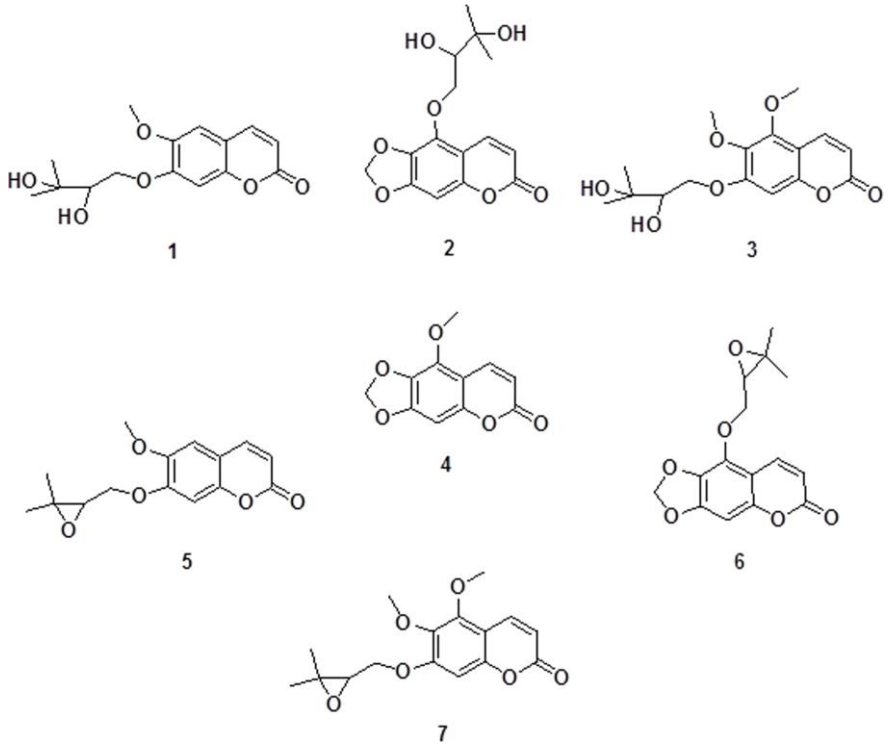


Fig. 1. Coumarins from *P. balansae.*

Through the use of HPLC/UV, Barata-Vallejo [20] identified 7-(2,3-dihydroxy-3-methylbutyloxy)-6-methoxycoumarin (**1**) and 5-methoxy-6,7-methylenedioxycoumarin (**4**) as the main compounds in the dichloromethane extract of *P. balansae*. Compound **2**, 5-(2,3-dihydroxi-3-methylbutyloxy)-6,7-methylenedioxycoumarin and the epoxy- substituted coumarins 7-(2,3-epoxy-3-methyl-3-butyloxy)-6-methoxycoumarin (**5**) and 5-(2,3-epoxi-3-methylbutyloxy)-6,7-methylenedioxycoumarin (**6**) were also reported as minor compounds. Coumarin **3** (5,6-dimethoxy-7-(3'-methyl-2',3'-dihydroxybutyloxy) coumarin) was first isolated from *P. balansae* by our research group, while the existence of coumarin **7** (5,6-dimethoxy-7-(2',3'-epoxy-3-methylbutyloxy) was never reported so far [17]. In this study, we performed the SC-CO_2 extraction of coumarins from the aerial parts of *P. balansae*, refining the conditions of extraction for the maximal recovery of these biologically active compounds.

2. Materials and Methods

2.1. Materials

Water (Mili-Q®), acetonitrile (Tedia, HPLC grade, USA) and acetic acid (Nuclear, Brazil) were used as the mobile phase in the chromatographic procedures. cLogP (calculated LogP) values of coumarins were assigned using ALOGPS (http://www.vcclab.org/lab/alogps/), a very reliable tool to predict LogP values of organic compounds [21]. Carbon dioxide 99.9% (Air Products) was used in the supercritical fluid extractions.

2.2. Plant material

Aerial parts of *P. balansae* were collected in the summer in Canoas, a city in the Rio Grande do Sul State, Brazil (February, 2013), dried at room temperature and powdered in a cutting mill. The species was identified by Dr. Sérgio Bordignon (Centro Universitário La Salle - Canoas, UNILASALLE, Brazil). Voucher specimen was deposited in the herbarium of the Universidade Federal do Rio Grande do Sul (ICN 157762). The collection of the plants was approved by the Ministério do Meio Ambiente – MMA, Instituto Chico Mendes de Conservação da Biodiversidade – ICMBio. Sistema de Autorização e Informação em Biodiversidade – SISBIO (number 38017-1).

2.3. Pterocaulon balansae extraction

SC-CO2 extraction was carried out on a pilot-scale automated equipment according to procedures previously described [22]. Powdered plant material (26.5 g DW) was extracted at constant temperature of 40 ^{o}C, while the pressure was successively increased from 90 to 200 bar. The extraction was performed according to the experimental procedure defined by Cargnin et al. [3] and the duration of the each condition was determined experimentally observing the increase of mass in the collector vessel, until a constant weight was observed. The extraction yield was determined by dividing the mass of extract by the mass of the dry material.

The supercritical carbon dioxide flow rate was $6.7x10^{-4}$ kg s^{-1} (through the extraction vessel) using a flowmeter assay (Sitraus F C Massflo 2100–Siemens) with accuracy of 0.1%. The pressure in the extractor was monitored with a digital transducer system, Novus 8800021600, acquired from Novus Produtos Eletrônicos (Brazil) with a precision of ±1.0 bar. The temperature controller was connected to the thermocouples (PT-100) with an accuracy of <0.5 ^{o}C.

2.4. Quantification of coumarins

The CO_2 extracts were treated with acetone to remove waxes and the acetone-soluble fractions were evaporated to dryness. For the HPLC analyses, samples were prepared in acetonitrile at concentration of 200 µg/mL. Analyses were performed using Schimadzu Proeminence LC-10A system (Kyoto, Japan) equipped with a model LC-20AT pump and a SPD-20AV ultraviolet-visible variable wavelength detector (327 nm). The separation of the compounds was developed in Phenomenex-C18 Synergi column (150 mm × 4.6 mm, 4 µm) coupled to a refillable pre-column filled with C18 silica, using a gradient of acetic acid 2% (A) and acetonitrile (B) filtered and degassed. LC system operated at a flow rate of 1 mL/min for 45 min at 30 °C with the injection volume of 20 µL. To build the calibration curve, we used a sample of the previously isolated 5-methoxy-6,7- methylenedioxycoumarin (**4**), as reported by Medeiros-Neves et al. [17].

2.5. Statistical analysis

Means difference among each condition of extraction was tested for significance by ANOVA using a probability value of $P < 0.05$. Tukey's test was used to ascribe the mean separation among these conditions (GraphPad Prism Software, version 5).

3. Results and Discussion

In line with our previous studies, in this paper we describe the extraction of coumarins from *P. balansae* using SC-CO_2 extraction. So far, the coumarins reported for this

species are usually hydrophobic compounds [17,20] and, accordingly, we hypothesized that supercritical CO_2 could be an ideal solvent to extract them. Although Bajerová et al. [23] reported that SC-CO_2 extraction was not suitable for isolation of coumarins, Oliveira et al. [11] demonstrated the opposite by achieving higher extraction yields with SC-CO_2 extraction when compared to the extraction with organic solvents using conventional maceration, maceration with sonication and Soxhlet extraction.

The conditions we used for the SC-CO_2 extraction were similar to those employed for the isolation of similar lipophilic compounds. The time of each extraction was experimentally determined by the absence of variation in total accumulated mass after three consecutive weighings. The yield of the extracts in the end of the process was 3.17 %. Table 1 illustrates the influence of each condition in the recovery of the crude extracts, highlighting an optimal yield for a pressure of 120 bar. The extraction performed at this condition was, about 1.5, 2 or 3 times more efficient than those performed at 90, 200 and 150 bar, respectively. The reduction of the extraction yield at 150 bar and the subsequent increment at 200 bar can be explained by the extraction of higher molecular weight and/or internalized compounds. The extraction rate decreased during the development of the process. This fact was expected, since the extractable material present in this plant decreased as the extraction was performed and the removal rate was proportional to that remaining amount.

Table 1. Yields (w/w%) and recovery rates (g/min) observed for the crude extracts from *P. balansae* using different pressure levels in the SC-CO_2 extraction.

Pressure (bar)	Yield (w/w%)	Recovery rate (g/min)	Extraction time (min)
90	0.81	0.0143	15
120	1.26	0.0083	40
150	0.40	0.0030	35
200	0.70	0.0018	105

The amount of the coumarins in the extracts was calculated by dividing the mass of each compound by the total mass of the plant (Table 2). The characterization of the

coumarins was performed by comparing their retention times in the HPLC with those previously reported by Medeiros-Neves et al. [17]. In Fig. 2 we present the chromatographic profile of the fraction obtained at 150 bar. As highlighted in Table 2, seven coumarins were identified in the SC-CO_2 extracts (corresponding structures are shown in Fig. 1**)**. The SC- CO_2 extraction performed at 120 bar displayed the optimal recovery rates for compounds **4**, **5**, **6** and **7**, the main coumarins isolated from the SC-CO_2 extracts.

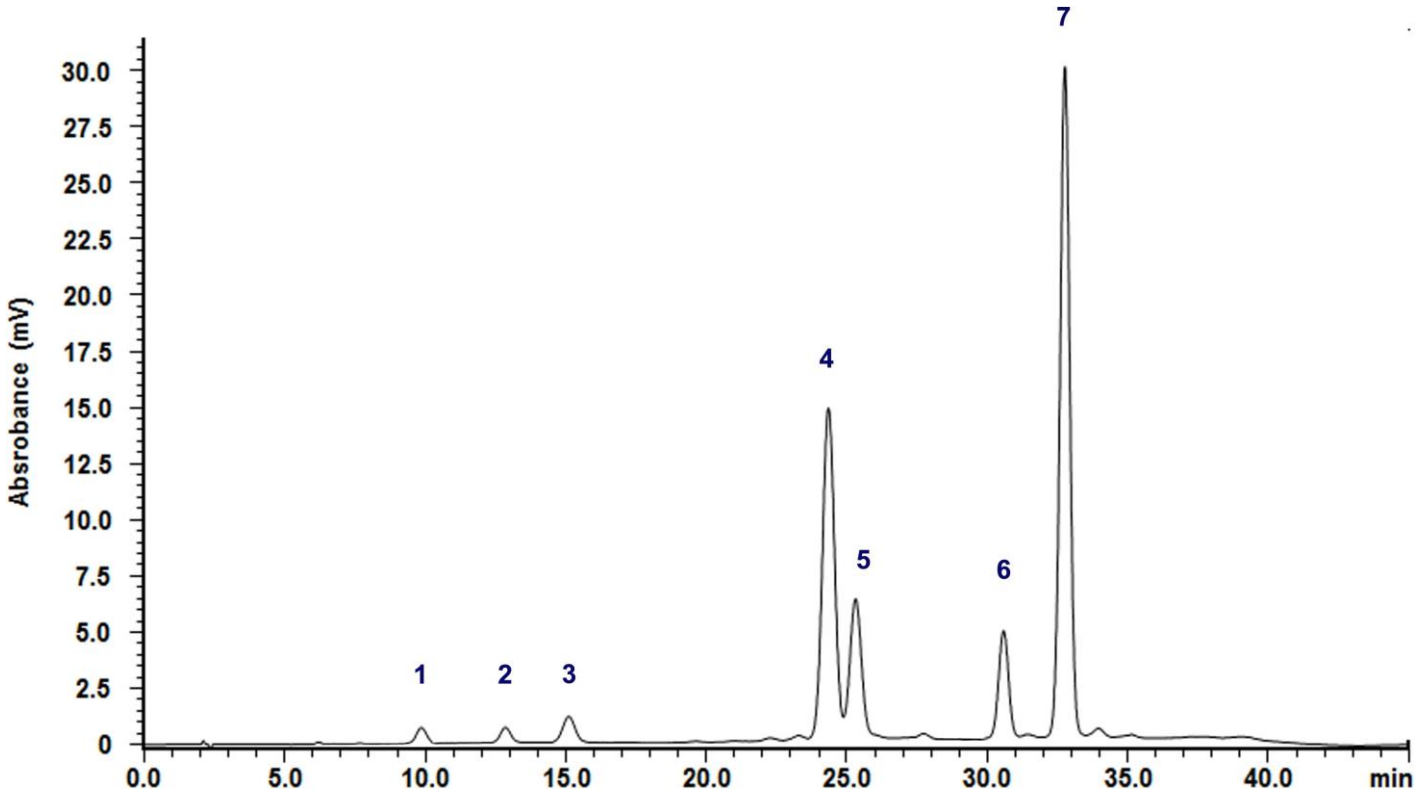


Fig. 2. HPLC chromatographic profile of the fraction obtained at 150 bar.

Table 2. Summary of the coumarin contents (mg/g*) in the SC-CO_2 extracts from *P. balansae*. $^{(a,b,c,d)}$. Different letters indicate significant differences among the different pressures ($P < 0.05$).

	90 bar	**120 bar**	**150 bar**	**200 bar**	**Total yield**
1	0.0462 ± 0.00035^{a}	0.0345 ± 0.0037^{b}	0.0210 ± 0.0002^{c}	0.046 ± 0.0004^{a}	0.1477 ± 0.0032
2	0.0287 ± 0.0003^{a}	0.0319 ± 0.0004^{b}	0.0166 ± 0.0002^{c}	0.034 ± 0.0004^{d}	0.1112 ± 0.0029
3	0.1361 ± 0.0008^{a}	0.1221 ± 0.0016^{b}	0.0454 ± 0.0006^{c}	0.073 ± 0.0011^{d}	0.3766 ± 0.0009
4	0.3436 ± 0.002^{a}	0.7195 ± 0.037^{b}	0.3166 ± 0.0011^{a}	0.539 ± 0.0062^{c}	1.9187 ± 0.0091
5	0.2693 ± 0.00034^{a}	0.6159 ± 0.0095^{b}	0.1718 ± 0.0015^{c}	0.246 ± 0.0021^{d}	1.303 ± 0.0032
6	0.2135 ± 0.0003^{a}	0.4087 ± 0.0005^{b}	0.1166 ± 0.0030^{c}	0.171 ± 0.0009^{d}	0.9098 ± 0.0005
7	1.5142 ± 0.0008^{a}	1.9001 ± 0.0013^{b}	0.4389 ± 0.000^{c}	0.688 ± 0.0011^{d}	4.5412 ± 0.0001
∑	**2.5516**	**3.8327**	**1.1269**	**1.7970**	**9.3082**

* Extraction yield (mg/g) = amount of target compound/plant mass

To highlight the selectivity of the SFE concerning the recovery of the coumarins, we expressed the quantification of the target compounds by the total mass of the extract (Table 3). According to these results, the total amount of coumarins in the extract was approximately 30%, being coumarin **7** the most prevalent compound independently of the system pressure. While the more lipophilic coumarins (**5**, **6** and **7**) were preferentially extracted at intermediate pressure conditions (i.e. 120 and 150 bar), a pressure of 200 bar was optimal for the extraction of more polar compounds (**1** and **2**).

Table 3
Summary of the coumarins selectivity (%) in SC-CO_2 extracts. Different letters (a,b,c,d) indicate significant differences among the different pressures ($P < 0.05$).

	90 bar	120 bar	150 bar	200 bar	Total
1	0.5703^{a}	0.2738^{b}	0.5250^{c}	0.6571^{d}	**0.47%**
2	0.3543^{a}	0.2532^{b}	0.4150^{c}	0.4857^{d}	**0.35%**
3	1.6802^{a}	0.9690^{b}	1.1350^{c}	1.0428^{d}	**1.19%**
4	4.2419^{a}	5.7103^{b}	7.9150^{c}	7.7000^{c}	**6.05%**
5	3.3247^{a}	4.8881^{b}	4.2950^{c}	3.5143^{d}	**4.11%**
6	2.6358^{a}	3.2436^{b}	2.9150^{c}	2.4428^{d}	**2.87%**
7	18.6938^{a}	15.0801^{b}	10.9725^{c}	9.8286^{d}	**14.32%**

* Selectivity (%) = amount of target compound/100g of extract

The chemical composition of the extracts we described here was somewhat divergent from the study reported by Barata-Vallejo [20] using chloroform as solvent. The authors isolated eight coumarins from *P. balansae*, being coumarin **1** the most abundant. On the other hand, Medeiros-Neves et al. [17] reported coumarin **3** as the major compound in the aqueous extract of *P. balansae*. To critically understand such differences, we must highlight that the epoxy-substituted coumarins **5**, **6** and **7** may yield their more polar analogues (**1**, **2** and **3**, respectively) via hydrolysis of the epoxy ring. In this context, using SC-CO_2 extraction we recovered about nine times more the

epoxy-substituted precursor **5** than its corresponding hydrolytic product **1**, the most prevalent coumarin from *P. balansae* according to Barata-Vallejo [20].

The lower amounts of epoxy-substituted coumarins in the aqueous extracts of *P. balansae* [17] when compared to their putative hydrolytic products suggests that SC-CO_2 extraction can protect these epoxy coumarins from degradation under the mild conditions necessary for the extraction. This high instability of the epoxy rings, which can be easily hydrolyzed, is due to the high constraint inherently associated with a three-member ring. According to Musto et al. [24], epoxy rings will open when exposed to ultraviolet radiation, weak acids or bases, water or even to high temperatures. Considering the conditions routinely performed for the isolation of natural products (e.g. acid-base extraction), it is very feasible to assume that coumarins **1**, **2** and **3** can be produced by the hydrolysis of their epoxy-substituted analogues **5**, **6** and **7**, respectively.

We must stress that SC-CO_2 extraction may not totally prevent the hydrolysis of epoxy compounds since CO_2 dissolves in water, then generating carbonic acid [25]. Therefore, supercritical CO_2 may react with moisture to form H_2CO_3 but, since the amount of the acid will be limited by the water content in the gas phase, labile compounds will be exposed to a much lower concentration of the acid in SC-CO_2 extraction than when they are subjected to an acid-base extraction, for example. Considering the possibility of acid catalysis both in SC-CO_2 extraction and conventional extraction procedures, we suggested the mechanism for the hydrolysis of coumarins **5** to yield coumarin **1** (Fig. 3).

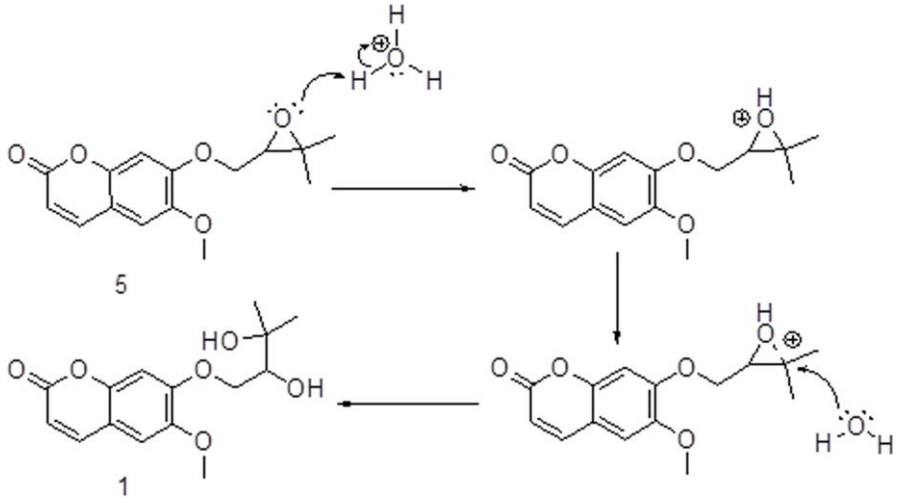


Fig. 3. Mechanism of epoxide ring opening under acid catalysis.

This mechanism includes the protonation of the epoxy oxygen, which favors the nucleophilic attack of water on the most substituted carbon because this is the carbon that holds the greatest degree of positive charge (i.e., due to inductive effect of the methyl groups, this is the carbon atom that will better accommodate the positive charge). Accordingly, the same mechanism is proposed for the conversion of coumarins **6** and **7** into their putative hydrolytic products, coumarins **2** and **3**, respectively. In fact, a similar behavior was observed for compound **2** (about eight times less recovered than its precursor **6**) and compound **3** (about 13 times less recovered than the precursor **7**) (Table 2).

The lower amounts of coumarins **1**, **2** and **3** in the SFE extracts could also be explained by their physicochemical properties, such as molecular weight and polarity. Due to the vicinal diols in their side chains, comarins **1**, **2** and **3** are expected to be more polar than their corresponding epoxy precursors (see average cLogP, Fig. 3). In fact, epoxy hydrolases found in mammals, fungi and bacteria provide a mechanism of detoxification by converting epoxides into their more hydrophilic diols derivatives [26]. For compounds with similar molecular weights, non-polar or slightly polar molecules are preferentially solubilized by SC-CO_2 extraction [27]. Therefore, it seems reasonable to expect a higher extraction of epoxy coumarins under SC-CO_2 extraction in comparison to the corresponding diols.

It can be observed from Fig. 4 that all coumarins have similar molecular weights, ranging from 276.28 to 324.33 g/mol, suggesting that this property probably does not have a significant impact in the extraction process. The influence of functional groups in the 2*H*- chromen-2-one nucleus concerning the solubility of coumarins in supercritical CO_2 was initially reported by Yoo et al. [28] and further corroborated by Choi et al. [29]. The authors investigated substitutions at the positions 4, 6 and 7, concluding that functional groups at position 7 tend to reduce the solubility in the following order: methyl > methoxy> hydroxyl, suggesting that as the polarity of the functional groups on the coumarin derivatives is increased, compounds become less soluble in supercritical CO_2. Moreover, the authors concluded that any substitution at the position 4 in the coumarin core decreases the solubility in supercritical CO_2.

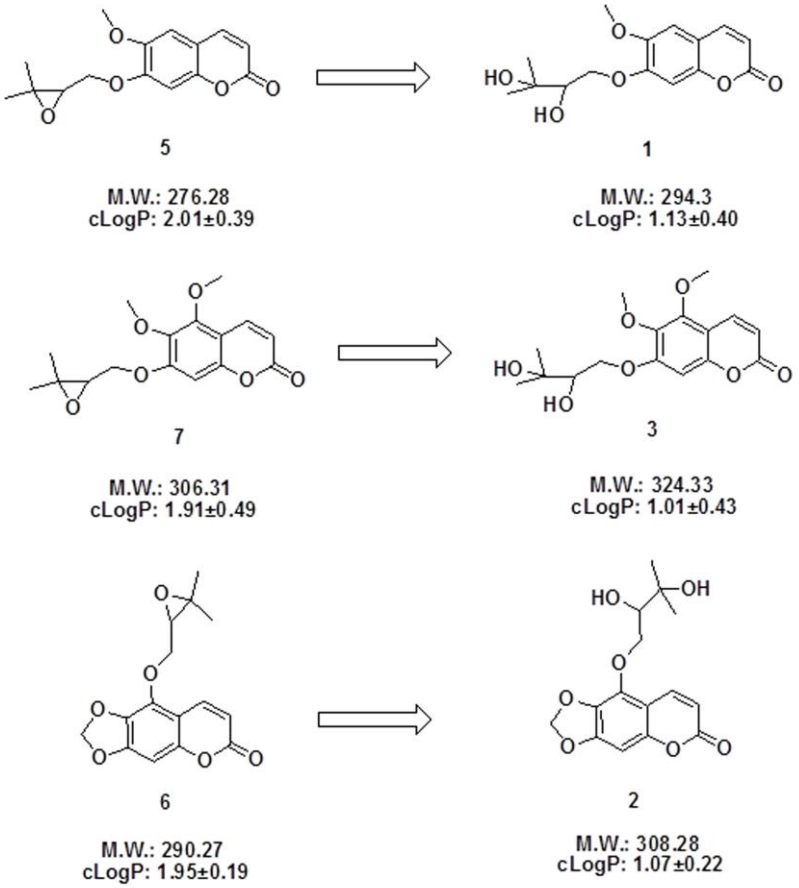


Fig. 4. Precursors **5**, **6** and **7** and their putative hydrolytic products **1**, **2** and **3**.

4. Conclusion

In summary, the physicochemical characteristics of SC-CO_2 provided good conditions for the isolation of the *Pterocaulon* coumarins, allowing mild extraction conditions to protect epoxy-substituted coumarins from hydrolysis into their diols derivatives. Considering the relevance of coumarins for the cosmetic and pharmaceutical industries, it is important to develop new methods for the extraction of these compounds. In this context, the extraction of coumarins of *P. balansae* with supercritical fluid was efficient and is in agreement with the current world demand for a clean technology since it does not use organic solvents and minimizes subsequent steps of solvent removal.

Acknowledgments

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Capítulo IV

Desenvolvimento de nanoemulsões de uso tópico contendo extrato de *Pterocaulon balansae* para o tratamento de infeções por *S. schenckii*

4.1. INTRODUÇÃO

Em continuidade aos estudos da tese, visando o tratamento tópico do fungo *Sporothrix schenckii*, este capítulo aborda a viabilidade de incorporação de extratos de *P. balansae* em nanoemulsões lipídicas. As nanoemulsões têm sido consideradas potenciais sistemas para administração tópica de moléculas, tanto de caráter lipofílico quanto hidrofílico (ROBERTS et al., 2017). Esses sistemas são dispersões nanométricas de gotículas oleosas em uma fase aquosa externa, estabilizada por um sistema tensoativo. Além disso, possuem uma ação promotora de absorção dos componentes das nanoemulsões (óleos e tensoativos), através do reduzido tamanho dessas estruturas, possibilitando a formação de um depósito mais uniforme sobre a pele e uma maior superfície de contato em comparação com os sistemas convencionais (PURI *et al.*, 2009; ZHAI & ZHAI, 2014; ROBERTS et al., 2017).

Em etapa anterior a esse capítulo, empregando novas técnicas verdes para extração de ativos naturais, realizamos um estudo de otimização de um extrato aquoso de *P. balansae* rico em cumarinas empregando o modelo matemático de Box Behnken Design (BBD). Os resultados apontaram para a condição ótima de extração do extrato aquoso com o maior conteúdo de cumarinas totais. A condição otimizada foi 4h07min, na proporção de 2% (material vegetal:solvente), a 65 ºC (MEDEIROS-NEVES, dados não publicados). Seguindo a linha de tecnologias verdes, recentemente publicamos um estudo apresentando uma nova técnica para extração de cumarinas de *P. balansae*, por meio da extração por fluido supercrítico. Foram testadas algumas condições de temperatura e pressão, das quais selecionou-se a pressão de 120 bar a 40 ºC, condição em que o extrato apresentou o maior conteúdo de cumarinas totais.

Neste capítulo, visando o tratamento das infecções subcutâneas causadas pelo fungo *S. schenckii*, desenvolvemos duas nanoemulsões pelo método de homogeneização à alta pressão incorporando esses diferentes extratos (extrato aquoso e extrato por fluido supercrítico). O perfil de permeação/retenção dessas cumarinas nas diferentes camadas da pele foi analisado em duas situações: na pele

intacta e na pele lesada. O capítulo a seguir está apresentado no formato de manuscrito.

4.3. ARTIGO

Manuscrito em preparação

Topical nanoemulsions as delivery systems for Pterocaulon balansae extracts aiming at the treatment of sporotrichosis

Topical nanoemulsions as delivery systems for *Pterocaulon balansae* extracts aiming at the treatment of sporotrichosis

Bruna Medeiros-Neves[a], Daiane Heidrich[b], Roselena Silvestri Schuh[a], Flávia N.S. Fachel[a], Eduardo Cassel[c], Rubem Mário Figueiró Vargas[c], Maria Lúcia Scroferneker[b], Gilsane Lino Von Poser[a], Helder Ferreira Teixeira[a,*]

[a] Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, CEP 90610-000, Porto Alegre, RS, Brazil.
[b] Department of Microbiology, ICBS, Universidade Federal do Rio Grande do Sul. Rua Sarmento Leite, Porto Alegre, RS, Brazil
[c] Faculdade de Engenharia, Departamento de Engenharia Química, Pontifícia Universidade Católica do Rio Grande do Sul. Av. Ipiranga, 6681-Prédio 30-Sala 277, 90619.900 Porto Alegre, RS, Brazil
*Corresponding author. Tel.: (+55) 51 3308 5231; fax: (+55) 51 3308 2165. E-mail address: helder.teixeira@ufrgs.br (Helder Ferreira Teixeira).

Abstract

Coumarins are benzopyrones found in several plant genera, including *Pterocaulon balansae*. According to studies, these compounds and their derivatives represent an important source for new treatments, especially as antimicrobial and antifungal agents. Recently, our research group demonstrated the incorporation of *P. balansae* extracts into nanoemulsions composed of an oil core of medium chain triglycerides (MCT), stabilized by phospholipids (egg-lecithin). In this context, the aim of this study was to evaluate the topical permeation/retention of coumarins from nanoemulsions containg *P. balansae* extracts, as well as assess their antifungal activity against *S. schenckii* strains. The nanoemulsions obtained by HPH display droplet size ranging from 127 to 162 nm and a viscosity of approximately 1.0 cP. PDI was lower than 0.14, ζ-potential was around -21mV, and pH was 5.0. Permeation/retention assays through porcine skin demonstrated shown that total coumarins are concentrated in the epidermis and dermis layers on intact skin (3.14 µg/cm² = NAE and 3.18 µg/cm² = NSFE). In the tests with impaired skin the amount of NAE and NSFE increase to 3.87 µg/cm² and 3.70 µg/cm², respectively. It is observed in the case of impaired skin a significant increase in the amount of total coumarins that reaches up to the receptor fluid 3 times for NAE and 4 times for NSFE. Antifungal activity was assessed, and MIC was 250 µg mL^{-1} for the formulations for all strains. The overall results demonstrated the feasibility of incorporating *P. balansae* extracts into nanoemulsions and shows a potential alternative for the treatment of sporotrichosis.

Keywords: *Pterocaulon balansae*, *Sporotrix schenckii*, permeation/retention assay, nanoemulsion, high-pressure homogenization

1. Introduction

Coumarins are aromatic secondary metabolites of the benzopyrone chemical class found in several plant genera. According to studies, these compounds and their derivatives represent an important source for new treatments, especially as antioxidant, antimicrobial, antineoplasic, and anti-inflammatory agents (BUBOLS et al., 2013; PENG; DAMU; ZHOU, 2013; RIVEIRO et al., 2010; SRIKRISHNA; GODUGU; KUMAR DUBEY, 2016; VENUGOPALA; RASHMI; ODHAV, 2013; ZHANG et al., 2013). Coumarins are the major compounds present in *Pterocaulon* Ell. extracts (MEDEIROS-NEVES et al., 2015; PANATIERI et al., 2017; RÓDIO et al., 2008; STEIN et al., 2007; TORRES et al., 2017), and recently, some reports have described the antifungal activity of extracts obtained from many *Pterocaulon* species (AVANCINI; WIEST, 2008; STEIN et al., 2005, 2006; STOPIGLIA et al., 2011).

Stopiglia et al. (2011) demonstrated the *in vitro* antifungal activity of five plant species belonging to the *Pterocaulon* genus (*P. balansae, P. cordobense, P. lanatum, P. lorentzii* and *P. polystachyum*) against 24 strains of *Sporothrix schenckii.* The methanolic extract at 10% concentration showed both inhibitory and fungicidal activity (STOPIGLIA et al., 2011). *S. schenckii* is the etiological agent of sporotrichosis, the most common subcutaneous mycosis, which can infect humans and animals. This disease has a worldwide distribution, although more frequently occurs in tropical and subtropical areas, with the main endemic areas found in Japan, India, South Africa, Mexico, Peru, Uruguay, and Brazil. In South America, the estimated annual incidence is 48 to 60 cases per 100,000 population. The fungus penetrates through the skin and may remain in the subcutaneous tissue or extend along the adjacent lymphatic vessels. This is a disease that is characterized by the appearance of skin lesions in the form of papules or pustules, followed by the formation of a subcutaneous nodules (BARROS; DE ALMEIDA PAES; SCHUBACH, 2011; MAHAJAN, 2014; VÁSQUEZ-DEL-MERCADO; ARENAS; PADILLA-DESGARENES, 2012). The first choice treatment

combines potassium iodide and itraconazole, which is generally effective, although the long duration of the therapeutic regimen and the occurrence of toxic side effects make it necessary to explore new alternatives for the treatment of severe infections.

The use of nanoemulsions as systems for incorporating plant extracts to enable their topical administration has been widely described, improving the characteristics of the compounds, such as promoting greater penetration into the skin layers. Recently, our research group developed an UFLC method which enables to quantify *Pterocaulon balansae* coumarins in different matrices. The feasibility of the incorporation of aqueous extract of *P. balansae* into nanoemulsions composed of an oil core of medium chain triglycerides (MCT), stabilized by phospholipids (egg-lecithin), was demonstrated (MEDEIROS-NEVES et al., data not published).

Following up on these results, the aim of this study was to evaluate the topical permeation/retention of coumarins from nanoemulsions containg *P. balansae* extracts, as well as assess their antifungal activity against *S. schenckii* strains.

2. Materials and methods

Plant Material

Aerial parts of *Pterocaulon balansae* Chodat. were collected in Canoas, RS, Brazil, in February 2016. The species was identified by the botanic Sérgio A. L. Bordignon (Centro Universitário La Salle - Canoas, UNILASALLE, Brazil). Voucher specimen was deposited in the herbarium of the Universidade Federal do Rio Grande do Sul (ICN157762). Plant collection was authorized by Ministério do Meio Ambiente (Nº 38017-1 - Sistema de Autorização e Informação em Biodiversidade).

Materials

The solvents acetonitrile and formic acid were obtained from Tedia (HPLC grade, US). Ultrapure water was obtained from a Milli-Q® Plus apparatus by Millipore (Billerica, US). Methanol, ethanol, isopropanol, polysorbate 80 (Tween® 80), dimethyl sulfoxide (DMSO), and sodium hydroxide were obtained from Vetec (BR). Medium chain triglycerides (TCM) and egg-lecithin (Lipoid® E80) were purchased from Lipoid GmbH (GER). Monobasic potassium phosphate was obtained from Dinâmica (BR). NBD-PE (N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)-1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine, Triethylammonium Salt) fluorescent labeled phospholipid was purchased from Thermo Fisher Scientific (US). The Roswell Park Memorial Institute 1640 broth medium (RPMI-1640), morpholinepropansulfonic acid (MOPS), [(tetra-zolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide)] (MTT) and Itraconazole were obtained from Sigma-Aldrich (US). Potato dextrose agar (PDA) was purchased from Acumedia (US). Tissue-Tek® O.C.T.™ was purchased from Sakura Finetechnical Co. (Japan). LogP (calculated LogP) values of coumarins were assigned using ALOGPS (http://www.vcclab.org/lab/alogps/) (Tetko et al., 2009).

Strains

The clinical isolates were obtained from the Mycology Collection at Pathogenic Fungi Laboratory (Department of Microbiology of the Institute of Basic Health Sciences of Universidade Federal do Rio Grande do Sul, BR). Four clinical isolates of *Sporotrix schenkii* (Santa Casa 1; MLS; 31 UCS; STT) and one agent were obtained from the American Type Culture Collection (ATCC, Rockville, MD, US), ATCC 201679.

2.1. *Pterocaulon balansae* extraction

The extracts were obtained from the aerial parts of *P. balansae*. The aqueous extract (AE) was obtained by the previously described methodology (Chapter 2). The supercritical fluid extract (SFE) was also obtained according to a previously described method (TORRES et al., 2017), at 40ºC and 120 bar. The samples were stored for subsequent studies.

2.2. Preparation of nanoemulsions

Three distinct formulations were prepared: (i) blank nanoemulsion (NB); (ii) aqueous extract-loaded nanoemulsion (NAE) and (iii) supercritical fluid extract-loaded nanoemulsion (NSFE). The nanoemulsions were prepared at the concentration of 1.6 mg/mL total coumarins. The nanoemulsions were obtained by high-pressure homogenization (HPH). Briefly, the oily phase (16% w/w MCT, 4% w/w egg lecithin) and the aqueous phase (1% w/w Tween 80® and 100% water) were mixed under magnetic stirring (15 min at room temperature) to form a coarse emulsion. For NAE, the AE was solubilized in the aqueous phase at room temperature due to the affinity; and for preparing the NSFE, the SFE was dispersed in the oil phase at 30ºC. The coarse emulsions were then mixed at 9,500 rpm for 2 min using an IKA® Ultra-Turrax T8 mixer (IKA® Works Inc., USA) to form crude pre-emulsions. Aiming to gradually decrease the droplet size, these pre-emulsions were subjected to HPH (EmulsiFlex-C3®, Avestin, CAN) at 750 bar (10000 psi) for 10 cycles, producing the nanoemulsions. The final composition of formulations are shown in **Table 1**.

Table 1. Composition of nanoemulsions.

Composition	**NB**	**NAE**	**NSFE**
Medium chain triglycerides (MCT) (%)	16.0	16.0	16.0
Egg lecithin (%)	4.0	4.0	4.0
Polysorbate 80 (%)	1.0	1.0	1.0
AE (mg mL^{-1})	-	2.02	-
SFE (mg mL^{-1})	-	-	2.02
Water *qs* to	100.0	100.0	100.0

NB: blank nanoemulsions; NAE: aqueous extract-loaded nanoemulsion; NSFE: supercritical fluid extract-loaded nanoemulsion. Content of coumarins adjusted by yield (AE: 9.5% and SFE: 42.26%).

2.3. Physicochemical properties of nanoemulsions

2.3.1. *Droplet size, polydispersity index (PDI), and ζ-potential*

The droplet size and PDI were determined by photon correlation spectroscopy (PCS) at 25 °C after dilution of the samples in water, previously filtered on a 0.22 μm membrane. The ζ-potential was determined by electrophoretic mobility at 25 °C after appropriate dilution with water. The measurements were performed using a Zetasizer Nano-ZS90® (Malvern Instruments, England) equipment. The results were expressed as mean of three independent determinations.

2.3.2. *pH and viscosity measurements*

The pH nanoemulsions was directly measured using a previously calibrated potentiometer (Model pH UtraBasic, Denver Instruments, US). The results were expressed as mean of three independent determinations. The viscosity of nanoemulsions was measured using capillary viscometry in an Ostwald viscometer at 23°C±0.1°C using a number 2 capillary, using 2 mL of each nanoemulsion. The flow time through the capillary and density of the formulations were taken into account to calculate the relative viscosity (cP). All determinations were performed in triplicate.

2.3.3. *NBD-PE labeled nanoemulsions*

The NBD-PE (N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)-1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine, Triethylammonium Salt) fluorescent labeled phospholipid (Thermo Fisher Scientific, USA) was dissolved in the oil phase of nanoemulsions in the proportion of 1 % of formulation. The preparation of the formulation continued as described on 2.2 section.

2.4. Permeation/retention assay in intact and impaired porcine skin

Porcine ears were obtained from a local slaughterhouse. The ears were removed from the animals before the scalding procedure, cleaned, and the full-thickness skin was excised from the outer region of the ear with a scalpel. After the removal of subcutaneous fat, the skin was wrapped in aluminum foil and stored at −20°C for a maximum period of 1 month. The permeation/penetration assay was carried out by the method of the Franz type diffusion cells using the porcine ear skin.

Throughout the experiment, the system was kept under a controlled temperature (32 ± 1.0 °C) and stirred at 480 rpm. The skin sections were hydrated with PBS pH 7.4 for 15 min at room temperature before being placed in the cell. The circular sections of porcine skin were deposited on the top of the cells, between the donor and receptor compartments of the Franz cell, on a surface area of 1.77 cm^2. The receptor compartment was supplied with a mixture of PBS:ethanol (60:40). A volume corresponding to 550 µL of NAE or NSFE formulations was then applied on the donor compartment. After eight hours, an aliquot of the receptor fluid was collected, and the skin was removed from the cell. The circular sections were cleaned, separated the epidermis from the dermis and cut in small pieces. The coumarins were extracted with methanol using an ultrasonic bath for 45 minutes. For the evaluation of impaired skin, tape stripping was performed by using 20 tapes (Scotch 750 tape, 3 M) before the experiment. The samples were analyzed by UFLC method (chapter 2) and the results expressed as µg of total coumarins per skin area.

2.5. Histological and confocal microscopy

After permeation assay, the skin cuts were cleaned with a cotton swab and immersed in a 10% buffered formalin solution. Subsequently, they were dehydrated, embedded in paraffin, and sectioned, with a thickness of 6 µm. The skin tissue specimens, after staining with hematoxylin and eosin, were photographed by optical microscopy (100x magnification). For confocal fluorescence experiments, a fluorescent dye, NBD-PE, was added during the preparation of nanoemulsions by HPH. Approximately 550 µL of each fluorescent nanoemulsion was placed in the donor compartment, and the permeation/retention study was performed under the same experimental conditions described in the "skin permeation/retention studies" section. After 1 h and 8 h, the skin cuts were cleaned and mounted with Tissue-Tek® O.C.T.™ into a metal sample holder and frozen at −20°C. Vertical slices of skin cuts of 30 µm thickness were obtained with a cryostat (CM 1,850; Leica Microsystems, Wetzlar, Germany), and the slides

were analyzed under a fluorescence microscope (Olympus BX51TF, JPN) (100x and 400x magnification).

2.6. UFLC analysis

The total coumarins (TC) content of extracts (AE, SFE), nanoemulsions (NAE, NSFE), porcine skin (epidermis and dermis), and receptor fluid after permeation studies were determined using a previously validated ultra-fast liquid chromatography (UFLC) method (chapter 3).

2.7. Antifungal activity

Antifungal susceptibility assays were performed using the broth microdilution method according to guidelines recommended by Clinical and Laboratory Standards Institute (CLSI) for filamentous fungi — M38-A2 (CLSI, 2008). Strains were subcultured in potato dextrose agar at 35ºC for 7 days. The surface was gently scraped with a sterile bent glass after flooding with sterile saline solution. Standard suspensions were adjusted by UV-visible spectrophotometry (Spectrum Instruments Co., China) to show absorbance at 0.09 of 0.13. Adjusted suspensions were diluted in RPMI-MOPS (1:50) to obtain a final inoculum of 10^4 CFU mL^{-1}, and 100 µL of the fungal suspensions were added to each microdilution well containing 100 µL of the samples (AE, SFE, NAE, and NSFE) at final concentrations ranging between 3.906 and 1,000 µg mL^{-1}. The same procedure was followed for the blank nanoemulsion (NB), positive control (itraconazole), growth control (untreated microorganisms), and sterility control (without microorganisms). The itraconazole stock solution (1,600 µg mL^{-1}) was prepared in DMSO and other solutions were diluted in RPMI-MOPS for obtaining the final concentrations ranging from 0.0313 to 16 µg mL^{-1}. Plates were incubated at 35 ºC for 4 days and analyzed.

2.7.1. *Minimum inhibitory concentration (MIC)*

The determination of MIC was visually performed by comparison with the growth control. The MIC was defined as the lowest concentration of treatments (extracts and nanoemulsions) which are able to completely inhibit fungal growth. All the experiments were conducted in triplicate.

2.7.2. *Hyphal damage assay with MTT*

To evaluate the ability of the treatments in causing hyphae damage, a colorimetric assay using MTT was performed (CHIOU et al., 2001). After determination of MIC, the same plates were used for the MTT assay. Initially, 150 µl of the supernatant was discarded, and 150 µl of MTT was added (0.005% w/v) to the wells, which were incubated for 3 h at 37°C. After removal of the MTT suspension, the MTT formazan crystals were extracted from the hyphae with 150 µl of isopropanol, homogenized and 100 µl of supernatant were transferred to a flat-bottom 96-well plate. Absorbance (A) was measured on a multiscan-enzyme-linked immunosorbent assay reader (Titertek MCC/340; Labsystems, Helsinki, Finland) at a dual wavelength of 570 and 690 nm. Percentage hyphal damage was calculated by the following equation:

$$Cell\ Demage\ (\%) = 1 - \frac{A570 - A690\ with\ drugs}{A570 - A690\ without\ drugs} x\ 100$$

The absorbance was adjusted for nonspecific absorption by subtracting absorbance at 690 nm from absorbance at 570 nm.

2.8. Statistical analyses

Significant statistical differences were determined by ANOVA with Tukey post hoc (*p<0.05, **p<0.001, ***p<0.0001). Parameters were analyzed by software GraphPad Prism (version 6.01, GrapPad software Inc, US).

3. Results and Discussion

3.1. Preparation and characterization of nanoemulsions

In the present study, we study two nanoemulsions loaded with different *P. balansae* extracts, aiming to evaluate their permeation/retention in intact and impaired porcine skin and investigating their antifungal activity against *S. schenkii*. Initially, the aqueous extract and supercritical fluid extract were obtained according to previously studies (TORRES et al., 2017) (Chapter 2). The extracts were obtained by green technology, which has been well received as environmentally friendly processing techniques and in some cases, an alternative to organic solvent-based extraction of natural products (DE MELO; SILVESTRE; SILVA, 2014; DESHPANDE et al., 2011). To date, the seven coumarins described to be found in this species are shown in Figure 1. According to the chromatographic profile of the NAE and NSFE formulations (**Figure 2**), it is possible to observe the different quantitative content of these coumarins.

In the chromatogram of the aqueous extract-loaded nanoemulsions (NAE), a greater amount of the coumarins 1-3 was observed, especially due to their chemical properties, which confer them a more hydrophilic character, confirmed by LogP values (1.01 - 1.13). In this sense, these compounds will be extracted in larger quantities when water is the solvent. In the case of the supercritical fluid extract-loaded nanoemulsions (NSFE), one can observe an opposite behavior, as this process enables the extraction of lipophilic coumarins, as can be observed in **Figure 2**, where there is a predominance of coumarins 4-7 in the formulation.

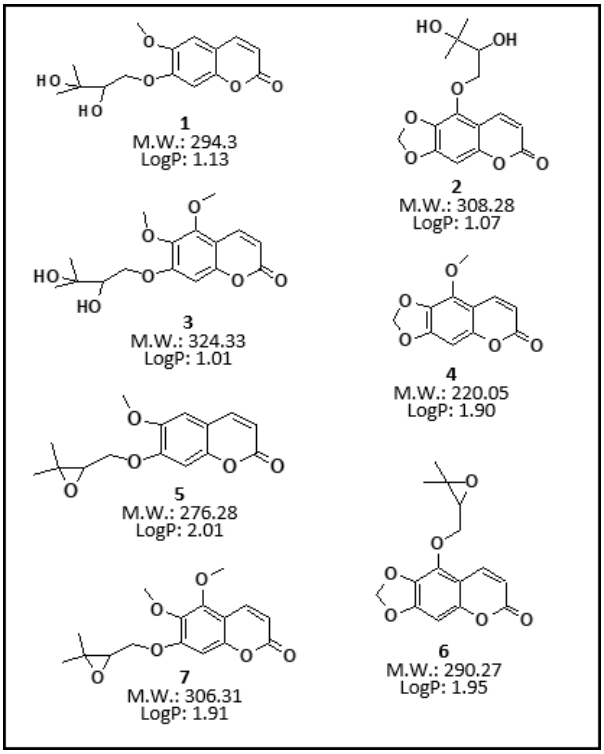

Figure 1. Structure, molecular weight, and LogP of the coumarins described in *P. balansae* extracts.

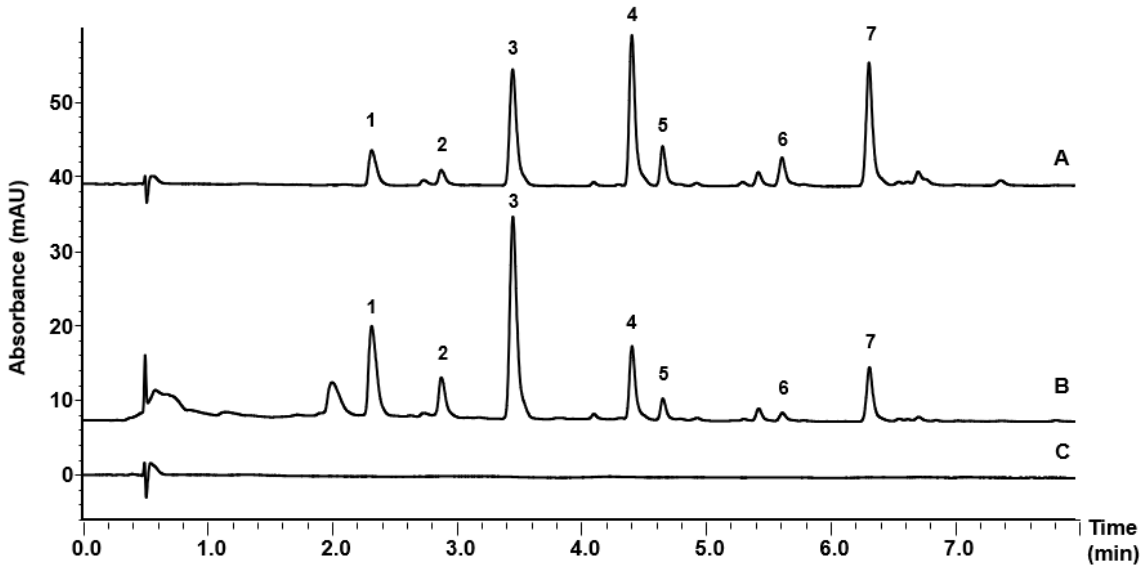

Figure 2. Chromatographic profile of A. Supercritical fluid extract-loaded nanoemulsion (NSFE); B. Aqueous extract-loaded nanoemulsion (NAE); and C. Blank nanoemulsions (NB).

The next step involved the incorporation of these extracts in nanoemulsions. Three formulations were prepared: (i) blank nanoemulsion (NB); (ii) aqueous extract-loaded nanoemulsion (NAE), and (iii) supercritical fluid extract-loaded nanoemulsion (NSFE) by HPH method. HPH is commonly used in the pharmaceutical industry and size reduction is achieved by forcing a coarse emulsion under high pressure through a homogenizing valve, thereby deforming and reducing the droplet size (RAI et al., 2018; SCHUH; BRUXEL; TEIXEIRA, 2014).

The total coumarins content in the formulations was higher than 90%, demonstrating the efficient association of coumarins to these systems, which were incorporated in the oil nucleus and/or adsorbed on the interface of lipid droplets.

Table 2. Physicochemical properties of nanoemulsions.

Parameter	NB	NAE	NSFE
Size (nm)	133.02 ± 3.87 [a]	162.02 ± 6.59 [ac]	127.49 ± 4.18 [c]
PDI	0.145 ± 0.02	0.111 ± 0.02	0.096 ± 0.03
ζ-potential (mV)	-38.92 ± 1.77 [b]	-32.62 ± 6.03	-21.20 ± 3.42 [b]
pH	4.69 ± 0.03 [ab]	5.33 ± 0.01 [ac]	4.46 ± 0.08 [bc]
Viscosity (cP)	0.96 ± 0.007 [ab]	1.08 ± 0.011 [a]	1.06 ± 0.005 [b]
Total coumarins content (%)	-	90.52 ± 0.04	91.30 ± 0.06

PDI: Polidispersity index; NB: blank nanoemulsion; NAE: aqueous extract-loaded nanoemulsion; NSFE: supercritical fluid extract-loaded nanoemulsion. Mean ± SD for three determinations (SD: standard deviation).
[a]Significantly different ($p<0.05$) for NB and NAE
[b]Significantly different ($p<0.05$) for NB and NSFE
[c]Significantly different ($p<0.05$) for NSFE and NAE

Table 2 presents the physicochemical properties of the nanoemulsions. The nanoemulsions obtained by HPH display droplet size ranging from 127 to 162 nm and a viscosity of approximately 1.0 cP. Droplet size is an important parameter, as it influences many properties, as viscosity, for example, where drops that are more spherical will typically flow easier than smaller or distorted droplets, which tend to coalescence (Schuh et al., 2014). Uniformity of droplet size distribution is measured by PDI, and nanoemulsions are generally referred to as 'monodisperse' if PDI is lower than 0.2 (SINGH et al., 2017). According to this parameter, all three

formulations are monodispersed, showing PDI lower than 0.145. In addition, the formulations exhibited pH above 5.0, compatible with the natural skin surface pH (CEVC; VIERL, 2010).

Regarding ζ-potential, nanoemulsions displayed negative values due to the presence of negatively-charged phospholipids in egg lecithin composition, such as phosphatidylethanolamine, phosphatidylserine, and phosphatidic acid, as described in previous literature (PIEMI et al., 1999). The values were (in modulus) ranging approximately from – 21 mV to – 39 mV, providing a good stability for the formulations. A higher ζ-potential was observed when the nanoemulsion was loaded with AE (NAE), suggesting the presence of some compounds at the o/w interface of this formulation.

3.2. Permeation/retention assay in intact and impaired skin

To assess the accumulative ability of *P. balansae* coumarins in the tissues in which *S. schenkii* establishes infections, total coumarins permeation/retention content in porcine skin was estimated. Permeation/retention studies were performed by using Franz-type diffusion cells to evaluate the distribution of coumarins present in the formulations (NAE and NSFE) through intact and impaired porcine skin. Once the spore infections affect the deeper layers of the skin, the experiments were also performed in damaged tissues, analyzing the total coumarins permeation/retention extent in this condition. Previous consolidated literature reports the use of the tape stripping technique to injure the skin, removing the stratum corneum (SC) (LADEMANN et al., 2009).

As can be seen in the **Table 3**, after 8 hours of experiment in the intact skin the major content of total coumarins was concentrated in the dermis, follow by the epidermis for the two formulations, without significant difference between them. The total coumarins found in the dermis and epidermis for intact skin count 3.14 μg/cm² for NAE and 3.18 μg/cm² for NSFE. A significant increase ($p<0.05$) in permeation of total coumarins from NAE and NSFE was detected in the dermis

and in the receptor fluid when the stratum corneum was removed. In the impaired skin the amount of total coumarins in the dermis and epidermis increased to 3.87 µg/cm² and 3.70 µg/cm² for NAE and NSFE, respectively. The removal of the most superficial layer of the skin, the stratum corneum, made possible a deeper reach of the formulations, these findings are important especially when the target of treatment is to combat subcutaneous fungi, such as *S. schenckii*. As observed in the dermis layer, the amount of coumarins that permeates to the receptor fluid increases when the skin is impaired, presenting significant differences. In the case, the concentrations of 0.70 µg/cm² (NAE) and 0.59 µg/cm² (NSFE) in the intact skin, increased to 1.76 e 2.24 µg/cm² in the impaired skin, respectively. These results represent a 3 and 4-fold increase in the permeation profile when the skin is impaired. This behavior occurs due to the removal of the upper layer of the skin (stratum corneum), which is intended to provide a physical barrier, protecting the skin (CEVC; VIERL, 2010). Once the skin is impaired, it may allow increased permeation and/or absorption of the compounds. In the case of infections caused by *S. schenckii*, which affect the inner layers of the skin, this study presents interesting treatment alternatives that are able to reach these layers.

Table 3. Distribution profile of total coumarins (TC) from NAE and NSFE in impaired and intact porcine ear skin layers after 8 hours of permeation/retention study.

		NAE Mean ± SD (DPR)	**NSFE** Mean ± SD (DPR)
		µg/cm²	µg/cm²
Intact skin	*Stratum corneum*	0,86 ± 0,23 (16,34%)	0,52 ± 0,06 (11,70%)
	Epidermis	1,16 ± 0,40 (17,59%)	1,03 ± 0,13 (13,50%)
	Dermis	1,98 ± 0,39 (12,02%) [a]	2,15 ± 0,14 (13,68%) [c]
	Receptor fluid	0,70 ± 0,11 (10,09%) [b]	0,59 ± 0,05 (11,20%) [d]
Impaired skin	*Epidermis*	1,02 ± 0,74 (15,89%)	0,91 ± 0,16 (17,26%)
	Dermis	2,85 ± 0,81 (16,40%) [a]	2,79 ± 0,42 (15,25%) [c]
	Receptor fluid	1,76 ± 0,51 (16,74%) [b]	2,24 ± 0,23 (10,41%) [d]

Statistically significant difference between impaired and intact skin ($p<0.05$). [a, b] for NAE and [c, d] for NSFE. NAE: aqueous extract-loaded nanoemulsion; NSFE: supercritical fluid extract-loaded nanoemulsion. Mean ± SD for three determinations (SD: standard deviation), ANOVA and Tukey post hoc.

3.3. Microscopy analyses

A confocal microscopy evaluation was carried out to shed light on the increased retention in the skin, and histological analyses were performed to assess the safety of the treatments. We used NBD-PE as a fluorescence marker because it is easily dispersed in the interface of nanoemulsions, and the confocal images revealed that the fluorescence was distributed throughout the skin layers. On the top of **Figure 3** it is possible to observe the confocal images and **Figure 4** shows the fluorescence intensity levels captured from the images. As it can be seen, a significant increase in fluorescence ($p<0.001$) was detected for impaired skin, for both formulations in one and eight hours, which corroborates with the results concerning coumarins retention in the previous experiment. There is also a difference ($p<0.001$) between the formulations at 1 h and 8 h for both intact and impaired skin. The NAE formulation presented higher fluorescence (approximately 550.3%) than the NSFE (approximately 413%) after 8 hours, corroborating the permeation assays, where higher amounts of coumarins were found in the receptor fluid collected from NSFE Franz cells. The tape stripping method used for the impairment of the skin was followed by a histological analysis (H&E), which was considered acceptable. In the skin, it was possible to differentiate the stratum corneum, the viable epidermis, and the dermis in intact skin, and the partial removal of the stratum corneum after tape stripping (Fig. 3, detailed images of controls stratum corneum, 400x magnification). Hystological analyses demonstrated that there were no signs of skin damage after the treatments (Fig.3, lower images).

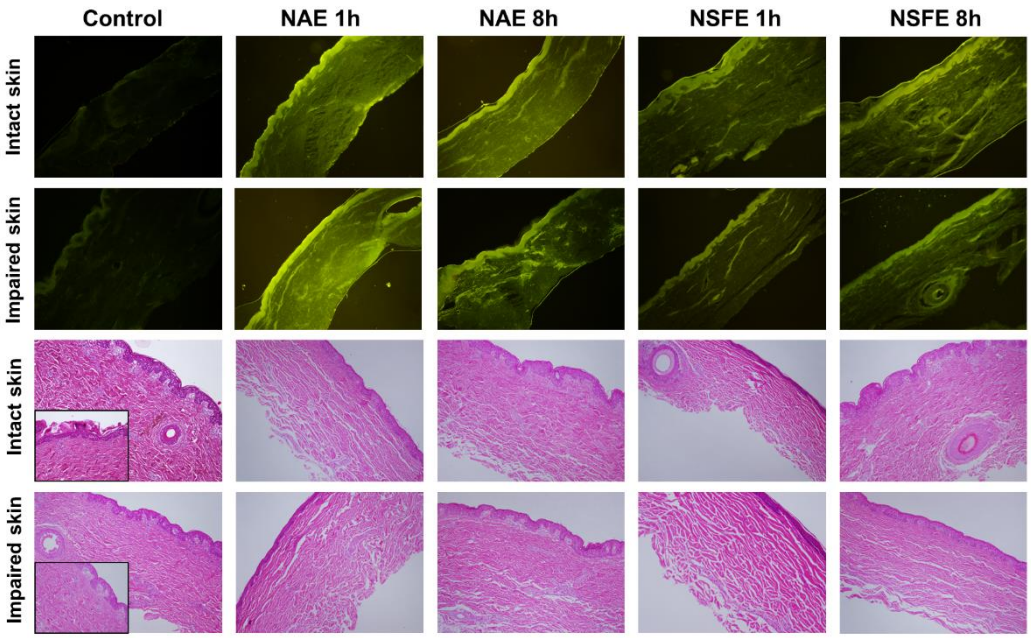

Figure 3. Representative hematoxylin/eosin-stained histological (lower images) and fluorescence (upper images) images of intact and impaired skin, as control, treated with NAE for 1h and 8h, and treated with NSFE for 1h and 8h.

Notes: Histological images show no skin damage after treatment with the nanoemulsions. The confocal images revealed that the fluorescence was distributed throughout the skin layers when the dye was incorporated into nanoemulsions. Images were obtained after 1 hour and 8 hours of permeation/retention studies using a Franz diffusion cell. Images were obtained at × 100 and 400 magnifications. NBD-PE was used as fluorescent dye in confocal images. NAE: aqueous extract-loaded nanoemulsion; NSFE: supercritical fluid extract-loaded nanoemulsion.

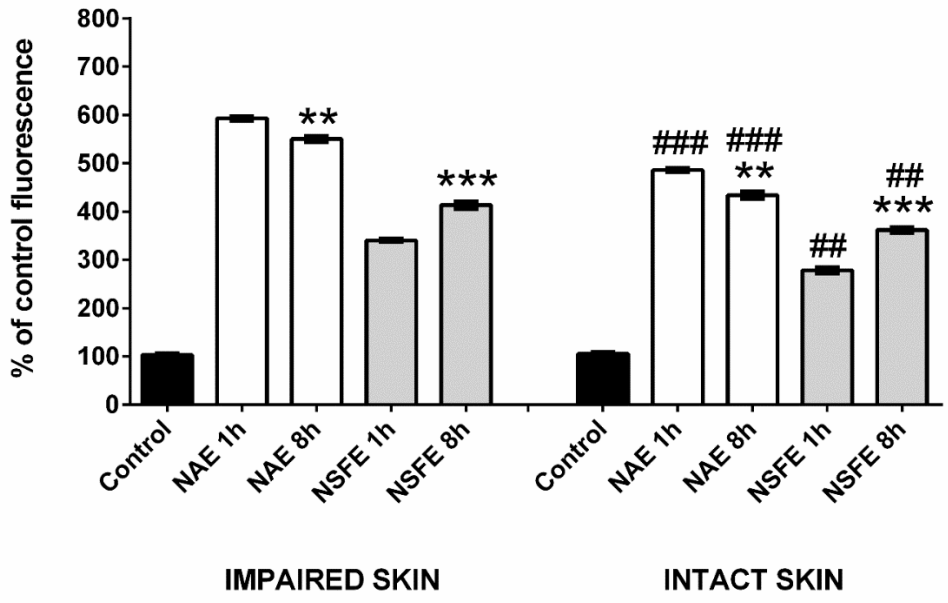

Figure 4. Fluorescent intensity levels detected for impaired or intact skin relative to the percentage of fluorescence emitted by the control tissue. Statistically significant differences were determined by ANOVA with Tukey post hoc (*p<0.05, **p<0.005, ***p<0.0005). Differences between intact and

impaired tissues were represented by ($^{\#}$), while differences between 1h and 8h of incubation with the same nanoemulsion were represented by (*).

3.4. Antifungal activity

Sporotrichosis is a subacute or chronic mycosis caused by the dimorphic species complex, *S. schenckii*. It is known that the probability of detecting antifungal activity in plants is higher when they have reported ethnopharmacological uses, and recently, a review of the *Pterocaulon* genus presented several biological activities, including antifungal activity (MEDEIROS-NEVES; TEIXEIRA; VON POSER, 2018). As with the ethnopharmacological use, a study carried out by Stopiglia et al. demonstrated the antifungal activity of different extracts of *Pterocaulon* against *S. schenckii* (Stopiglia et al., 2011), showing to be an interesting alternative for this kind of infections. In this sense, this study aimed at evaluating the antifungal activity of the *P. balansae* extracts-loaded nanoemulsions against some strains of this species. The nanoemulsions were tested against five strains of *S. schenckii*, which included 31 UCS, MLS, Sta Casa 1, STT, and ATCC 201679.

Table 3. Antifungal activity (MICs in μg mL^{-1}) of *P. balansae* extracts and nanoemulsions.

Samples	***Sporothrix schenkii***				
	Ss 31 UCS	**Ss MLS**	**Ss Sta Casa 1**	**Ss ATCC 201679**	**Ss STT**
AE	> 1000	> 1000	> 1000	> 1000	> 1000
SFE	> 1000	> 1000	> 1000	> 1000	> 1000
NB	500	500	500	500	500
NAE	250	250	250	250	250
NSFE	250	250	250	250	250

Notes: Values the MIC to Itraconazole (positive control) for *S. schenckii* 31 UCS (2.0); MLS (2.0); Sta Casa 1 (>16.0); ATCC 201679 (2.0); STT (>16.0). AE: aqueous extract; SFE: supercritical fluid extract; NAE: aqueous extract-loaded nanoemulsion; NSFE: supercritical fluid extract-loaded nanoemulsion.

The geometric mean of MICs, and the MIC ranges of the evaluated sporothricosis agents are presented in Table 3. The results showed a MIC value above 1,000 μg mL-1 for both extracts (AE and SFE) in all strains analyzed. In a previous study, values ranging from 312 to 1,250 μg mL^{-1} were also found for plant extracts (STOPIGLIA et al., 2011).

However, those extracts were obtained with organic solvents (hexane, dichloromethane). When incorporated into a lipid release system, MIC values decreased 4-fold, with MIC values of 250 µg mL^{-1}. This was the lowest antifungal concentration found for this species (*P. balansae*) when treating *S. schenckii* strains. The markedly pronounced activity of the nanoemulsions when compared to the extracts may be linked to the composition of those formulations, which comprise phospholipids that may interact and destabilize the cellular wall of these fungi, provoking cell death (HAMOUDA et al., 2001).

4. Conclusion

This study showed the feasibility of incorporating aqueous and supercritical fluid *P. balansae* extracts in nanoemulsions, which demonstrated satisfactory physicochemical properties. These formulations were capable of releasing coumarins content through impaired and intact skin, with larger amount of coumarins detected in the receptor fluid and the dermis, reaching the inner layers of skin. Consequently, the formulations demonstrated an important antifungal activity against *S. schenckki* strains, and more studies may be conducted as this is an interesting alternative treatment for the widespread sporotrichosis infections.

5. References

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Discussão Geral

Nas últimas décadas, aproximadamente 49% dos medicamentos aprovados para uso são de origem natural ou moléculas semi-sintéticas derivadas dessas matérias primas (NEWMAN & CRAGG, 1997, 2003, 2007, 2012, 2016). Esse número expressivo destaca a importância do estudo com produtos naturais na busca por novos fármacos. Este importante dado está relacionado ao fato do reino vegetal ser uma fonte abundante para o isolamento de novos compostos (COS et al., 2006; MAREGESI et al., 2008). Dentre as possibilidades de estudos, a probabilidade de se detectar atividades biológicas em plantas é mais elevada quando existem relatos de usos etnofarmacológicos (SVETAZ, 2010).

Nesse contexto, o tema geral da tese envolve a investigação da espécie *Pterocaulon balansae*. Diversos estudos vêm sendo realizados com esta planta, demonstrando resultados promissores em avaliações das atividades antifúngica, antibacteriana, antiparasitária e antitumoral. Além disso, estudos recentes têm demonstrado a presença de cumarinas em diferentes tipos de extratos, e considerando-se que normalmente são os compostos majoritários, é provável que as ações farmacológicas desta espécie estejam relacionadas a sua presença (STEIN et al., 2005; STEIN et al., 2006; STOPIGLIA et al., 2011; VIANNA et al., 2012, PANATIERI et al., 2016; TORRES et al., 2017).

A fim de aprofundar o conhecimento acerca da espécie em estudo nesse trabalho (*Pterocaulon balansae*), a primeira etapa da tese foi dedicada a uma revisão da literatura sobre o gênero *Pterocaulon* Elliot. Através deste estudo foi possível compilar informações sobre a composição química, dados etnofarmacológicos e atividades biológicas descritas até o presente momento para este gênero. A busca (1968-2017) foi realizada nas bases de dados *Science Direct*, *Web of Science*, *Scopus* e *Pubmed*. Trabalhos anteriores a 1968 referem-se somente à descrição e classificação botânica do gênero, desde quando foi identificado por Elliot em 1823. O gênero *Pterocaulon*, pertencente à família Asteraceae, foi revisado por Bean (2011), que descreveu a existência de 26 espécies, que são divididas em americanas e não americanas. De forma geral, as espécies apresentam altura em torno de um metro, caule alado e capítulos sésseis terminais, os quais formam densos glomérulos, ou estão arranjados em densas

espigas (CABRERA and RAGONESE, 1978; LIMA & MATZENBACHER, 2008; BEAN, 2011).

Em relação à composição química dos representantes deste gênero, já foram isolados e identificados até o momento cumarinas, ácidos fenólicos, flavonoides, terpenos e poliacetilenos. Confirmando o descrito na literatura, os compostos mais relatados são as cumarinas, consideradas marcadores quimiotaxonômicos do gênero. Ao todo já foram identificadas 41 cumarinas, originarias das espécies *P. intermedium*, *P. balansae*, *P. lanatum*, *P. virgatum*, *P. serrulatum*, *P. purpurascens*, *P. rugosum*, *P. alopecuroides* e *P. redolens*. Os compostos são 6,7 dioxigenados (16 compostos), 6,7,8 trioxigenados (8 compostos), 5,6,7-trioxigenados (14 compostos) e 5,6,7,8 tetraoxigenados (3 compostos). Foram encontrados também ácidos fenólicos e flavonoides em 7 espécies (*P. virgatum*, *P. purpurascens*, *P. sphacelatum*, *P. serrulatum*, *P. reodolens*, *P. balansae* e *P. alopecuroides*), somando um total de 26 compostos.

Vale ressaltar que no gênero *Pterocaulon* a maioria dos flavonoides isolados até o momento ocorre em sua forma aglicona. Já os terpenos foram encontrados numa quantidade equivalente (28 compostos) aos ácidos fenólicos e flavonoides, mas concentrados em 4 espécies (*P. virgatum*, *P. serrulatum*, *P. balansae* e *P. polystachyum*). Para os poliacetilenos foram encontrados somente dois trabalhos na literatura (BOHLMANN et al.,1981 e MAGALHÃES et al., 1989), que identificaram a presença de 13 compostos nas espécies *P. alopecuroides*, *P. balansae*, *P. lanatum*, *P. rugosum* e *P. virgatum*. Ao todo, encontramos 108 compostos químicos já relatados para o gênero *Pterocaulon*, identificados em 11 das 26 espécies existentes.

Em continuidade, os estudos etnofarmacológicos encontrados estão concentrados em países como a Argentina, Austrália, Madagascar, Martinica e Brasil. No total, foram relacionadas 13 espécies de *Pterocaulon*, sendo mais relatado o uso das partes aéreas em forma de decocto. A grande maioria relata o uso dessas plantas para o tratamento de problemas de pele de diferentes etiologias. É importante ressaltar que o segundo maior uso encontrado para essas espécies é para o tratamento de desordens do fígado, seguido

do tratamento de problemas respiratórios. Por fim, o capítulo I apresenta uma ampla relação de estudos com atividades biológicas para o gênero, contemplando as atividades antibacteriana, antifúngica, antiviral, antioxidante, antiparasitária, inseticida e antitumoral. Foram encontrados ao todo 32 estudos que relatam diversas atividades biológicas e toxicidade.

Os estudos confirmaram que várias espécies de *Pterocaulon* citadas nos estudos etnofarmacológicos contêm moléculas bioativas que podem explicar os efeitos benéficos e promotores da saúde observados. A maior parte das atividades tem sido atribuída às cumarinas, abundantes no gênero. Outro dado relevante observado neste tópico foi a utilização de solventes orgânicos na obtenção dos extratos e/ou compostos isolados.

Esse estudo tornou possível conhecer melhor o gênero *Pterocaulon*, e confirmar, por meio das publicações, a importância deste gênero como fonte de novas moléculas, principalmente de cumarinas, que são promissoras para uma série de aplicações terapêuticas. No entanto, para desenvolvimento de produtos para a saúde, bem como durante os ensaios analíticos e bioanalíticos, é de extrema importância um método quantitativo que assegure a confiabilidade dos resultados obtidos através da sua comparabilidade e rastreabilidade (ANVISA, 2003; PASCHOAL, 2008; ANVISA, 2012).

Para a análise das cumarinas presentes na espécie *P. balansae*, nosso grupo de pesquisa desenvolveu um método de CLAE que possibilitou a análise de um extrato aquoso, onde foram identificadas sete cumarinas (MEDEIROS-NEVES et al., 2015). No entanto, para analisar essa matriz complexa, são necessários 45 minutos no CLAE, o que consome muito tempo na rotina de trabalho, além de gastar uma grande quantidade de solvente. A alternativa encontrada foi o desenvolvimento de um novo método que permitisse uma análise rápida e eficiente das cumarinas presentes na espécie *P. balansae*. Optou-se pela utilização da técnica de CLUE, que permite trabalhar a pressões superiores à do CLAE, possibilitando o uso de colunas cromatográficas com menor tamanho de partículas. Dessa forma, apresenta como vantagen direta a redução do tempo

de análise, aumentando a eficiência e a redução do consumo de solventes (GAIKWAD et al., 2010; GANGADASU et al., 2015).

Face ao exposto, o Capítulo II apresentou o desenvolvimento de um método por CLUE, bem como a validação analítica e bioanalítica de duas metodologias (CLAE e CLUE) para a determinação de cumarinas presentes no extrato aquoso de *P. balansae* em diferentes matrizes. Os equipamentos utilizados foram o CLAE, acoplado a espectro UV (CLAE-UV) e o CLUE com detecção de arranjo de diodos (CLUE-DAD). A amostra utilizada para a avaliação da separação dos compostos foi o extrato aquoso de *P. balansae*, obtido pela metodologia descrita por Medeiros-Neves e colaboradores (2015). Por meio de um programa disponibilizado pela Shimadzu, foi realizada inicialmente a adaptação da técnica de CLAE para a técnica por CLUE. Foi realizada uma série de modificações na metodologia, visando uma melhor resolução entre os picos ($Rs > 1.5$), número de pratos teóricos adequados ($N > 2000$), menor fator de cauda ($T < 1.5$) e menor tempo de análise até chegar à condição desejada.

A fase estacionária selecionada para os estudos foram as colunas Phenomenex-C_{18} RP para CLAE-UV e Shim-pack XR ODS para CLUE-DAD. A fase móvel foi constituída por 0,1% de ácido fórmico (A) e acetonitrila (B) usando gradiente de eluição, fluxo e temperatura de forno para CLAE (1 mL/min; 30 ºC) e CLUE (0,55 mL/min; 55 ºC) por 45 e 8 min, respectivamente. Ambas análises foram realizadas a 327 nm, comprimento de onda referente ao máximo de absorção no UV para esse grupo de cumarinas (MEDEIROS-NEVES et al., 2017). Após o desenvolvimento do método por CLUE, ambas metodologias (CLAE e CLUE) foram validadas conforme guias internacionais (ICH, 2005; EMA, 2011; FDA, 2013) para a análise quantitativa das cumarinas presentes em diferentes matrizes. As matrizes utilizadas no trabalho foram o extrato aquoso de *P. balansae*, a nanoemulsão contendo o extrato bem como as camadas da pele suína (derme, epiderme e estrato córneo) para estudos posteriores de permeação/retenção cutânea *in vitro*.

Após a etapa de seleção dos parâmetros cromatográficos, as matrizes foram analisadas quanto à especificidade. As matrizes nanoemulsão branca, epiderme/derme,

estrato córneo e fluído receptor mostraram ausência de picos no mesmo tempo de retenção das cumarinas de *P. balansae*, demonstrando que os métodos empregados são específicos para a análise simultânea de cumarinas de *P. balansae* nas matrizes analisadas. A quantificação da 5MMDC por ambos os métodos apresentou regressão linear na faixa de 0,1 a 7,5 µg/mL em todas as matrizes avaliadas. As curvas padrão não apresentaram desvio da linearidade após análise de ANOVA ($p>0,05$) e apresentaram coeficiente de correlação adequado para determinação analítica e bionalítica da 5MMDC. As metodologias também se mostraram precisas na determinação da 5MMDC, com valores de DPR menor que 2% nos experimentos intra-dia e inter-dia. Os métodos também foram considerados exatos, considerando-se a complexidade das diferentes matrizes, na faixa de 99.31-102 % para o CLAE e 102-106 % para o CLUE. Para os estudos bioanalíticos, as matrizes da pele (epiderme/derme; estrato córneo) foram contaminadas com uma solução padrão de 5MMDC em diferentes concentrações e determinada a capacidade de recuperação após a extração da 5MMDC destas matrizes. A taxa de recuperação ficou na faixa de 85 – 92%, com desvio padrão relativo menor que 8.83%, dentro do preconizado pelo FDA (2013). Os resultados apresentaram baixo efeito de matriz durante as análises. Posteriormente foram realizadas pequenas alterações nos parâmetros cromatográficos dos métodos, através de um desenho experimental de Plackett-Burman, a fim de avaliar a robustez das metodologias. Foram modificados fatores como concentração inicial de acetonitrila, temperatura do forno, fluxo inicial da fase móvel e concentração de ácido fórmico. De acordo com os resultados obtidos, as modificações nos fatores estudados não interferiram na determinação da 5MMDC nas diferentes matrizes avaliadas.

Após a etapa de validação das metodologias foi analisada a aplicabilidade dos métodos. Inicialmente foram preparadas nanoemulsões contendo 5MMDC e o extrato aquoso de *P. balansae* na concentração de 0,5 mg/mL. As formulações foram compostas por lecitina de gema de ovo, TCM, polissorbato 80 e água. Essas nanoemulsões foram, então, submetidas a estudos de permeação/retenção cutânea em pele de orelha suína. Após as 8 h de experimento, observou-se diferença significativa das formulações testadas em relação ao controle, retendo na pele uma quantidade de aproximadamente 3 µg/cm² de cumarinas em ambos os métodos. Também foram encontradas quantidades

de 5MMDC no fluido receptor, demonstrando a capacidade de permeação desses compostos quando incorporados a um sistema nanoestruturado. Da mesma forma, a permeação das cumarinas a partir das nanoemulsões foi superior ao controle, assim como também houve diferença significativa entre as nanoemulsões (NE_{AE} e NE_{5MMDC}). Estes resultados preliminares sugerem que a quantidade de 5MMDC retida na pele não foi influenciada pela presença dos outros compostos do extrato, no entanto, a quantidade de 5MMDC permeada é reduzida na presença destes compostos.

De maneira geral, as metodologias propostas mostraram ser lineares, precisas e exatas para estimar cumarinas em extratos aquosos de *P. balansae*, sem diferenças significativas entre os dois métodos (CLAE e CLUE). No entanto, o método por CLUE foi mais rápido (quase 4 vezes), consumindo menos solvente, sendo considerado *eco-friendly* em comparação com o CLAE.

Na linha de pensamento *eco-friendly*, o terceiro capítulo da tese focou na otimização de uma nova ferramenta para extração das cumarinas da espécie *P. balansae* utilizando tecnologias verdes (*green technology*). Esse trabalho foi realizado em uma parceria com o pesquisador Fernando Torres, que no âmbito da sua tese de doutorado também investigou as cumarinas presentes no gênero *Pterocaulon* focando também na síntese desses compostos. Essa parte da tese contou com a colaboração do Laboratório de Operações Unitárias da Pontifícia Universidade Católica do Rio Grande do Sul que disponibilizou o equipamento de fluido supercrítico para a realização dos processos de extração. A ideia inicial foi submeter as partes aéreas de *P. balansae* à extração por fluido supercítico (SFE) usando CO_2 como solvente. Uma extensa revisão, publicada em 2016, apresentou a extração por fluido supercítico como uma alternativa verde para a extração de compostos ativos, classificando como um método com boa recuperação de ativos, baixa degradação e maior facilidade de remoção do solvente. Na lista apresentada, das 33 extrações apenas uma utilizava propano e todas as outras utilizavam CO_2 como solvente no processo (DA SILVA; ROCHA-SANTOS; DUARTE, 2016).

Considerando as informações, o solvente escolhido para realizar as extrações foi o dióxido de carbono (CO_2). Inicialmente, foram fixadas algumas condições como o

solvente e a temperatura de 40 ºC e avaliado o efeito da pressão crescente (90, 120, 150 e 200 bar), sobre o rendimento em cumarinas totais e na preservação das características estruturais dessas cumarinas. Os resultados encontrados apontaram para a condição de 120 bar como a melhor pressão para obtenção dos maiores rendimentos de cumarinas. Nesta condição, os rendimentos foram 1,5; 2 ou 3 vezes maiores que as pressões de 90, 200 e 150 bar, respectivamente. As amostras foram analisadas por HPLC e observou-se que nessas condições de processo extraiu-se majoritariamente as cumarinas mais lipofílicas (4 – 7), ou seja, aquelas que apresentavam anel epóxi como substituição na posição 7 do anel cumárico, conforme apresentado na **Figura 1**.

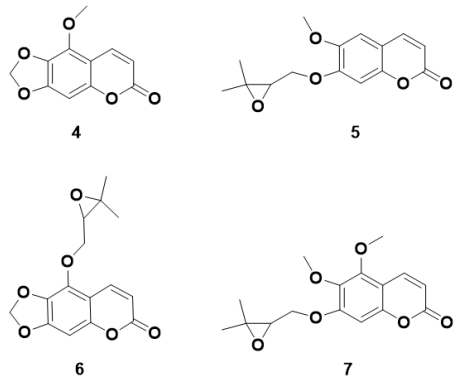

Figura 1. Cumarinas majoritárias encontradas no extrato por fluido supercrítico (SFE) das partes aéreas de *P. balansae*.

Essa condição de extração (40º C, 120 bar) mostrou-se seletiva para as cumarinas mais lipofílicas, com menor tempo de extração (40 min) e utilizando solvente *eco-friendly* com menor geração de resíduos na natureza.

O perfil de extrato encontrado no SFE mostrou-se diferente daquele observado para o AE avaliado em estudo anterior (MEDEIROS-NEVES et al., 2015). Era de se esperar que uma extração utilizando água como solvente tivesse mais afinidade pelos compostos mais hidrofílicos, como foi possível comprovar. O perfil do AE apresentou majoritariamente os compostos apresentados na **Figura 2**. Considerando os perfis distintos desses dois extratos (AE e SFE), optou-se por investigar a incorporação de ambos em nanoemulsões para testar suas atividades biológicas e determinar se as mesmas estavam atreladas aos tipos de cumarinas encontradas em cada extrato.

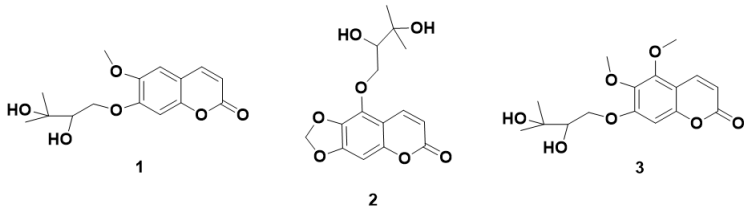

Figura 2. Cumarinas majoritárias encontradas no extrato aquoso (AE) das partes aéreas de *P. balansae*.

Após a validação das metodologias de análises cromatográficas optou-se por dar segmento ao método por CLUE para analisar a composição quantitativa das cumarinas presentes, devido às vantagens da redução no tempo de análise e consequentemente menor gasto de solvente. Nesse sentido, visando o tratamento tópico do fungo *Sporothrix schenckii*, o capítulo IV apresenta estudos de viabilidade da incorporação dos extratos de *P. balansae* em nanoestruturas lipídicas, o perfil de permeação/penetração destes compostos nas camadas da pele e a atividade antifúngica *in vitro* destas formulações frente a diferentes cepas do fungo *S. schneckii*.

O fungo *S. schenckii,* agente etiológico da esporotricose, é um fungo que pode afetar seres humanos, com lesões usualmente limitadas à pele e tecidos subcutâneos (DIXON et al., 1991; DA ROSA et al., 2005). Visando a permeação das cumarinas até camadas mais profundas da pele (por exemplo, derme) analisamos as características físico-químicas das nanoemsulões contendo extratos de *P. balansae* através de estudo de permeação/retenção cutânea em pele de orelha suína e posterior determinação da atividade antifúngica destas formulações frente a cepas do fungo *S. schenckii*.

O conjunto de resultados obtido demonstra que a técnica de homogeneização à alta pressão foi eficaz na produção das nanoemulsões contendo os extratos AE (NAE) e SFE (NSFE). O método de preparo gerou nanoemulsões monodispersas, com tamanho de gotícula de 162 nm e 127 nm, e potencial zeta de -32 e -21 mV, respectivamente. As cumarinas permearam nas camadas mais profundas da pele, principalmente quando havia remoção parcial do estrato córneo. Através das micrografias da pele observou-se que as formulações não danificaram os tecidos. Após a análise do perfil de permeação/retenção das formulações deu-se seguimento à etapa de análise *in vitro*

dessas formulações frente a diferentes cepas de *S. schenckii*, sendo duas cepas resistentes aos tratamentos convencionais. Os valores de MIC encontrados para os extratos puros (SFE e AE) foram acima de 1000 µg mL^{-1}, enquanto para as nanoemulsões esses valores foram 4 vezes menores, aproximadamente 250 µg mL^{-1} para todas as formulações. Os resultados apresentados demontraram que a incorporação dos extratos de *P. balansae* nos sistemas nanoestruturados intensificam a atividade antifúngica dos extratos.

Conclusões

— Desenvolveu-se um método por CLUE para realizar a análise das cumarinas presentes no extrato aquoso de *P. balansae*.

— Realizou-se a validação analítica e bioanalítica dos métodos por CLAE e CLUE para a quantificação da 5MMDC, que se mostrou específico, linear, preciso, exato e robusto para a análise da 5MMDC nas diferentes matrizes (extrato aquoso, nanoemulsão branca, derme/epiderme, extrato córneo e fluido receptor).

— Otimizou-se o processo de extração por fluido supercrítico em função de maiores rendimentos de cumarinas. As melhores condições encontradas foram solvente dióxido de carbono (CO_2), 40º C de temperatura e pressão de 120 bar.

— Demonstrou-se a viabilidade de incorporação dos dois diferentes extratos de *P. balansae* (AE e SFE) em um sistema nanoestruturado com propriedades físico-químicas adequadas para produtos de uso tópico.

— Os estudos de permeação/retenção cutânea *in vitro* demonstraram que as cumarinas da *P. balansae* permanecem retidas na epiderme da pele intacta, enquanto na pele lesada as cumarinas permeiam até o fluido receptor.

— Os extratos quando incorporados às nanoemulões apresentaram maior atividade antifúngica (MIC = 250 µg mL^{-1}) frente a diferentes cepas de *S. schenckii*.

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