UNIVERSIDADE DO ALGARVE

FACULDADE DE CIÊNCIAS E TECNOLOGIA

PHYTOCHEMICAL STUDIES AND BIOLOGICAL ACTIVITY OF CARNIVOROUS PLANTS FROM THE MEDITERRANEAN REGION

(Tese para a obtenção do grau de doutor no ramo de Ciências Biotecnológicas, especialidade de Biotecnologia Vegetal)

TOMÁS GREVENSTUK

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Orientador: Prof. Doutora Anabela Romano

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DECLARATION

This thesis contains results that were, or will be published in collaboration with A Romano, S Gonçalves, J Vervoort, JJJ van der Hooft, P de Waard, C Quintas, N Gaspar, AL Escapa, N Coelho, S Almeida, JMF Nogueira and N Neng.

I hereby declare that this thesis is a presentation of original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions.

Faro, September 2010

Tomás Grevenstuk

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δ	chemical shift
1D	one-dimensional
2D	two-dimensional
AAPH	2,2'-azobis-2-methyl-propanimidamide dihydrochloride
ABTS	2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid
$ABTS^{\bullet+}$	ABTS radical cation
amu	atomic mass units
ANOVA	analysis of variance
AOC	antioxidant capacity
ATCC	American type culture collection
AUC	area under curve
BA	6-benzyladenine
CFU	colony forming units
COSY	correlation spectroscopy
DAD	diode array detector (or diode array detection)
DMSO	dimethyl sulfoxide
DQF	double-quantum-filtered
EI	electron impact
ESI	electrospray ionization
F-C	Folin-Ciocalteu
g, mg, µg	gram, milligram, microgram
GAE	gallic acid equivalent
h, min, s	hour, minute, second
HAT	hydrogen atom transfer
HMBC	heteronuclear multiple bond coherence
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence
HTS	high throughput screening
Hz, kHz, MHz, GHz	Hertz, kiloHertz, megaHertz, gigaHertz
i.d.	internal diameter
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
INADEQUATE	Incredible Natural Abundance Double Quantum Transfer Spectroscopy
IZD	inhibition zone diamters
K	Kelvin
Kin	kinetin
L, mL, µL	liter, milliliter, microliter
cm, mm, nm	centimeter, millimeter, nanometer
m/z	mass to charge ratio
MAE	microwave-assisted extraction
mAU	milli absorbance units
MDR	multidrug resistance pumps
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	minimum inhibitory concentration
mM, µM	milllimolar, micromolar

MPa	megaPascal
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectrometer (or mass spectrometry)
MS	Murashige and Skoog culture medium
NAA	2-naphthaleneacetic acid
NCCLS	national committee for clinical laboratory standards
NMR	nuclear magnetic resonance
NOESY	nuclear-Overhauser-effect spectroscopy
^{o}C	degree Celsius
ORAC	oxygen radical absorption capacity
P	statistical probability
PCA	plate count agar
PGR	plant growth regulator
ppm	part per milion
ROESY	rotational frame nuclear Overhauser effect spectroscopy
ROS	reactive oxygen species
rpm	revolutions per minute
S/N	signal to noise ratio
SE	standard error
SET	single electron transfer
SFE	supercritical fluid extraction
SPE	solid phase extraction
SPSS	statistical package for the social sciences
TE	trolox equivalent
TEAC	trolox equivalent antioxidant capacity
TOCSY	total-correlation spectroscopy
TOF	time of flight
t_R	retention time
UAE	ultrasound assisted extraction
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
Zea	zeatin

ABSTRACT

In this thesis several studies were conducted with four carnivorous plant species which occur on Portuguese territory: *Pinguicula lusitanica*, *Pinguicula vulgaris*, *Drosera intermedia* and *Drosera rotundifolia*. Most habitats of these plants are threatened and natural populations are scarce, therefore micropropagation protocols were developed to produce biomass for the subsequent studies. Efficient micropropagation protocols were developed for *P. lusitanica* and *D. intermedia* enabling large scale biomass production, while protocols for the other two species have still to be optimized (in Chapter 2). The *in vitro* established cultures represent active germplasm collections of Portuguese natural populations and contribute therefore for their conservation. In Chapter 3 extracts prepared from micropropagated plant material were analyzed using state of the art HPLC-ESI-MS and HPLC-SPE-NMR equipment which enabled the identification of the major secondary metabolites produced by *P. lusitanica* and *D. intermedia*, directly from essentially crude extracts. The metabolites identified in *P. lusitanica* belong to the iridoid glucosides and caffeoyl phenylethanoid glycosides and *D. intermedia* was shown to produce mainly flavonoid glucosides, ellagic acid derivatives and the naphthoquinone plumbagin. The evaluation of the biological activities of these extracts, compiled in Chapter 4, showed that the methanol extract of *P. lusitanica* has considerable antioxidant activity and that the *n*-hexane extract of *D. intermedia* has high antimicrobial potential. In Chapter 5 a method for the extraction of plumbagin from micropropagated *D. intermedia* plants was optimized and its potential as an alternative for bioprospection evaluated. It was shown that the commercial exploitation of plumbagin from *D. intermedia* cultures might be viable and that UAE with *n*-hexane followed by an SPE purification step is an efficient procedure for obtaining large quantities of high purity plumbagin. It is hoped that this study represents an enrichment of the knowledge on these plants and contributes to their conservation and valorisation.

Keywords: *Pinguicula*; *Drosera*; micropropagation; conservation; hyphenated analytical techniques; antioxidant activity; antimicrobial activity; bioprospection; plumbagin.

Nesta tese apresentam-se resultados de diversos estudos realizados em quatro espécies de plantas carnívoras que ocorrem naturalmente em Portugal continental: *Pinguicula vulgaris*, *Pinguicula lusitanica*, *Drosera rotundifolia* e *Drosera intermedia*. As plantas carnívoras mantêm todas as características de qualquer outro ser vivo do reino vegetal: são plantas verdes onde ocorre fotossíntese, contudo estas plantas desenvolveram a capacidade única de capturar e digerir pequenas presas, pertencentes essencialmente ao grupo dos artrópodes. A maior parte das plantas carnívoras terrestres ocorre em turfeiras ou pântanos, onde persistem condições desfavoráveis constantes. Nestes habitats os solos encontram-se frequentemente submersos ou saturados em água e são de natureza ácida e relativamente pobres em relação ao teor de nutrientes disponíveis. Tendo em conta que o hábito carnívoro nas plantas surgiu em várias famílias distintas de forma independente, crê-se que representa uma adaptação aos factores de stress típicos destes habitats.

As plantas carnívoras despertaram o interesse dos biólogos desde longa data, devido à sua morfologia peculiar e aos seus hábitos carnívoros, no entanto, poucas espécies têm sido estudadas em relação aos metabolitos secundários que produzem e às suas potenciais aplicações farmacológicas. As plantas são organismos sésseis desprovidos de sistema imunitário e portanto desenvolveram estratégias alternativas que envolvem a produção de compostos orgânicos bioactivos capazes de dissuadir ataques de herbívoros ou infecções por parte de microorganismos. Milhares de anos de evolução resultaram na imensa diversidade de metabolitos secundários produzida actualmente pelas plantas. Apesar do desenvolvimento das técnicas de modelação molecular e síntese química, as plantas continuam a ser uma fonte importante de novas drogas e estruturas químicas, fornecendo pistas importantes para o tratamento de varias doenças. Estima-se que aproximadamente um quarto das drogas actualmente em uso clínico tenham sido isoladas directamente ou derivadas de fitoquímicos. É de salientar que as estruturas químicas provenientes de plantas para além de serem usadas directamente podem servir de precursores para novos medicamentos por processos de modelação química. Deste modo, o estudo da composição química de extractos preparados a partir de plantas,

aliado à avaliação das suas propriedades biológicas, torna-se muito importante no âmbito da identificação de substâncias bioactivas com interesse farmacológico.

O estado de conservação dos habitats das espécies estudadas neste trabalho é precário e as populações naturais não suportam a colheita de exemplares para a realização de um trabalho de investigação deste âmbito. Desta forma, no Capítulo 2 descrevem-se os protocolos de micropropagação desenvolvidos para as espécies em estudo, garantindo a produção de biomassa para os estudos subsequentes. A aplicação de técnicas de cultura *in vitro* é muito importante para alcançar os objectivos deste trabalho porque permite a obtenção, de forma rápida, de grandes quantidades de material a partir de quantidades reduzidas de tecido vegetal inicial. As culturas das quatro espécies foram iniciadas a partir de rebentos provenientes de germinantes produzidos *in vitro* de forma a evitar a recolha de exemplares do campo, reduzir a probabilidade de contaminações e manter uma diversidade genética elevada nas culturas estabelecidas. Em geral obtiveram-se percentagens de germinação relativamente baixas, pelo que poderá ser interessante testar o efeito de algumas técnicas de estratificação. No entanto, os germinantes obtidos demonstraram elevada capacidade de proliferação e permitiram avaliar os parâmetros de crescimento das espécies em vários meios de cultura.

O efeito da concentração do meio MS sem reguladores de crescimento foi testado em todas as espécies tendo-se verificado, de uma forma geral, que as culturas têm uma preferência para meios de cultura com baixas concentrações de macronutrientes, o que vai de encontro com as condições naturais em que as plantas carnívoras prosperam. No caso da espécie *P. lusitanica* testaram-se meios de cultura com três concentrações de meio basal (MS total, ½MS e ¼MS) suplementados com citocininas (BA, Kin e Zea) ou auxinas (NAA, IBA, IAA) a duas concentrações (0.2 e 0.5 mg/mL) e verificou-se que na grande maioria dos casos as culturas produziam simultaneamente novos rebentos e raízes, independentemente da composição do meio em reguladores de crescimento. Desta forma determinou-se que o protocolo de micropropagação para esta espécie dispensa uma fase de enraizamento adicional e que plântulas enraizadas podem ser produzidas num único passo. As culturas de *P. lusitanica* responderam melhor em meio MS suplementado com citocininas na concentração mais alta (0.5 mg/mL), obtendo-se cerca de 26 novos rebentos por explantado inicial e percentagens de enraizamento superiores a 90 % em todos os casos. O facto de se terem registado percentagens de

enraizamento superiores em meios suplementados com citocininas do que com auxinas é pouco comum e deve ser investigado. No entanto, as culturas também mostraram capacidade de proliferação e enraizamento elevadas em meios sem reguladores de crescimento, o que pode indicar níveis endógenos elevados de citocininas e auxinas. Meios de cultura suplementados simultaneamente com citocininas e auxinas não promoveram a proliferação de rebentos.

O desenvolvimento de um protocolo de micropropagação para a espécie *P. vulgaris* foi uma tarefa mais complicada devido à fragilidade e pouca viabilidade dos rebentos produzidos *in vitro*. Os rebentos mostraram-se muito susceptíveis ao passo de individualização no início de cada ensaio e portanto a quantidade de meios testada para esta espécie foi inferior. Em primeiro lugar foi testado o efeito da concentração de meio basal na capacidade de resposta das culturas usando as concentrações de MS total e ¼MS e posteriormente testou-se o efeito da adição de 0.1 mg/mL das citocininas BA e Zea individualmente, ou em combinação com a auxina IBA a 0.01 mg/mL, ao meio basal mais adequado. Os resultados demonstraram que o meio MS total influenciou negativamente o crescimento das culturas de *P. vulgaris* e que não se verificaram diferenças entre o controlo e meio de cultura suplementado com citocininas, em termos de rebentos produzidos ou percentagens de enraizamento. No entanto, do ponto de vista morfológico, os rebentos produzidos em meio de cultura sem reguladores de crescimento eram mais vigorosos enquanto os rebentos produzidos em meios suplementados com BA ou Zea mostraram por vezes sinais de necrose e reduzido desenvolvimento. Tal como no caso de *P. lusitanica*, a combinação de citocininas e auxinas não promoveu a proliferação das culturas de *P. vulgaris*. Desta forma, dos meios de cultura testados, o mais indicado para a micropropagação de *P. vulgaris* é o meio ¼MS sem reguladores de crescimento. No entanto, este protocolo deverá ser optimizado uma vez que a baixa viabilidade das culturas produzidas não permite produção de biomassa em larga escala.

Ao contrário das outras espécies estudadas, as percentagens de germinação de *D. intermedia* foram bastante elevadas. Curiosamente, registaram-se percentagens de germinação superiores no ensaio controlo do que no tratamento de estratificação a frio, ao contrário do que tinha sido observado para outras plantas do mesmo género. Na espécie *D. intermedia* foram testados meios com as concentrações de meio basal ¼MS,

½MS e MS total sem reguladores de crescimento ou suplementados com Kin 0.1 mg/L. A resposta de *D. intermedia* foi semelhante à de *P. vulgaris* na medida em que a redução da concentração de macronutrientes melhorou a proliferação dos rebentos e que a adição de Kin não induziu diferenças significativas na resposta das culturas. Quando cultivadas em meio ¼MS sem reguladores de crescimento, as culturas de *D. intermedia* produziram em media 15.8 rebentos ao fim de 8 semanas e em todos os casos os explantados iniciais formaram raízes, bem como mais de 80% dos novos rebentos formados. Como seria difícil melhorar qualquer um dos parâmetros de crescimento não foram testados outros meios. Enquanto os protocolos de micropropagação de *P. lusitanica*, *P. vulgaris* e *D. intermedia* foram desenvolvidos pela primeira vez neste trabalho, a cultura *in vitro* da espécie *D. rotundifolia* já tinha sido descrita previamente. No entanto, nestes estudos em vez de sementes, foram usados explantados recolhidos de exemplares de campo como material de partida. Para esta espécie testaram-se apenas dois meios, nomeadamente ¼MS e MS total. Apesar de se ter obtido um número de rebentos razoável nos dois meios testados, os rebentos eram de tamanho reduzido, muitas vezes difíceis de contabilizar e demasiado pequenos para iniciar novas culturas. Esta dificuldade foi também referida por outros autores, pelo que deve ser considerada uma fase de alongamento e uma fase de indução de raízes de forma a desenvolver um protocolo eficiente.

As plântulas micropropagadas de *P. lusitanica*, *P. vulgaris* e *D. intermedia* com sistemas radiculares bem desenvolvidos foram aclimatizadas com sucesso às condições *ex vitro* apresentando um desenvolvimento normal, sem aparentes anomalias morfológicas e com folhas funcionais capazes de capturar insectos, podendo ser usadas em programas de reintrodução em populações naturais. Em suma, foram desenvolvidos protocolos de micropropagação eficientes para *P. lusitanica* e *D. intermedia*, que permitiram a produção de material suficiente para as fases subsequentes deste trabalho. Os protocolos de micropropagação de *P. vulgaris* e *D. rotundifolia* têm que ser optimizados, no entanto, as culturas estabelecidas *in vitro* representam uma colecção activa de germoplasma que se pode tornar valiosa na eventualidade de extinção das populações.

No Capítulo 3 deste trabalho procedeu-se à caracterização química de extractos preparados a partir de material micropropagado de *P. lusitanica* e *D. intermedia*. Neste

capítulo discutiu-se também a possível relevância ecológica dos metabolitos secundários identificados bem como a sua significância taxonómica. Em projectos de descoberta de produtos naturais bioactivos é vantajoso realizar uma caracterização química numa fase precoce do trabalho para evitar o estudo alargado de compostos já conhecidos e caracterizados. Para o efeito recorreu-se às técnicas hifenadas HPLC-ESI-MS e HPLC-SPE-NMR. A espectroscopia de massa é uma ferramenta extremamente útil na identificação de compostos em amostras naturais, que para além da sua elevada sensibilidade, permite obter a massa molecular com grande precisão e portanto inferir a fórmula molecular, e em alguns casos até informação estrutural por análise do padrão de fragmentação. Em termos de informação estrutural a técnica de NMR é a mais valiosa, dando informações sobre distâncias intramoleculares entre átomos e grupos funcionais e acerca da orientação espacial de substituintes em torno de centros quirais, permitindo assim determinar a estrutura completa de uma molécula bem como relações de estereoisomerismo. O maior impedimento para o uso mais alargado da técnica de NMR, para além do custo elevado, está relacionado com a relativa baixa sensibilidade. No entanto, para além da miniaturização das células de fluxo, bobinas arrefecidas a temperaturas criogénicas e campos magnéticos cada vez mais potentes, o acoplamento de uma unidade automatizada entre o HPLC e o equipamento de NMR capaz de extrair e concentrar picos cromatográficos em cartuchos de SPE, tem contribuído muito para o ganho de sensibilidade. Desta forma, a utilização destas técnicas avançadas permitiram identificar os compostos maioritários directamente a partir de extractos sem ter que recorrer ao isolamento por técnicas de cromatografia preparativa para obter informação estrutural.

A espécie *P. lusitanica* não foi previamente estudada do ponto de vista bioquímico, tornando impossível a utilização de padrões. No entanto, explorando as potencialidades destas técnicas hifenadas foi possível a identificação dos seus metabolitos maioritários que pertencem a dois grupos de compostos naturais: iridóides e feniletanóides glicosídicos. A partir do extracto metanólico de *P. lusitanica* identificaram-se os iridóides ácido mussaenosídico, globularina e scutellarioside II e os feniletanóides acteoside, R/S campneoside I e R/S campeneoside II. Por análise das intensidades dos sinais dos protões anoméricos, determinou-se de forma aproximada que os compostos presentes em maior quantidade são o ácido mussaenosídico e acteoside. O extracto metanólico continha também um composto cuja estrutura foi impossível de determinar

conclusivamente, porque foi co-extraído juntamente com scutellarioside II e alguns dos seus sinais encontravam-se sobrepostos no espectro de NMR. No entanto, os dados preliminares indicam que o composto poderá não ter sido identificado previamente, podendo revelar-se interessante proceder ao seu isolamento.

No caso da espécie *D. intermedia* identificaram-se vários flavonóides glicosídicos (quercetina-3-*O*-galactopiranosídeo, quercetina-3-*O*-glucopiranosídeo, quercetina-3-*O*-(2’’-*O*-galoil)-galactopiranosídeo), miricetina-3-*O*-glucopiranosídeo e miricetina-3-*O*-(2’’-*O*-galoil)-galactopiranosídeo) e derivativos de ácido elágico (ácido elágico, 3-*O*-acido metil-elágico, ácido 3,3'-di-*O*-metil-elágico e ácido 3,3'-di-*O*-metil-elágico 4-*O*-glucopiranosídeo), para além de uma naftoquinona que poderá possivelmente ser hidroplumbagina di-1,4-*O*-glucopiranosídeo. O extracto aquoso de *D. intermedia* não foi investigado e o extracto hexânico era constituído exclusivamente por um composto, que foi analisado directamente por NMR e identificado como a naftoquinona plumbagina. Grande parte dos metabolitos secundários identificados foram atribuídos pela primeira vez a *D. intermedia*, fazendo com que este trabalho represente também uma contribuição para a melhor compreensão da bioquímica desta espécie. Do posto de vista taxonómico, o perfil de metabolitos secundários obtido para *P. lusitanica* e *D. intermedia* corroboram estudos realizados previamente noutras espécies das famílias Lentibulariaceae e Droseraceae, respectivamente.

Sendo impraticável testar um grande número de actividades biológicas em simultâneo neste trabalho, como ponto de partida decidiu-se avaliar a actividade antioxidante e antimicrobiana dos extractos preparados a partir de *P. lusitanica* e *D. intermedia*. A determinação da actividade antioxidante de um extracto é um dado importante na medida em que pode indicar potenciais actividades contra outros alvos biológicos. A avaliação da actividade antimicrobiana é igualmente importante devido à necessidade urgente de encontrar novas fontes de agentes anti-sépticos em resposta ao desenvolvimento de resistências múltiplas aos antibióticos em uso clínico. Quando possível, tentaram estabelecer-se relações de estrutura-actividade entre os compostos identificados previamente nos extractos e os resultados obtidos nos ensaios. Os resultados mostraram que o extracto com maior actividade antioxidante foi o extracto metanólico de *P. lusitanica*, possivelmente devido a um dos seus compostos maioritários, acteoside. Os dois grupos catecol desta molécula conferem uma elevada

predisposição para reagir com e estabilizar radicais livres, e evitar a propagação de reacções em cadeia. O facto de terem sido comprovadas actividades contra vários alvos biológicos e ser considerada não tóxica, torna esta molécula interessante para estudos posteriores. O extracto metanólico de *D. intermedia* também apresentou actividade antioxidante considerável, que pode ser explicada pela combinação de flavonóides e derivativos de ácido elágico presente no extracto.

Os extractos de *D. intermedia* foram mais eficazes nos ensaios de actividade antimicrobiana, especialmente o extracto hexânico, que inibiu o crescimento de todas as estirpes incluídas no painel de microorganismos seleccionados, à excepção de *P. aureginosa*. Esta actividade foi atribuída ao composto maioritário do extracto, plumbagina, para o qual já tinha sido comprovado elevada actividade antimicrobiana. A actividade desta naftoquinona parece estar relacionada com a sua capacidade de se ligar covalentemente a biomoléculas tornando-as inactivas, mas também com a sua capacidade de produzir radicais livres em sistemas biológicos. Porém, a possível pequena margem de segurança da plumbagina tornam a sua aplicação farmacológica incerta. O extracto metanólico de *D. intermedia* também inibiu o crescimento de grande parte dos microorganismos testados incluindo, curiosamente, a estirpe multirresistente *P. aeruginosa*. Como os antibióticos activos contra esta estirpe são escassos seria interessante estudar em maior detalhe o mecanismo subadjacente. Por sua vez, o extracto de *P. lusitanica* mostrou possuir reduzida actividade antimicrobiana. Apesar deste estudo não ter identificado potenciais candidatos para o desenvolvimento de novos fármacos é importante a continuação de programas com o intuito de escrutinar aplicações de extractos vegetais contra alvos biológicos, tendo em conta o enorme contributo que o Reino das plantas tem dado à medicina moderna.

As análises efectuadas no Capítulo 3 demonstraram que era possível obter, de uma forma relativamente simples, quantidades significativas de plumbagina de elevada pureza a partir de material micropropagado de *D. intermedia*. Desta forma, aliado ao valor comercial desta naftoquinona, decidiu-se avaliar o potencial de prospecção de plumbagina a partir de culturas *in vitro* de *D. intermedia*. A produção de biomassa foi monitorizada ao longo do tempo para determinar o período de crescimento máximo e o material micropropagado foi extraído por várias metodologias: maceração com agitação, extracção Soxhlet, extracção assistida por ultrasons (UAE) e extracção com fluidos

supercríticos (SFE). Os resultados demonstraram que, para além da taxa de produção de biomassa ser bastante elevada, as quantidades de plumbagina produzidas por *D. intermedia* são superiores em relação à actual fonte de prospecção de plumbagina, as plantas do género *Plumbago*. A comparação dos métodos de extracção levou a concluir que a melhor alternativa para a extracção de plumbagina é aplicar ultrasons à matriz vegetal colocada no solvente de extracção (*n*-hexano). Este procedimento traz vantagens em relação ao tempo de operação e permite obter rendimentos de extracção superiores, assim como maiores concentrações de produto, um factor importante para os passos subsequentes de purificação. Em alternativa, o material vegetal pode também ser extraído com fluidos supercríticos. Apesar de esta metodologia ter produzido resultados inferiores à técnica de UAE, os rendimentos foram consideráveis para uma primeira abordagem. A possibilidade de evitar o uso de solventes orgânicos nocivos é uma grande vantagem do ponto de vista ambiental. Na segunda parte do desenvolvimento do processo de extracção de plumbagina avaliou-se a potencialidade de usar colunas de SPE para concentrar e purificar os extractos. Os resultados demonstraram que usando esta abordagem é possível remover grande parte das impurezas co-extraídas num único passo com pequenas perdas de produto. Aplicando a purificação por SPE ao extracto obtido por UAE é possível produzir plumbagina em grandes quantidades com uma pureza final próxima dos 100%.

Neste trabalho pretendeu-se estudar a composição química e avaliar as propriedades biológicas de extactos preparados a partir de algumas espécies de plantas carnívoras que existem em Portugal. Para tal foi imperativo o desenvolvimento de técnicas de micropropagação que permitiram também optimizar um processo de bioprospecção de um metabolito secundário de valor. Espera-se que este trabalho tenha, de forma geral, contribuído para a conservação e valorização das espécies estudadas.

Palavras-chave: *Pinguicula*; *Drosera*; micropropagação; conservação; técnicas analíticas hifenadas; actividade antioxidante; actividade antimicrobiana; bioprospecção; plumbagina.

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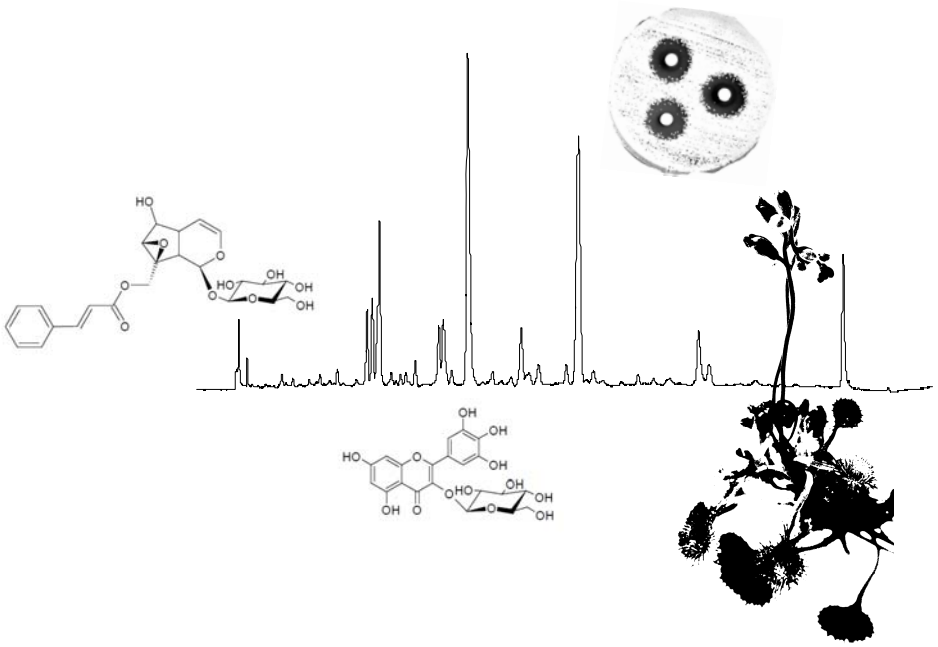
CHAPTER 1

GENERAL INTRODUCTION



1.1. Current status of drug discovery from natural products

Although the modern pharmaceutical industry was born from natural product research, synthetic approaches to drug discovery have become standard. The role of natural products in drug discovery has recently been diminished by the advent of structure activity-guided organic synthesis, combinatorial chemistry, and computational (*in silico*) drug design (Schmidt et al., 2008). This trend is in great part due to the increased compatibility of these synthetic approaches with high throughput screening (HTS) methods, whereby a large number of samples (up to 100000 in 24 h) can be screened for a single activity using molecular targets (Bindseil et al., 2001; Gurib-Fakim, 2006; Schmidt et al., 2008). Natural extracts, being comprised of a complex mixture of compounds are difficult to implement in HTS platforms (Bindseil, 2001). However, this modern approach has led to a decline in new drug development in the past two decades (Butler, 2004; Rishton, 2008).

Comparative analysis of structural diversity in natural product mixtures and combinatorial libraries suggests that nature still has an edge over synthetic chemistry. Despite the fact that combinatorial libraries use superior elemental diversity, this does not compensate for the overall molecular complexity, scaffold variety, stereochemical richness, ring system diversity, and carbohydrate constituents of natural product libraries (Lee and Schneider, 2001; Feher and Schmidt, 2003; Newman, 2008). It is generally believed that the complexity of plant-produced secondary metabolites and the vast number of natural products will constitute a resource beyond the capacity of current synthetic chemistry for a long time (Koch et al., 2005). In addition, natural products, characterized as small-molecule secondary metabolites that originate from terrestrial and marine plants, microorganisms and animals, tend to present more structurally diverse ‘‘drug-like’’ and ‘‘biologically friendly’’ molecular qualities than pure synthetic compounds at random, and are an important source of novel lead structures for the synthetic and combinatorial chemistry aspects of drug discovery (Bindseil et al., 2001; Vuorelaa et al., 2004; Pan et al., 2010). This is because the importance of natural product molecules to medicine lies not only in their pharmacological effects but also in their role as template molecules for the production of new drug substances. Morphine from the opium poppy, for example, which continues to be used as a highly effective analgesic for the relief of terminal pain, has also served as a template molecule for the

design of numerous drugs including analgesics such as pethidine and pentazocine and the cough suppressant dextromethorphan (Philipson, 1994). An organic chemist considering the structure of morphine would be quick to point out that such a molecule would never have been conceived of by medicinal chemists engaged in a rational drug design program for pain. Without morphine as a small molecule tool for pharmacology and without its unique chemical structure for inspiration, drug discovery scientists might never have developed an analogously effective therapy for pain (Rishton, 2008).

Industrial funding for natural product-based drug discovery has been declining (Bindseil et al., 2001), yet the percentage of natural product-derived small molecule patents has remained relatively unchanged and there has also been a steady introduction of new natural product and natural product-derived drugs (Butler, 2004; Koehn and Carter, 2005). Between 2000 and 2003 a total of 15 drugs were launched which included new drug types such as the antimalarial arteether (Graul, 2001), the antifungal caspofungin (Graul, 2002), the anti-Alzheimer's drug galantamine (Heinrich and Teoh, 2004) and the antibacterial lipopeptide daptomycin (Frantz, 2004). On the contrary, while the investment in R&D and clinical development using current drug discovery approaches has skyrocketed, the output of newly launched drugs has fallen (Butler, 2004). Surprisingly, to date, there has been only one drug approved by the US Food and Drug Administration (sorafenib for renal carcinoma in 2005) resulting from high-throughput screening of combinatorial chemistry libraries followed by the optimization of hits (Newman, 2008). This way, it can be stated that the major achievements of natural product research of the past decades have clearly demonstrated that natural products represent an unparalleled reservoir of molecular diversity to drug discovery and development, and are complementary to combinatorial libraries (Pieters and Vlietink, 2005).

1.2. Drug discovery from plants

Plants continue to serve as a valuable source of therapeutic compounds because of their vast biosynthetic capacity. It is estimated that plant-derived natural products represent more than 25% of all drugs in clinical use in the world (Rates, 2000; Gurib-Fakim, 2006). Examples of important drugs obtained from plants are digoxin from *Digitalis* spp., quinine and quinidine from *Cinchona* spp., vincristrine and vinblastine from

Catharanthus roseus, atropine from *Atropa belladonna* and morphine and codeine from *Papaver somniferum* (Rates, 2000). However, the potential of higher plants as a source for new drugs is still largely unexplored. Among the estimated 250000-500000 plant species, only a small percentage has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even smaller (Hamburger and Hostettmann, 1991).

The wide molecular diversity of metabolites throughout the plant kingdom represents an extremely rich biogenic resource for the discovery of novel drugs and for developing innovative drugs. Not only do plant species yield raw material for useful compounds but knowledge on their biochemistry also provides pointers for rational drug development (Phillipson, 2007). Plant constituents have a key position in the advancement of knowledge on biological activity because bioactive plant compounds are themselves products of metabolism, and hence function in life processes in a similar way to compounds that operate in humans and animals (Gurib-Fakim, 2006). Most of the plant compounds that have been found to be medicinally useful and interesting tend to be secondary metabolites.

1.2.1. The biogenetic significance of secondary metabolites

A typical character of plants is the production and storage of usually complex mixtures of secondary metabolites. Although the function of most is unknown, and only limited numbers of secondary metabolites have been studied in detail in terms of physiology, biochemistry and ecology, it is safe to assume that secondary metabolites are not functionless waste products (as suggested earlier in the 20th century), but important for the plants in an ecological context (Wink, 2008).

Despite the uses that mankind may have for secondary metabolites, they are compounds that have important functions in the organism that produces them (Macías et al., 2007). Ever since their existence, plants had to cope with infectious diseases and animals which tried to feed on them, and although being obvious, it is important to note that plants cannot run away when challenged by a herbivore nor do they have an elaborate immune system to fight off a microbial infection. As a common defence measure, plants and other sessile organisms evolved bioactive natural products, which repel, deter or

poison herbivores and which can inhibit growth and development of bacteria, fungi and even viruses (Wink, 2008). Some of the defense compounds are constitutive (phytoanticipins) while others can be induced under stress conditions (phytoalexins) and are synthesized *de novo* when a plant is challenged by bacteria, fungi or viruses (Macías et al., 2007). Because plants have to compete with other plants for light, water and nutrients, secondary metabolites often also serve as mediators in plant-plant interactions, termed as allelopathy. During evolution, secondary metabolites were apparently optimized in such a way that they did not only exhibit defensive but also additional non-defence functions: some have additional physiological and ecological functions (for example, as nitrogen storage compounds or UV protectants) or serve as signal compounds to attract pollinating or seed dispersing animals and can mediate the interactions between symbiotic bacteria and their plant hosts (Wink, 2008).

The metabolic system of a plant may be regarded as being constituted of regulated processes within which biochemical conversions and mass transfer take place. The metabolic performance of living organisms can be distinguished into primary metabolism and secondary metabolism. Primary metabolism is associated with fundamental life processes common to all plants. It comprises processes such as photosynthesis, pentose cycle, glycolysis, the citric acid cycle, electron transport, phosphorylation and energy regulation and management. Secondary metabolites are therefore termed as group of compounds that are not directly involved in the normal growth, development or reproduction of organisms. Primary and secondary metabolisms are interconnected in the sense that the biosynthesis of accumulating secondary metabolites can be traced back to ubiquitous primary metabolites. However, in contrast to primary metabolites, secondary metabolites represent features that can be expressed in terms of ecological, taxonomic and biochemical differentiation and diversity (Gurib-Fakim, 2006). Secondary metabolites are often restricted to a narrow set of species within a phylogenetic group and can therefore provide a basis for chemosystematics.

1.2.2. The importance of biotechnological approaches

Many higher plants which are used as sources of pharmaceuticals and are of value in drug discovery are rare or threatened with extinction (Phillipson, 1994). In addition, drug discovery programs require large quantities of material which cannot be simply

harvested from nature. Quantitative considerations regarding the average yield of active compounds and the amount of starting crude plant material required for the discovery, development and launch of a new drug on the market emphasize the urgency of using alternative procedures by which it can be obtained: 50 kg of raw material are necessary to provide 500 mg of pure compound for bioassays, toxicology, and *in vivo* evaluation; and full pre-clinical and clinical studies can require 2 kg of pure compounds obtained from 200 ton of raw material (Rates, 2000).

Placlitaxel is a good example of the application of biotechnological strategies to this field. Placlitaxel is one of the most important natural product-derived antitumor agents found in the recent past and was initially isolated from *Taxus brevifolia*. However, the biggest obstacle to its clinical use was obtaining the material, considering that in order to produce 2.5 kg of taxol, 27000 tons of *T. brevifolia* bark were required and 12000 trees had to be cut down. Due to the high demand, this species of *Taxus* would soon be extinct if no alternative source could be developed (Hamburger and Hostettmann, 1991). The antitumour agent contains 11 chiral centres with 2048 possible diastereoisomeric forms so its synthesis *de novo* on a commercial scale appears to be unlikely (Phillipson, 1994). Currently the drug is produced by plant cell fermentation and *Taxus* trees are no longer used in the process. Plant biotechnology offers the possibility of improved production methods of cultivated medicinal plants as well as alternative approaches to the production of natural products for the preparation of pharmaceuticals (For further details see Sections 2.1.2 and 5.1.1.).

1.2.3. Strategies for drug discovery

Different approaches to drug discovery using higher plants can be distinguished; however, all have the final goal of isolating new bioactive products or lead structures with novel structures and novel mechanisms of action. In all cases plants can be either selected randomly or as a follow-up of bioactivity reports or ethnomedical uses (Cos et al., 2006). The most common approach and the one accessible to most laboratories consists on performing biological assays using essentially crude extracts and selecting the most promising extracts for chemical analysis with the intent of trying to identify the active compounds. The entire extract can be chemically characterized and the active components can be identified by confirming the activity with commercial standards of

the identified compounds. However, following this approach the generated new knowledge is limited as only the activity of known compounds is determined, although possibly in a new bioactivity context. Alternatively, following the approach of bioactivity-guided fractionation, the plant extracts can be sequentially fractionated and the active components can be identified by subjecting each fraction to bioassay (Verpoorte, 1989). The compound or compounds of the active fraction can then be purified by chromatographic methods and structurally characterized by spectroscopic methods. However, this approach also presents some limitations. Bioactivity-guided fractionation may exclude compounds with relevant pharmacological activities when the effect is not caused by a single compound, but rather by a combination of compounds, as a result of pharmacodynamic synergism. A good example of this is *Panax ginseng* in which the whole plant or its saponin fractions are more active than the isolated compounds (Hamburger and Hostettmann, 1991). In addition, when only one activity is considered in pharmacological screens the possibility exists of missing out compounds with interesting activities for which the assay does not test for. *Catharanthus roseus* was initially studied for its anti-diabetic activity described in folk medicine, but was then shown to produce the powerful anti-tumour compounds, vincristine and vinblastine (Rates, 2000). Another issue concerns the possibility of isolating already known and characterized compounds after the laborious and time consuming efforts to isolate and determine the structure of the active compound.

Another strategy consists on performing a chemical screening prior to biological assays and submitting only new or structurally interesting compounds to bioassays. The process of rapidly indentifying known compounds is known as dereplication and ensures that novelty is brought into the isolation process and that no time is wasted on re-investigating existing and known molecules (Sprogøe et al., 2007). The high sensitivity and efficiency of current hyphenated techniques such as HPLC-UV, HPLC-MS and HPLC-NMR allow for the rapid identification of known compounds in an early stage of the procedure, and identification of enough of an unknown structure to prioritise or conduct an isolation (Wolfender et al.; 2003, Butler, 2004). This can mean either a full identification of a natural product after only partial purification, or partial identification to the level of a family of known compounds after which the most promising lead-structures are selected for further investigation.

A more radical approach consists on generating libraries of pure natural products which are compatible with HTS and can be tested against a large number of molecular targets in a reduced time (Bindseil et al., 2001). Based on the principle that natural products offer structural diversity that is not rivalled by the creativity or synthetic ingenuity of synthetic chemists, it is suggested that the most important paradigm shift for natural product chemistry is the general change from activity-guided extract screening to pure-compound screening, which implies activity-independent compound isolation and characterization. Despite being obvious that pure compound isolation includes significant investments before screening, the overall process from screening to a validated lead is much faster, as well as significantly less expensive, when pure natural-compound libraries are used as basic raw materials, and not crude extracts. Resulting from a collaboration between the pharmaceutical companies Aventis Pharma AG (Vitry sur Seine, France) and AnalytiCon Discovery (Berlin, Germany), a library of 4000 pure natural products was generated and in most cases, the natural product libraries showed superior hits to in-house combinatorial libraries (Bindseil et al., 2001). This confirms the potential of natural products in modern drug discovery and it is believed that this strategy will be responsible for the revival of natural product research.

1.3. Plant description, taxonomy and biology

1.3.1. The trait of carnivory in plants

Carnivorous plants have acquired the unique ability to capture prey and to absorb nutrients from the captured animals. The majority of terrestrial carnivorous plants grow in bog and fen soils, where they endure persistent unfavourable conditions, i.e., the soils are usually wet or waterlogged, mostly acidic, and poor in available mineral nutrients (Adamec et al., 2005). The multiple, independent evolution of carnivory in diverse plant families suggests that it is an adaptation to the stress factors typical of these habitats (Givnish et al., 1984; Ellison and Gotelli, 2001; Adlassnig et al., 2005). Givnish et al. (1984) proposed a cost-benefit model that predicts that carnivory is adaptive only in nutrient-poor environments that are well lit and moist, because the photosynthetic costs to carnivory are thought to exceed the benefits in either shady or dry habitats. Thus, in these habitats carnivory confers an important competitive advantage in the ability to

obtain nutrients without an overwhelming cost to photosynthesis (Ellison and Gotelli, 2001).

The prey of carnivorous plants range from unicellular organisms to small mammals, although the most common prey are insects (Darnowski et al., 2006), and therefore these plants are often referred as insectivorous plants. In order to be carnivorous, a plant must attract, trap and digest prey, followed by nutrient absorption. To attract prey the plants secret nectar or exhale a sweet odor or exhibit bright colors (Adlassnig et al. 2005). A variety of active and passive mechanisms exist for trapping prey. A well-known example of an active mechanim is that of the Venus flytrap, *Dionaea muscipula* (Droseraceae), which possesses modified leaves, the lobes of which close on prey when trigger hairs are touched. A passive example is found in the pitcher-plants (*Sarracenia* spp. and *Heliamphora* spp; Sarraceniaceae) which present pitfall traps filled with water or digestive fluid.

Drosera spp. (Droseraceae) use traps generically termed as fly-paper traps, which are partly passive in their action, consisting of highly specialized leaves bearing two types of glands, stalked glands which attract insects by their distinctive red coloured head and by their mucilage secretions rich in carbohydrates, and sessile glands which produce digestive enzymes (Figure 1.1.1). Rost and Schauer (1977) found that the mucilage of *Drosera capensis* is composed of a 4% aqueous solution of a complex polysaccharide containing xylose, mannose, galactose, and glucoronic acid with no protein and a pH around 5, at which value the viscosity is maximal. When prey adheres to a tentacle and struggles, there is an initial rapid movement of the individual tentacle, followed by the slower inflection of the leaf lamina itself ensuring that the prey is held. Leaves are induced by the presence of trapped insects to secrete enzymes for digestion of the prey (Matusikova et al., 2005). One to two weeks after prey has been caught, the leaf opens and the blackened remains, mostly chitinous pieces of legs and wings, fall off or blow away, allowing for the leaf to make repeated captures (Crowder et al., 1990). Other genera of carnivorous plants that use the same basic mechanism include *Byblis* (Bylidaceae), *Drosophyllum* (Drosophyllaceae), *Pinguicula* (Lentibulariaceae) and *Triphyophyllum* (Dioncophyllaceae) (Darnowski et al., 2006).

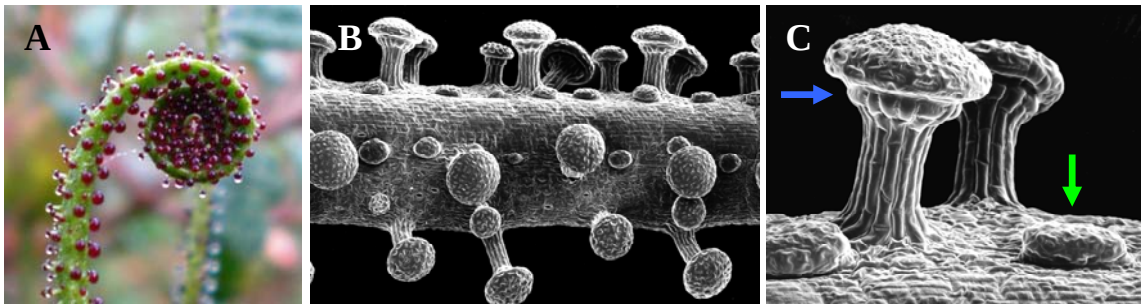


Figure 1.1.1 - Example of specialized glands of a carnivorous plant using the fly-paper mechanism (*Drosophyllum lusitanicum*): leaf extremity bearing distinctive red coloured stalked glands (A); Scanning electron microscopy of leaf (B) and detail of stalked (blue arrow) and sessile gland (green arrow) (C). (Photographs by S Gonçalves, used with permission).

A considerable number of these carnivorous plant species are included in the pharmacopeias (Blumenthal et al., 1998), however, very few have been studied from a chemical and pharmacological perspective. In Portugal, three genera of terrestrial carnivorous plants are represented, the genus *Drosera* (*D. rotundifolia* and *D. intermedia*), *Pinguicula* (*P. vulgaris* and *P. lusitanica*) and *Drosophyllum* (*D. lusitanicum*). *D. lusitanicum* has been previously studied at the Plant Biotechnology Lab and besides the development of a micropropagation protocol (Gonçalves and Romano, 2005), extracts prepared from this species were investigated for their biological activities (Gonçalves et al., 2009) and chemical composition (Grevenstuk et al., 2008), and the encouraging results obtained incentivized the study of other carnivorous plant species. The distribution in Portugal of the plant species under study in this thesis are depicted in Figure 1.1.2.

1.3.2. The genus *Pinguicula*

1.3.2.1. Taxonomy and geographical distribution

Pinguicula is one of the three genera that together with *Genlisea* and *Utricularia* compose the Lentibulariaceae family. The genus *Pinguicula* consists of some 85 currently accepted species (Cieslak et al., 2005) which are present on all continents except Australia and in Africa is limited to the extreme north-west of the continent (Heslop-Harrison, 2004). The greatest concentration of *Pinguicula* spp. is in the humid mountainous regions of Central America and South America, where they probably originated as it is the centre of diversity. In Europe, 12 species were described by

Casper (1962) and nine species occur on the Iberian Peninsula, of which five are endemics (Blanca et al., 1999). The representatives of the genus in the Iberian Peninsula belong to the three subgenera into which it has been subdivided: subgenus Pinguicula, Isoloba and Micranthus. The Lentibulariaceae family is placed in the Lamiales order which has been supported by cladistic analysis (APG, 1998).

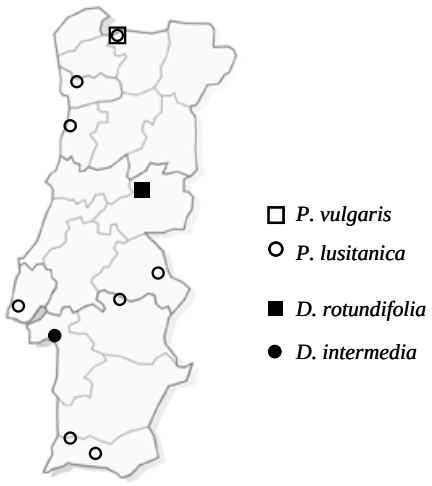


Figure 1.1.2 - Distribution of *P. lusitanica*, *P. vulgaris* (Blanca et al., 1999), *D. rotundifolia* and *D. intermedia* (Crowder et al., 1990) in continental Portugal.

1.3.2.2. Biology, morphology and ecology

The *Pinguicula* spp., commonly known as butterworts, are herbaceous, relatively short-lived perennials (although occasionally behaving as annuals) and of rosette habit in active growth, while some overwinter as resting buds (hibernacula). As well as sexual reproduction by seed, many reproduce vegetatively by means of bulbils or buds which later take root. Unlike members of the other two genera in the family (*Genlisea* and *Utricularia*), all species of *Pinguicula* bear true roots, which are generally fibrous, tufted, and ephemeral. The leaves, which in most species lie appressed to the ground, are occasionally heterophyllous and the later formed ones may be larger and semi-erect (whereby the plant can tolerate more shaded conditions). The leaves are adapted for insectivory and bear stalked and sessile glands on the upper surfaces. The stalked glands carry permanent mucilaginous droplets giving the characteristic greasy feel and its generic name which is derived from the Latin *pinguis* - fatty or greasy to the touch.

However, unlike the *Drosera* spp., the leaves of most *Pinguicula* spp. are sessile and therefore the capture system is entirely passive (Blanca et al., 1999). As with almost all carnivorous plants, the flowers of the butterworts are held far above the rest of the plant by a long stalk, in order to reduce the probability of trapping potential pollinators.

Pinguicula plants are restricted to nutrient-poor habitats, such as bogs and swamps, which remain sunny and moist at least during the growing season (Blanca et al., 1999). Despite the rarity of such sites in the Mediterranean ecosystems, many *Pinguicula* species are known from the Mediterranean basin (Casper, 1962). In these regions, where plant growth is greatly limited by water availability, suitable habitats for the *Pinguicula* species are scattered. Populations of the same species are often separated from one another by large distances and the isolation of the populations might have played an important role in the speciation processes. Since the current aridity of the Mediterranean basin has made small, isolated populations vulnerable to extinction, there is an urgent need to ensure the conservation of these species (Zamora et al., 1996).

1.3.2.3. *P. vulgaris*

Pinguicula vulgaris (L.) Linneaus, or the common butterwort, is a perennial plant consisting of a rosette of 4-7 leaves lying close to the ground, shallowly anchored by a tuft of fine, fibrous roots and overwinters as a hibernaculum (Figure 1.1.3 A). The leaves are bright, yellowish-green, and fleshy in texture with the margin somewhat involute and the upper surface covered with stalked glands. Each plant can produce 1-8 scapes in succession in the growing season which increases in length as the fruit develops (Figure 1.1.3 B). *P. vulgaris* produces violet, solitary, bisexual and zygomorphic flowers (Figure 1.1.3 C). Normally the flower assumes a horizontal posture at anthesis, but is occasionally held more or less erect (Heslop-Harrison, 2005). Besides seed formation, *P. vulgaris* also reproduces vegetatively by means of buds formed in the axils of the last foliage leaves of the season (Blanca et al., 1999). Plants produce flowers, after being grown from seed or bulbils, usually in their third year, i.e. after their second season of vegetative growth. Flowering and subsequent seed set are usually then of annual occurrence, if conditions are favourable. Seedling establishment in the wild is precarious because the tiny seed size provides negligible food reserves, and suitable wet sites free from competition by other species are rare. Although small in

number, the axillary buds probably provide an effective method of reproduction because these can draw on relatively large starch reserves stored in the bud scales (Heslop-Harrison, 2005).

P. vulgaris has a northern circumpolar distribution. The species is widespread in the northern and upland parts of Europe, extending into Corsica, Italy and Macedonia and across Siberia into north Asia but it thins out eastwards to Ukraine. Its most northerly limit is on the east coast of Greenland and southwards into central Spain and north Portugal. In North America it extends from Alaska in the north, as far south in the USA as northern New York State, the southern limit being roughly equivalent to that in Europe (Heslop-Harrison, 2005). It occurs mainly in seepage channels in the less acid parts of bogs, mires, calcareous fens and flushes, wet heaths and on wet rocks and seems to be indifferent to soil type (Blanca et al., 1999). A high humidity requirement during the growing season limits the number of suitable habitats available for the species, and it can survive only some degree of desiccation as a hibernaculum. Leaf extracts of *Pinguicula* spp. were found by early herbalists to be effective in giving spasmodic relief in cases of whooping cough, asthma, tuberculosis and spasms of intestinal pain (Christen, 1961; Hegnauer, 1966; in Heslop-Harrison, 2005). Biochemical data indicates that *P. vulgaris* produces iridoid glucosides (Damtoft et al., 1985; 1994; Marco et al., 1985; Section 3.1.2).

1.3.2.4. *P. lusitanica*

Pinguicula lusitanica (L.) is a small herbaceous plant consisting of a horizontal rosette of 5-12 leaves, lying close to the ground, shallowly anchored by a tuft of fine, fibrous roots (Figure 1.1.3 D). Unlike most species of *Pinguicula* in Europe, *P. lusitanica* belongs to the subgenus Isoloba and its morphological, vegetative and floral characteristics are closer to the species centred in the Gulf of Mexico, rather than to the other European ones (Casper, 1962). The leaves are oval or oblong-oval and sometimes the margins are inrolled exposing little of the lamina which is pale green with dark red veins and covered with stalked red-headed glands on the upper surface (Heslop-Harrison, 2005). In general, *P. lusitanica* plants flower successively over a period of months from May until August, but it can vary significantly according to climate as variations, as they are able to flower as early as April in temperate climates, until as late

Figure 1.1.3 - Illustrations of the carnivorous plant species under study. *P. vulgaris*: rosette detail (A), flowering plants (B), flower detail (C); *P. lusitanica*: rosette detail (D), individual plant and reproductive structure at early stage of development (E), flower detail (F); *D. rotundifolia*: mature plant (G), upper leaf detail (H), detail of inflorescence and flowers (I), captured insect (J); *D. intermedia*: flowering plant (K), leaf detail (L).

October in the Bristish Isles. A single, slender, erect and glandular scape is produced per leaf axil, reaching 1-6 in number per season, which lengthens as the fruit develops to a maximal length of 15 cm (Figure 1.1.3 E). The flowers are small, covered with glands and pale lilac in colour (Figure 1.1.3 F) (Blanca et al., 1999). *Pinguicula lusitanica* does not form a hibernaculum at the end of the growing season. When conditions remain relatively mild the plant overwinters as a rosette, or occasionally it behaves as an annual (Heslop-Harrison, 2005).

P. lusitanica is a native of bogs, wet heaths and is restricted to the oceanic, relatively frost-free areas wherever the substratum is favourable and sufficient moisture is available. It is found along coastal western Europe from Scotland to Iberia and Morocco in north-western Africa (Heslop-Harrison, 2005). In continental Europe *P. lusitanica* is found in sites from 10 m to 1200 m in altitude (Blanca et al., 1999) and its maximum limits are approximately only half those of *P. vulgaris*, possibly due to its habit and distinctive method of perennation. As opposed to *P. vulgaris*, it does not reproduce vegetatively and reproduction is entirely dependent on seed production. A single plant does not persist for longer than 2–3 years, and flowering is usually annual, although in unfavourable conditions flowering may be delayed until the following year. Seed set is very good as large numbers of seeds are released from the capsules, however, establishment is limited because seedlings do not withstand competition (Heslop-Harrison, 2005). In addition, *P. lusitanica* is particularly susceptible to trampling because of the brittle and very thin nature of its leaves and the weak anchorage afforded by its short and shallow fibrous root system. It is mainly restricted to areas which have never been cultivated, and recent drainage and other man-made operations have resulted in the destruction of many of its previously existing sites. There is no biochemical data available for *P. lusitanica*, possibly due to its scarce existence.

1.3.3. The genus *Drosera*

1.3.3.1. Taxonomy and geographical distribution

The genus *Drosera* belongs to the Droseraceae family together with the genera *Aldrovandra* and *Dionaea*. It comprises nearly 150 species with a worldwide distribution, however the vast majority of species are found in the Southern

Hemisphere, especially in south-western Australia (Rivadavia et al., 2003). In Europe only three species exist, *Drosera anglica*, *D. intermedia* and *D. rotundifolia* (Crowder et al., 1990). Droseraceae is classified in the order Caryophyllales, in a clade with three other families containing carnivorous genera, the Nepenthaceae, Drosophyllaceae, Dioncophyllaceae, and one non-carnivorous family, the Ancistrocladaceae.

1.3.3.2. Biology, morphology and ecology

Plants of the *Drosera* genus bear leaves with pin-shaped tentacles covered at the tip with glistering drops of mucilage that resemble drops of morning dew and to which they owe their scientific name (from Greek *droseros*, dewy) and their common name of sundew (Crowder et al., 1990). Sundews are short-lived perennial (or rarely annual) herbaceous plants which in most cases form prostrate or upright rosettes. In Australia however, many have long aerial stems, a few are bushy and up to 1 m tall, while others have scrambling stems up to 1.5 m long. The mature leaves vary in shape according to species but all are characterized by the above mentioned glandular tentacles that cover the upper part of their laminae. In most parts of the world the plants are confined to wet or damp sites, but in Australia many live in seasonally dry sites and perennate as tubers (Crowder et al., 1990). Most species are rather strongly light demanding, but some species with particularly large leaves occur in eucalypt and rain forest in southern Australia and northern Queensland (Australia), respectively (Lavarack, 1979). Many species of sundews are self-fertile and flowers will often self-pollinate upon closing but they also show varying degrees of vegetative reproduction. The seeds of most species germinate in response to moisture and light, while seeds of the tuberous species require a hot, dry summer period followed by a cool, moist winter to germinate.

Drosera species have long held the interest of botanists and horticulturists because of their unique biology and carnivorous habit. However, apart from their ornamental value, *Drosera* plants have medicinal significance and due to uncontrolled collection, natural populations are becoming increasingly scarce, especially in Europe where the *Drosera* species are included in the European Red List of Threatened Plants (Kawiak et al., 2003). In addition, according to a report of the Portuguese Instituto da Conservação da Natureza (ICN, 2006) the natural habitats of the *Drosera* species occurring in Portugal

are severely threatened. Dewatering, drainage in order to create productive land, trampling and cattle grazing are indicated as main threats to these habitats.

1.3.3.3. *Drosera rotundifolia*

Drosera rotundifolia (L.) is one of the most broadly distributed *Drosera* species, occurring throughout much of the Holarctic (Wolf et al., 2006). *D. rotundifolia* is an herbaceous perennial plant consisting of a prostrate rosette of long, flat, narrow, petioled, pubescent leaves which are attached spirally to the base (Figure 1.1.3 G,H). The species' root structure is fibrous, fine, and blackish with two or three slightly divided branches (Crowder et al., 1990). The inflorescence of *D. rotundifolia* is a single, one-sided raceme that terminates in a naked, scape 5 to 12 cm in height. The flowers are white, 10 to 12 mm in diameter, radially symmetrical and 15 to 25 flowers occur on each flowering scape (Figure 1.1.3 I). The flowers are hermaphroditic and blossom from June to August (Wolf et al., 2006). *Drosera rotundifolia* can reproduce both sexually and asexually. Asexual reproduction occurs when leaf buds form plantlets or when axillary buds found below the rosette can form a secondary rosette, with two genetically identical individuals resulting from the decay of the joining stem. Sexual reproduction is achieved almost exclusively through self-pollination of the hermaphroditic flowers (Crowder et al., 1990). Cross-pollination and genetic recombination are rare, so nearly all reproduction results in offspring that are either genetically identical to the parent (via vegetative reproduction) or that contain an equal, or slightly reduced, genetic variability compared to the parent generation (via sexual self-pollination) (Wolf et al., 2006).

Throughout the world *D. rotundifolia* occurs on peat, particularly on living *Sphagnum* moss, but it can occur on floating logs or damp acidic sand near ponds or streams (Crowder et al., 1990). Herbalists prescribe *D. rotundifolia* as a diuretic, a laxative, and a treatment for a variety of kidney, stomach, and liver problems (Wolf et al., 2006). Biochemical studies have shown that *D. rotundifolia* produces naphthoquinones (plumbagin and 7-methyljuglone) and flavonoids (Buzianowski, 1997; Paper et al., 2005). The beneficial effects of Drosera Herba, a commercial formulation prepared from *D. rotundifolia* plants to treat convulsive or whooping cough, have been attributed to its content in naphthoquinones, but this issue is controversial since studies have shown that the drug contains only trace amounts of naphthoquinones (Krenn et al.,

1998) and that the *in vitro* anti-inflammatory and spasmolytic effects of an ethanol extract of *Drosera madagascariensis* was correlated to the flavonoid content (Melzig et al., 2001).

1.3.3.4. *Drosera intermedia*

Drosera intermedia (H.) Hayne, or spoonleaf sundew, is a perennial herb which forms a semi-erect stemless rosette of spatulate leaves (Figure 2.1.3 K,L). It is slightly larger than *D. rotundifolia* and owes its name because it is intermediate in size between *D. rotundifolia* and *D. anglica* (Crowder et al., 1990). *D. intermedia* has a much more restricted distribution than *D. rotundifolia*. It is widespread in eastern north America and has a scattered distribution in the north and north-west America and western Europe (Wolf et al., 2006). Just as *D. rotundifolia*, *D. intermedia* is an obligate wetland species that requires continuously moist or saturated soils and is found in sites with shallow water depths (Crowder et al., 1990). *D. intermedia* is a lowland plant and is found over a narrow range of macroclimates, it is primarily a plant of sites that are flooded in winter, and subjected to drying out in summer, but it is also found in persistent pools. An adult plant can withstand periods of very low water levels but can also survive prolonged submergence. *D. intermedia* forms adventitious roots with few short hairs, similar to those of *D. rotundifolia*. Plants in temperate regions hibernate as small winter buds, from which new leafs expand and develop in the growing season. A mature ramet consists of a rosette of 10-30 leaves and blooms from June to August forming one to three flower stalks bearing 3-8 flowers each (de Ridder and Dhondt, 1992a). *D. intermedia* produces self compatible flowers and is also able to reproduce vegetatively by production of side rosettes during the growing season, regeneration of plantlets on senescing tissue and formation of axillary buds in autumn (de Ridder and Dhondt, 1992b). *D. intermedia* is known to produce plumbagin (Budzianowski, 1996) but the biochemical description of this species in literature is poor (Section 3.1.2). *D. intermedia* has been used as an infusion or a tincture for asthma, pulmonary catarrh and whooping cough (Crowder et al., 1990).

1.4. Objectives

Carnivorous plants are species with very particular habits which are becoming increasingly scarce and whose potentialities are largely unexplored. The objectives of this study are to increase the knowledge on these plants hoping simultaneously to contribute to their conservation. This way, in this study it is proposed to:

i) develop micropropagation protocols for *P. vulgaris*, *P. lusitanica*, *D. rotundifolia* and *D. intermedia*;

ii) identify the major secondary metabolites produced by these species by analysing extracts prepared from them;

iii) evaluate the biological properties of the prepared extracts;

iv) develop a method for the extraction of the secondary metabolite plumbagin from micropropagated *D. intermedia*.

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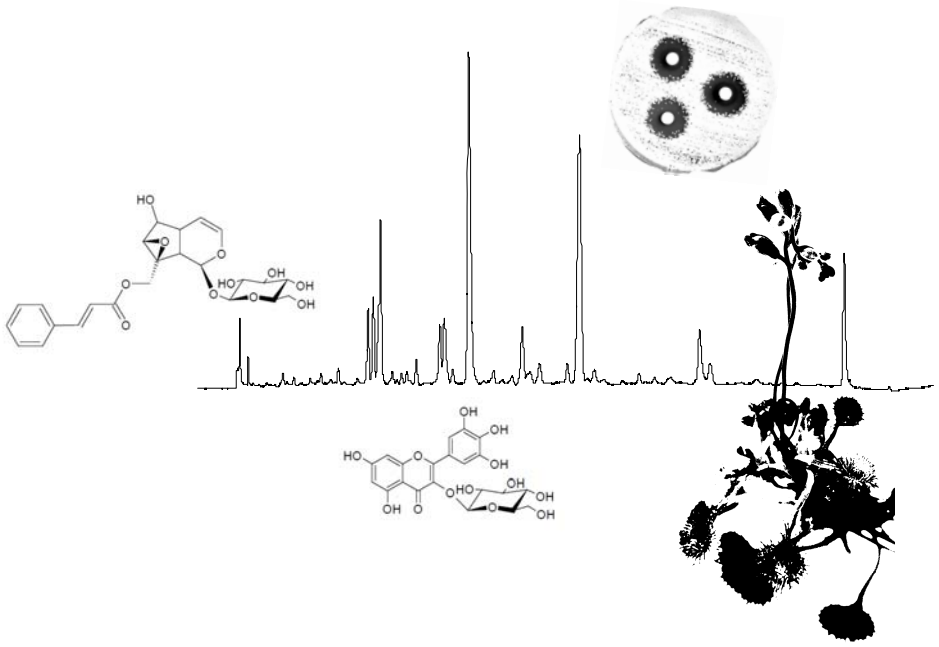
CHAPTER 2

MICROPROPAGATION OF *P. VULGARIS*, *P. LUSITANICA*, *D. ROTUNDIFOLIA* AND *D. INTERMEDIA*



2.1. INTRODUCTION

2.1.1. The importance of micropropagation for the conservation of carnivorous plants

Overharvesting of carnivorous plants together with a loss of their natural habitats has led to the protection of many species. Although species conservation is achieved most effectively through the management of wild populations and natural habitats (*in situ* conservation), *ex situ* techniques can be used to complement *in situ* methods and may in some instances be the only option (Sarasan et al., 2006). Micropropagation can contribute to the conservation of a species following a multifaceted approach, offering the possibility of plant regeneration for reintroduction in the wild, *in vitro* storage of germplasm, and biomass production for biological assays and chemical screening purposes, avoiding the collection of rare field specimens. As a result there has been a greater research into the micropropagation of carnivorous plants as a conservation strategy and the use of *in vitro* grown plants as alternative sources of biomass (Jang et al., 2003; Kim and Jang, 2004; Gonçalves and Romano, 2005).

In vitro culture allows for the provision of plant material for DNA analyses and autecological studies, and the development of successful storage methods enables the establishment of extensive basal collections, with representative genetic diversity (Sarasan et al., 2006). Material that has been propagated *in vitro* can be viewed as a parallel collection to a seed bank, and has been termed an *in vitro* active gene bank (Fay, 1992). In the interest of reducing labour and minimizing the risk of genetic drift and somaclonal variation, techniques have been developed for the long-term storage of this material. This storage can be of two types, reduced growth and cryopreservation. Slow growth techniques have been used successfully with cultures of rare plants, normally by reducing the incubation temperature (Fay, 1992).

Apart from the importance of micropropagation techniques to the conservation of threatened species, efficient propagation protocols allow for the continuous supply of large quantities of material with food value (Cassells et al. 1999; Choi et al., 2002). In addition, *in vitro* propagation techniques hold tremendous potential for the production of high-quality plant-based medicines, considering that these produce plants that are

genetically identical to the donor plants, allowing for the selection of high producing genotypes and clonal propagation for optimal product recovery (Bourgaud et al., 2001). For further details on *in vitro* culture techniques for secondary metabolite production see Section 5.1.1.

2.1.2. Advantages of micropropagation

Micropropagation can be considered in a broader sense a vegetative propagation technique. These techniques emerged from the necessity to produce plants genetically equal to the mother plant in order to retain its more favourable characteristics, avoiding the genetic recombination of gametes and therefore the phenotypic diversity of the plant. Vegetative propagation techniques are based on the totipotency theory, which states the capacity of a single cell to regenerate the genotype of the original organism and originate a new plant (Debergh and Zimmerman, 1991). However, not every plant species responds positively to the conventional methods due to the impossibility of controlling several environmental conditions, which led to the development of *in vitro* techniques. Micropropagation can be defined as the *in vitro* propagation of plants from cells or organs in a controlled environment and in a defined medium, through the employ of aseptic techniques and the use of proper containers (Debergh and Zimmerman, 1991). Micropropagation has many advantages over conventional methods of vegetative propagation and the most significant merits offered by micropropagation are: the possibility of producing large number of plants starting from a single explant in a relatively short time and space; propagation throughout the year; production of pathogen-free material, considering that tissue cultured plants are generally free from fungal and bacterial diseases, and virus eradication and maintenance of plants in a virus-free-state are also readily achieved in tissue culture (Debnath et al., 2006).

2.1.3. Development of micropropagation protocols

Micropropagation techniques can be grouped into three types, namely, organogenesis, somatic embriogenesis, and meristem culture. Somatic embryogenesis involves the formation of bipolar structures while in organogenesis formation of shoots or roots are induced. Organogenesis and somatic embryogenesis can occur indirectly or directly, depending on whether an intermediary *callus* induction stage is involved or not,

respectively. The induction of *callus* growth and subsequent differentiation, organogenesis and somatic embryogenesis are accomplished by the differential application of plant growth regulators (PGRs) and the control of conditions in the culture medium. With the stimulus of endogenous growth substances or by addition of exogenous growth regulators to the nutrient medium, cell division, and cell growth and tissue differentiation are induced (Debnath et al., 2006). However, when working with plants of conservation importance, it is generally considered wise to avoid methods likely to induce somaclonal variation, like *callus* cultures, and to try and maintain genetic integrity in tissue culture (Fay, 1992). Meristem cultures are widely used as these express best the culture regeneration capacity, considering that the meristems have non-differentiated cells which are more reactive.

An *in vitro* propagation protocol can be divided into five main stages: collection and preparative stage; culture initiation; culture maintenance and multiplication; elongation and root induction or development; and acclimatization (Debergh and Zimmerman, 1991). Micropropagation begins with the selection and preparation of plant material to be propagated. When the cultures are to be initiated from plant explants, it is important to raise the mother plant or its parts under more hygienic conditions, by the use of sterilization agents and growing it in greenhouse, to fight possible contaminations before initiation of culture. During this phase it is also important to manipulate certain parameters to make an explant more suitable or more reliable as starting material. Temperature, light, photoperiod and growth regulators are controlled to obtain explants more reactive to *in vitro* growth (Debergh and Zimmerman, 1991).

In the next stage the objectives are to establish cultures in aseptic conditions and to produce a considerable amount of explants with high proliferation capacity. Contamination, of both exogenous and endogenous origin, is a major obstacle for *in vitro* culture of plants. It is particularly important when dealing with threatened taxa for which the source material is often limited and usually located in the wild. Plant material is routinely initiated into axenic culture via a variety of sterilization procedures. These vary considerably from single-step to more complex protocols and utilize a huge variety of chemicals depending on the nature of tissue used. Contamination from internal sources can be potentially serious in culture as many plants harbour endophytic bacteria or fungi (Sarasan et al., 2006). The developmental stage, physiological age and size of

the initial explant are very important in this stage and can determine the success of the *in vitro* cultures (Debergh and Zimmerman, 1991).

As an alternative to the use of explants as starting material, micropropagation can be initiated from seeds. Seeds are the ideal starting material for *in vitro* propagation when species conservation is a concern because a wider genetic base can be maintained (Fay, 1992). In addition, more aggressive sterilization procedures can be used when disinfecting seeds, increasing the chances of initiating aseptic cultures. The activation of the metabolic machinery of the seed embryo leading to a new seedling plant is known as germination. For germination to be initiated the seeds must be viable and subjected to the appropriate environmental conditions, as seeds are often under a dormancy state which prevents immediate germination and regulates the time, conditions and place in which germination will occur in order to enhance seedling survival in natural conditions (Hartmann et al., 1990). The use of specific stratification treatments, like temperature or chemical treatment, can be used to break the dormancy state and to consequently induce germination.

The induction and multiplication of shoots can be difficult for some species, particularly woody plants. Many plant species have very specific *in vitro* requirements for multiplication and, therefore, substantial variation in culture medium formulations exists. The medium should include a support material consisting of a semisolid or liquid medium; a mineral salt mixture with essential major and minor elements; an energy source, normally sucrose; and vitamin supplements (Hartmann et al., 1990). In general, mineral salt requirement varies from one plant group to the other. Similarly, specific growth hormones or supplements greatly enhance regeneration and growth in many cases. The most often used growth regulators in this phase are cytokinins, as they promote the formation and development of axillary shoots. Metabolism of phenolic compounds might be stimulated at this time in the explants due to stress of being isolated from the mother plant and to adaptation to the new conditions. As a result these products provoke blackening of the explants and medium, leading to a growth inhibition of the explants and consequently their death (Debergh and Zimmerman, 1991). After several weeks in culture, depending upon the plant, the mass culture is divided and subcultured onto fresh medium and this process is repeated until a uniform, well-growing culture is produced (Hartmann et al., 1990).

Once cultures are successfully initiated, the following stage is characterized by the fragmentation and distribution of the plant material into new media to exponentially increase the number of shoots with high proliferation capacity. The culture medium is essentially the same as the one used in the initiation stage, but often the growth regulators supplement is increased (Hartmann et al., 1990). The concentration and ratio of cytokinin to auxin are especially important during this stage; their adjustment will promote the development of axillary shoots, reduce terminal shoot elongation and diminish rooting potential (Hartmann et al., 1990; Debergh and Zimmerman, 1991). Multiplication may be repeated several times to increase the supply of material to a predetermined level for subsequent rooting and transplanting (Hartmann et al., 1990).

The third stage consists in the elongation of the produced shoots and their rooting. Frequently elongation can be obtained by transfer of isolated shoots from the multiplication medium to an appropriate elongation medium, devoid of cytokinins (Debergh and Zimmerman, 1991). Rooting can be particularly problematic for *in vitro*-reared woody and recalcitrant taxa, requiring novel approaches and methods (Sarasan et al., 2006). Rooting can be attained *in vitro*, whether combined with elongation or not, or *ex vitro*. Usually rooting involves a medium in which the auxin level is increased and the cytokinin is decreased or suppressed (Hartmann et al., 1990). There is an increasing interest for rooting *ex vitro* due to its many advantages, essentially its simplicity and lower costs (Debergh and Zimmerman, 1991). This procedure consists in the direct rooting of the plantlet in the acclimatization substrate, after dipping the shoot's base into a rich auxin solution.

Plantlets growing *in vitro* are largely heterotrophic since they obtain their energy from sucrose present in the medium and therefore their photosynthetic activity is very low. These plantlets are also exposed to a very high relative humidity. All together, these aspects make the *in vitro* plantlets very sensitive to *ex vitro* transplantation. Therefore, in the final stage of micropropagation, the plantlets should be kept in very high humidity conditions and gradually exposed to a natural environment (Hartmann et al., 1990). Addition of a carbon source is a primary requirement in conventional micropropagation systems for most plants. However, for some species supplementation with a carbon source is unnecessary and these issues can be circumvented (Sarasan et al., 2006). In comparison to plantlets produced by conventional systems, those produced

by photoautotrophic systems with a sugar-free medium showed, in many cases, better growth, higher quality, lower contamination rate *in vitro*, and higher percentage survival *ex vitro* (Zobayed et al., 2004). Successful acclimatization of plants is fundamental to the micropropagation of threatened species for collection and conservation. Once rooted and acclimatized, plants can be used for re-establishment programs or for botanic garden living collections (Sarasan et al., 2006). Several species grown *in vitro* have been successfully reintroduced into the wild (Decruse et al., 2003; Martin, 2003; Garcia-Rubio and Malda-Barrera, 2010).

2.1.4. Objectives

This thesis deals with the chemical investigation and evaluation of biological activities of four carnivorous plant species occurring in Portugal. The development of efficient micropropagation protocols is imperative for this work because the natural populations of the species under study cannot withstand collection of field specimens in sufficient amounts to allow the evaluation of their biological and chemical properties. The specific objectives of this chapter are to:

i) Initiate *in vitro* cultures of *P. vulgaris*, *P. lusitanica*, *D. rotundifolia* and *D. intermedia* from seeds;

ii) Establish micropropagation protocols for these species to provide plant biomass for the subsequent studies;

iii) Contribute to the conservation of these species by maintaining an active germplasm collection *in vitro*.

2.2. EXPERIMENTAL

2.2.1. Seed collection, seed germination and establishment of cultures

The species under study in this thesis are carnivorous plants which grow naturally on Portuguese territory. Seeds of *P. lusitanica* (Figure 2.2.1 A) were collected from plants growing in a natural population close to Algoz (Algarve region) by Dr. Jorge Jesus and *P. vulgaris* seeds (Figure 2.2.1 B) were gently obtained from Dr. Henrique Pereira of the Institute for Nature Conservation and Biodiversity (ICNB). Seeds were collected by Mr. António Rebelo from a population near Cagarouço in Peneda Gerês National Park (Trás-os-Montes e Alto Douro region). *D. intermedia* seeds were collected from a population near the Sado estuary (Estremadura region) and *D. rotundifolia* seeds from a population found at a peat bog near the highest point of the Serra da Estrela mountain range (Beira Alta region). Information on the location of these two populations was kindly provided by Dr. Miguel Porto.

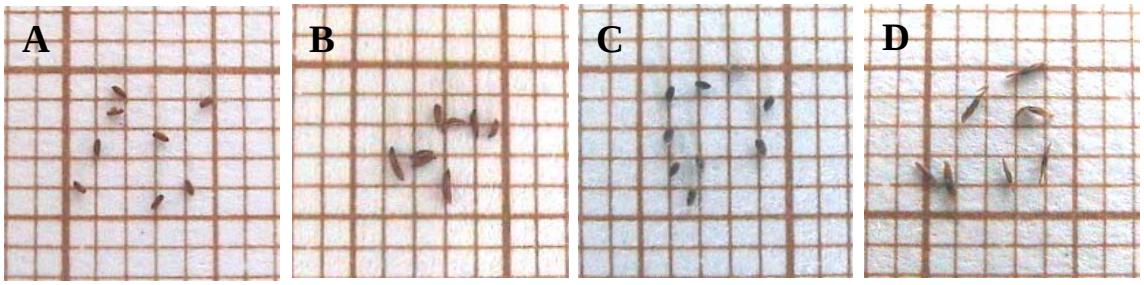


Figure 2.2.1 - Seeds of *P. lusitanica* (A), *P. vulgaris* (B), *D. intermedia* (C), and *D. rotundifolia* (D).

Specimens of *P. lusitanica* and *D. intermedia* were authenticated by Dr. Ana Isabel Correia of the Botanical Garden of the University of Lisbon and voucher specimens were deposited under the number LISU215272 and LISU231581, respectively. The obtained seeds were surface sterilized with commercial bleach at 15% (v/v) (5% of sodium hypochlorite) with a few drops of Tween-20 for 15 min, and washed 3 times in sterile water. A subset of *D. intermedia* seeds was subsequently stored at 5ºC during one week prior to inoculation to determine the effect of cold stratification on the germination rate. For each treatment 3 replicates of 10 seeds were tested. Seeds were aseptically transferred into test tubes containing 10 ml ¼MS medium (Murashige and Skoog, 1962) without growth regulators. Sucrose (2%, w/v) was used as carbon source

and media were solidified with 1% (w/v) agar. Media pH was adjusted to 5.75 before autoclaving at 121 ºC and 1.1 Kg/cm for 20 min. All cultures were incubated under a 16 h photoperiod provided by cool-white fluorescent lights at a photon flux density of 60 μmol m^{-2} s^{-1} at a temperature of 25 ± 2 ºC.

2.2.2. Proliferation and rooting

Two months after germination roots were discarded and the entire shoot was sub-cultured onto fresh ¼MS medium (*P. lusitanica* and *P. vulgaris*) or full strength MS medium (*D. intermedia* and *D. rotundifolia*) without PGRs in order to obtain sufficient number of shoots for the subsequent assays. After 8 weeks, shoots with identical size were separated and used in the different assays. In the case of *P. lusitanica*, the effect of three concentrations of MS macronutrients (¼MS, ½MS and total MS), without PGR or supplemented with 0.2 or 0.5 mg/L of cytokinin (BA - 6-benzyladenine, Kin - kinetin and Zea - zeatin), on shoot proliferation was evaluated. Afterwards, the effect of combining Kin (0.5 mg/L) and indole-3-butyric acid (IBA) (0.25 or 0.5 mg/L) in ½MS or ¼MS medium was also evaluated. To induce rooting, shoots were isolated individually and transferred to MS, ½MS or ¼MS basal medium containing 0.2 or 0.5 mg/L indole-3-acetic acid (IAA), IBA or 2-naphthaleneacetic acid (NAA). In the proliferation and rooting assays, 10 shoots were inoculated per assay and repeated 5 times. Since after 8 weeks of culture simultaneous proliferation and rooting was observed in almost all tested media both proliferation and rooting results were recorded. The proliferation rate was assessed as the total number of shoots produced per culture and rooting expressed in terms of rooting frequency, root number and the longest root length per plantlet.

The same procedure was employed for the remaining species with variations in the tested culture media. The effect of two concentrations of MS macronutrients (¼MS and total MS) and addition of cytokinin (BA and Zea at 0.1 mg/L) supplemented to ¼MS medium on the proliferation and rooting of *P. vulgaris* was evaluated. In addition, the effect of combining the auxin IBA (0.01 mg/L) and the cytokinins BA and Zea (0.1 mg/L) in ¼MS basal medium was also evaluated. *D. intermedia* shoots were cultured in three concentrations of MS macronutrients (total MS, ½MS and ¼MS) without cytokinins or supplemented with 0.1 mg/L of Kin and *D. rotundifolia* shoots in PGR-

free MS and ¼MS medium. For *P. vulgaris*, *D. intermedia* and *D. rotundifolia* cultures, 4 repetitions with 10 explants were performed for each culture medium. Additional parameters were monitored for the three last cited species: the proliferation frequency, which corresponds to the percentage of explants that were able to regenerate new shoots; and the rooting percentage of the new produced shoots. No root formation was observed in the *D. rotundifolia* cultures and therefore the rooting response was not recorded.

2.2.3. Plantlet acclimatization

Plantlets with well developed roots were selected, removed from the culture flasks and the roots cleared of agar to prevent pathogenic contamination. The plantlets were placed in plastic pots containing a mixture of peat and vermiculite (3:1, v/v) and acclimatised inside transparent polyethylene boxes, and maintained in a growth room for 3 weeks under controlled conditions (16 h photoperiod, 60 μmol m^{-2} s^{-1} and 25 ± 2ºC), and then gradually exposed to reduced relative humidity by progressively opening the plastic covers over a period of 3 weeks.

2.2.4. Statistical analysis

The data were subjected to analysis of variance (ANOVA) to assess treatment differences and interactions using the SPSS statistical package for Windows (release 15.0, SPSS INC.). Significance between means was tested by Duncan's New Multiple Range Test (P = 0.05). To analyze the data on rooting percentages, arcsin square root transformation was used.

2.3. RESULTS AND DISCUSSION

2.3.1. *P. lusitanica*

Seeds are the preferred starting material for establishing cultures of rare species as this ensures that a wide genetic base is maintained (Benson et al., 2000). Moreover, the use of seeds for the establishment of primary cultures can prevent most of the contamination problems that are often associated with explant establishment. Seeds of *P. lusitanica* were successfully sterilized and 40% germinated *in vitro* producing normal seedlings. Explants obtained from seedlings were cultured in several media with different basal media, and PGR type and concentrations, in order to determine the best conditions to propagate *P. lusitanica in vitro*. Despite the low germination rate, the seedlings had a great proliferation capacity even in PGR-free medium producing enough number of explants for the subsequent experiments (Figure 2.3.2 A, B).

Interestingly, simultaneous proliferation and rooting was observed for the *P. lusitanica* shoots in media without PGR, media supplemented with cytokinins, auxins, or both. Thus, all the media tested were evaluated simultaneously in terms of proliferation and rooting response. The results concerning the proliferation rate and rooting frequency are shown in Figure 2.3.1 and the remaining parameters regarding rooting response, namely root number and root length, are shown in Table 2.3.1. Since the *P. lusitanica* shoots grow like a small horizontal rosette, it was impossible to evaluate shoot length, and therefore shoot proliferation was only evaluated by the proliferation rate, expressed as the mean number of shoots produced by each explant at the end of the subculture period. Figure 2.3.1 shows that the *P. lusitanica* cultures had a great proliferation capacity even in medium without PGR or supplemented with auxins only. The mean number of shoots was significantly affected by the MS macronutrients concentration ($P < 0.001$), the type ($P < 0.001$) and concentration ($P < 0.001$) of PGR and by the interaction between these factors ($P < 0.001$). Regardless of the type of PGR or its concentration, ½MS medium was more effective in inducing proliferation ($P < 0.001$). These results are in agreement with those obtained for other carnivorous plants in which shoot proliferation was promoted in media with low concentration of macronutrients (Jang et al. 2003; Kim and Jang 2004; Gonçalves et al. 2005) and seems to be a characteristic to this group.

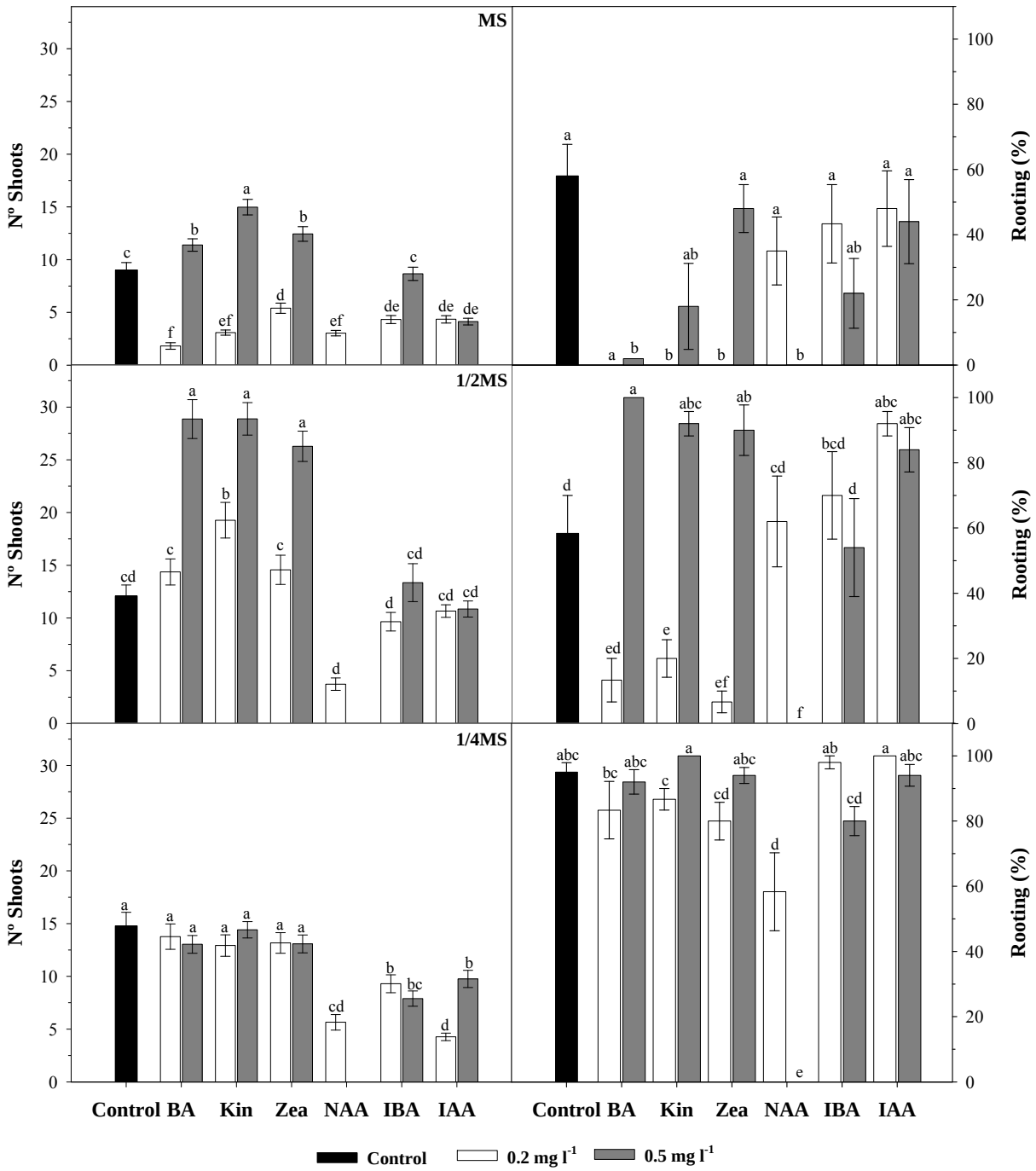


Figure 2.3.1 - Effect of MS medium concentration, cytokinin and auxin type and concentration on proliferation rate and rooting frequency of *P. lusitanica* shoots. Control: media without growth regulators. Values represent means ± SE of 5 replications with 10 shoots. In each graph columns with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

This appears to be related with the requisites for growth in their natural habitats, considering that carnivorous plants are adapted to subsist in nutrient poor soils. The highest proliferation rates were observed in ½MS medium supplemented with the cytokinins BA, Kin or Zea at 0.5 mg/L, for which values of 28.88 ± 1.54, 28.86 ± 1.84

and 26.28 ± 1.44 were scored, respectively (Figure 2.3.1, 2.3.2 C). However, high proliferation rates were also obtained in the absence of PGR and in auxin containing media, particularly IBA. The hormonal content of explants is a very important factor directing *in vitro* responses (Centeno et al., 1996; Baroja-Fernández et al., 2002). Therefore, the obtained results could be due to high levels of endogenous cytokinins in *P. lusitanica* shoots, as previously reported in other species (Centeno et al., 1996; Malá et al., 2005). It is unclear however, why shoots grown in full strength MS medium supplemented with cytokinins at 0.2 mg/L showed considerably lower proliferation rates than shoots supplemented with cytokinins at 0.5 mg/L, but also than shoots grown in PGR-free medium. For instance, shoots grown in control medium showed a proliferation rate of 9.03 ± 0.70, while the proliferation rates of shoots grown in media supplemented with 0.2 and 0.5 mg/L of Kin corresponded to 3.09 ± 0.25 and 14.48 ± 0.74, respectively. It is also interesting to see that, as opposed to the shoots grown in full strength MS and ½MS media, the proliferation rate of the shoots grown in ¼MS medium showed no response to supplementation with cytokinins.

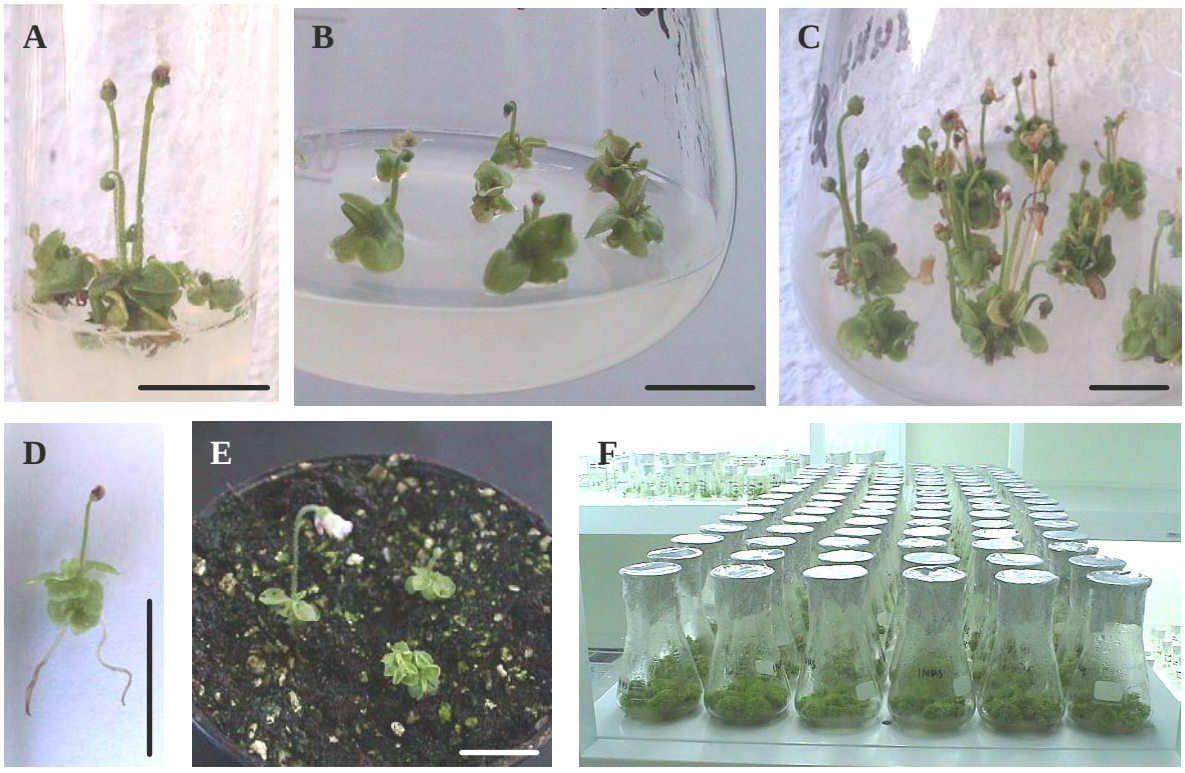


Figure 2.3.2 - Micropropagation of *P. lusitanica*: seedling explants used in the assays (A); shoots at the beginning of proliferation phase (B); shoots during proliferation phase (C); rooted shoot after 8 weeks of culture (D); plants during acclimatization after 2 months in *ex vitro* conditions (E); large scale biomass production for biological assays and chemical analysis (F). Bars = 1 cm.

The rooting of *P. lusitanica* shoots was significantly influenced by MS medium strength ($P < 0.001$) and the highest rooting response was observed in ¼MS medium (Figure 2.3.2 D). Rooting response was also significantly affected by PGR type ($P < 0.001$) and concentration ($P < 0.05$). There is substantial evidence that auxins play a crucial role on adventitious root formation (de Klerk et al., 1999), although responses can vary depending on the auxin concentration, form of auxin application and the sensitivity of tissues to absorb or utilise the exogenous auxin (Kooi et al., 1999; Chhun et al., 2004). Nevertheless, in this study higher rooting frequencies (58-95%) were attained in PGR-free media, probably due to available endogenous auxins. Unexpectedly, high rooting frequencies were also achieved in cytokinin containing medium, especially at the higher tested concentration (0.5 mg/L) (Figure 2.3.1). For instance, rooting percentages of 100.00 ± 0.00, 92.00 ± 3.74 and 90.00 ± 7.75 were obtained for shoots growing in ½MS medium supplemented with 0.5 mg/L of BA, Kin and Zea, respectively. Again, it is unclear why shoots growing in the same basal medium supplemented with the lower concentration of cytokinin (0.2 mg/L), showed considerably lower rooting percentages than shoots growing in medium supplemented with cytokinins at 0.5 mg/L or in control medium. It seems that in some cases, at lower concentrations, the cytokinins compromise the induction of shoot multiplication and rooting of *P. lusitanica*.

The results presented in Figure 2.3.1 show that overall, the rooting response of *P. lusitanica* shoots is higher in the presence of cytokinins. This result is very unusual and needs to be further studied and explained. Auxins are intimately involved in the process of adventitious root formation and the interdependent physiological stages of the rooting process are associated with changes in endogenous auxin concentrations (Gaspar et al., 1997). However, studies by Soh et al. (1998) and de Klerk et al. (2001) demonstrated that cytokinins, particularly at low concentrations, may be essential at early stages of the root induction process. Noteworthy, better rooting was observed in cytokinin supplemented medium than in NAA containing medium. In fact, the use of 0.5 mg/L NAA totally suppressed rooting response (Figure 2.3.1). Similarly, NAA strongly suppressed root formation in shoots of the carnivorous plant *Dionaea muscipula* (Jang et al., 2003). The auxin seems to have a general negative affect on the viability of the shoots of *P. lusitanica*, considering that the highest concentration NAA also suppressed shoot multiplication completely (Figure 2.3.1).

Table 2.3.1. Effect of basal medium and PGR type and concentration on mean root number and length (mm) of *P. lusitanica* micropropagated shoots.

Basal medium	PGR	Concentration (mg/L)	Mean number of roots	Longest root length (mm)
MS	Control	-	2.76 ± 0.30 a	3.90 ± 0.34 a
	BA	0.2	-	-
		0.5	-	-
	Kin	0.2	-	-
		0.5	2.67 ± 0.65 a	4.11 ± 0.39 a
	Zea	0.2	-	-
		0.5	2.25 ± 0.37 a	4.63 ± 0.33 a
	NAA	0.2	1.93 ± 0.22 a	3.00 ± 0.31 a
		0.5	-	-
	IBA	0.2	2.54 ± 0.35 a	3.69 ± 0.59 a
		0.5	3.09 ± 0.56 a	4.45 ± 0.58 a
	IAA	0.2	2.42 ± 0.26 a	3.79 ± 0.31 a
		0.5	2.59 ± 0.25 a	3.64 ± 0.27 a
½MS	Control	-	2.49 ± 0.22 c	3.71 ± 0.25 cd
	BA	0.2	1.25 ± 0.25 c	7.25 ± 1.80 b
		0.5	14.08 ± 1.39 a	4.40 ± 0.12 c
	Kin	0.2	1.50 ± 0.22 c	6.17 ± 0.70 b
		0.5	9.41 ± 1.01 b	4.20 ± 0.14 c
	Zea	0.2	1.50 ± 0.13 c	7.50 ± 0.39 a
		0.5	5.16 ± 0.57 bc	4.27 ± 0.18 c
	NAA	0.2	2.81 ± 0.16 c	2.71 ± 0.26 d
		0.5	-	-
	IBA	0.2	2.83 ± 0.17 c	4.20 ± 0.24 c
		0.5	4.59 ± 0.81 c	3.37 ± 0.30 cd
	IAA	0.2	3.80 ± 0.49 c	3.65 ± 0.22 cd
		0.5	4.24 ± 0.49 c	4.24 ± 0.23 c
¼MS	Control	-	3.26 ± 0.25 de	3.97 ± 0.20 cde
	BA	0.2	5.16 ± 0.74 cd	7.24 ± 0.58 a
		0.5	5.17 ± 0.50 cd	3.22 ± 0.12 ef
	Kin	0.2	4.35 ± 0.48 de	6.19 ± 0.50 b
		0.5	9.40 ± 0.78 a	3.76 ± 0.12 def
	Zea	0.2	3.52 ± 0.69 de	5.83 ± 0.61 b
		0.5	5.11 ± 0.49 cd	4.30 ± 0.20 cd
	NAA	0.2	3.14 ± 0.22 e	3.06 ± 0.31 f
		0.5	-	-
	IBA	0.2	3.55 ± 0.30 de	4.84 ± 0.20 c
		0.5	3.45 ± 0.42 de	3.45 ± 0.42 def
	IAA	0.2	6.52 ± 0.60 bc	4.64 ± 0.17 c
		0.5	7.77 ± 0.83 ab	4.70 ± 0.18 c
Basal medium (A)			***	ns
PGR type (B)			***	***
PGR concentration (C)			***	***
A × B × C			***	ns

Values represent means ± SE of 5 replications with 10 shoots. *, **, ***: significant at $P < 0.05$, at $P < 0.01$ and at $P < 0.001$, respectively (Three-way ANOVA). For each variable, values followed by the same letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

The mean number of roots was strongly affected by the basal medium ($P < 0.001$), the type and concentration of PGR ($P < 0.001$) and the interactions between the three factors ($P < 0.001$) (Table 2.3.1). Root length was only affected by the type and concentration of PGR ($P < 0.001$) (Table 2.3.1). The highest mean number of roots was obtained in ½MS medium containing 0.5 mg/L BA (14.08 ± 1.39). However, at this cytokinin concentration root elongation was suppressed in comparison to the lower concentration (0.2 mg/L) (Table 3.2.1). The longest roots were observed in ½MS containing 0.2 mg/L Zea (7.50 ± 0.39 mm) and ¼MS containing the same concentration of BA (7.24 ± 0.58 mm).

To date, the only micropropagation protocol available for a plant belonging to the *Pinguicula* genus is the one developed by Adams et al. (1979) for *P. moranensis* H.B.K. The author used leaf explants as starting material and found that the highest plantlet number and growth rate were obtained using $^1/_5$Linsmaier-Skoog medium supplemented with a combination of BA at 0.02 mg/L and NAA at 0.01-0.10 mg/L. Although several authors have reported a synergistic effect of cytokinins and auxins during the proliferation of various species (Beena et al., 2003; Faisal et al., 2007), in this study this positive effect was not observed when Kin and IBA were used. In fact, this combination was less effective than the application of each PGR separately (Table 2.3.2).

Table 2.3.2. Effect of Kin (0.5 mg/L) in combination with IBA (0.25 or 0.5 mg/L) on the mean number of shoots, rooting frequency and mean root number and length (mm) of *P. lusitanica* shoots.

Basal medium	IBA (mg/L)	Mean number of shoots	Rooting (%)	Mean number of roots	Longest root length (mm)
½MS	0.25	4.86 ± 0.40 b	100.00 ± 0.00 a	2.42 ± 0.36 b	3.29 ± 0.38 ab
	0.5	4.16 ± 0.36 b	70.00 ± 0.00 c	2.71 ± 0.40 b	4.07 ± 0.34 a
¼MS	0.25	7.37 ± 0.53 a	46.67 ± 6.67 d	7.31 ± 0.81 a	3.29 ± 0.16 ab
	0.5	5.38 ± 0.54 b	96.67 ± 3.33 b	7.79 ± 1.11 a	2.79 ± 0.24 b

Values represent means ± SE of 5 replications with 10 shoots. For each variable, values followed by the same letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

The produced plantlets did not show any apparent morphological variation and great number of them flowered spontaneously *in vitro* (Figure 2.3.2 C). Sixty per cent of regenerated plantlets were successfully acclimatized to *ex vitro* conditions and flowers produced capsules and seeds normally (Figure 2.3.2 E).

Overall the best conditions for the micropropagation of *P. lusitanica* were obtained with ½MS cytokinin supplemented medium at 0.5 mg/L as these media afforded the highest proliferation rates (> 26 explants), rooting percentages (> 90 %) and root formation (> 5 roots per shoot). However, the higher cytokinin concentration compromised root elongation. Using ½MS medium supplemented with BA at 0.5 mg/L an average of 28.9 rooted plantlets can be obtained from one single shoot in 8 weeks of culture. The protocol established in this study can be used to produce plant material to evaluate the biological properties of the plants' secondary metabolites and for chemical screening purposes, avoiding the collection of field specimens and reducing the pressure of wild stock (Figure 2.3.2 F). In addition, the protocol can be used to generate plantlets with the objective of replenishing the declining populations in the wild.

2.3.2. *P. vulgaris*

Seeds of *P. vulgaris* were all free from contaminations after the applied sterilization procedure and 24.24% of the inoculated seeds germinated (Figure 2.3.4 A, B). The low germination rates might be explained by the low viability of the seeds or by the need of a specific treatment to break dormancy (Heslop-Harrison, 2004). Due to the reduced number of available seeds it was not possible to test the effect of stratification treatments on germination. The development of a micropropagation protocol for *P. vulgaris* proved to be a more difficult task in comparison to *P. lusitanica*. During the proliferation phase the shoots were very susceptible to the individualization step due to their delicate nature. In most cases a large number of explants dried out or lost viability during the course of the 8 weeks culture period. Due to the limiting amount of available plant material, the cultures were subjected to a reduced number of assays. Initially, the influence of two basal medium concentrations was tested: full strength and ¼MS medium. In addition to the number of produced shoots, the multiplication percentage of the explants was also monitored, which represents the ability of each explant to regenerate new shoots and is therefore also a measure of viability.

The results show that most of the parameters were negatively influenced by the higher concentration of macronutrients. More specifically, the multiplication percentage (Figure 2.3.3 A), the rooting percentage of the new formed shoots (Figure 2.3.3 D), the number of roots of the initial explant (Figure 2.3.3 E) and the maximal root length of

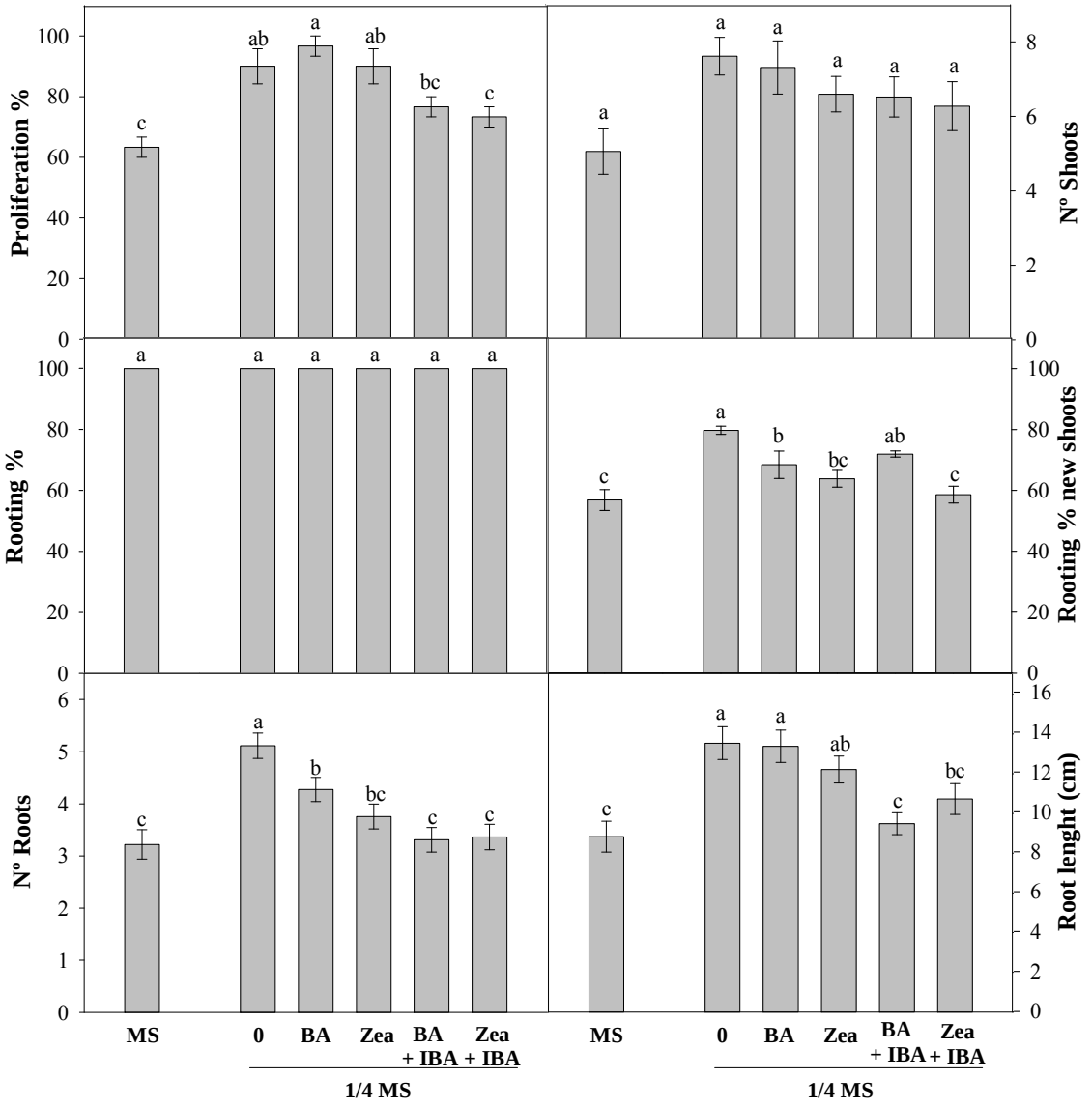


Figure 2.3.3 - Effect of MS medium concentration, cytokinin type (BA and Zea at 0.1 mg/L) and combination of cytokinin (0.1 mg/L) and auxin (IBA at 0.01 mg/L) on proliferation and rooting of *P. vulgaris* shoots: proliferation frequency (A), mean number of developed shoots per initial explant (B), rooting percentage of initial explant (C), rooting percentage of new produced shoots (D), mean number of developed roots per initial explant (E), longest root length of initial shoot (F). Control: media without growth regulators. Values represent means ± SE of 3 replications with 10 shoots. In each graph columns with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

shoots (Figure 2.3.3 F) cultured in full strength MS (63.33 ± 3.33, 56.87 ± 3.44, 3.22 ± 0.28, 8.83 ± 0.77, respectively) showed decreased values in comparison to shoots cultured in medium with lower macronutrients concentration (90.00 ± 5.77, 79.74 ± 1.35, 5.11 ± 0.24, 13.54 ± 0.82, respectively) ($P < 0.05$), which is in agreement with the results obtained for *P. lusitanica* and other carnivorous plants. Despite the difference

not being statistically significant, the number of produced shoots per initial explant (Figure 2.3.3 B) is also larger for *P. vulgaris* shoots cultured in ¼MS medium (5.06 ± 0.61 in full strength MS and 7.62 ± 0.51 in ¼MS medium).

Following the results obtained for *P. lusitanica*, the *P. vulgaris* shoots were cultured in media supplemented with two cytokinins, BA and Zea at 0.1 mg/L. The results presented in Figure 2.3.3 show that no significant differences were observed between treatments regarding the proliferation frequency, number of produced shoots and rooting percentage of the initial explant ($P \geq 0.05$). For these culture media, the proliferation frequencies were on average above 90.0%, the number of produced shoots over 6.6, and the rooting percentage of the initial explant was 100% in all cases. Noteworthy, all shoots grown in the other tested media rooted as well, independently of the basal media strength or supplementation with PGR (Figure 2.3.3 C).

However, some differences for the other rooting parameters could be observed between the ¼MS PGR-free medium and the cytokinin supplemented media. In general, the rooting of the *P. vulgaris* was favoured by the absence of cytokinins. The highest rooting percentage of new shoots (79.74% ± 1.35) ($P < 0.05$) and number of produced roots (5.11 ± 0.24) ($P < 0.05$) were obtained for the shoots grown in ¼MS PGR free medium, while the length of the longest root was not significantly affected by cytokinin supplementation ($P < 0.05$), scoring values on average above 12.2 mm.

It can be seen from Figure 2.3.3 that the proliferation of the *P. vulgaris* shoots was not greatly influenced by the tested cultured media. However, in the case of this species, the numerical values are not a correct representation of the results obtained from a morphological point of view. In general, the cultures grown in PGR free medium produced vigorous and healthy looking shoots, while the cultures grown in cytokinin supplemented medium displayed in some cases signs of necrose, underdevelopment and curdled leaves with a brittle texture, especially in the case of shoots grown in medium supplemented with Zea (Figure 2.3.4 C, D, E). This shows that in some cases growth parameters such as multiplication rate and rooting response alone do not represent the real efficiency of the experimental conditions.

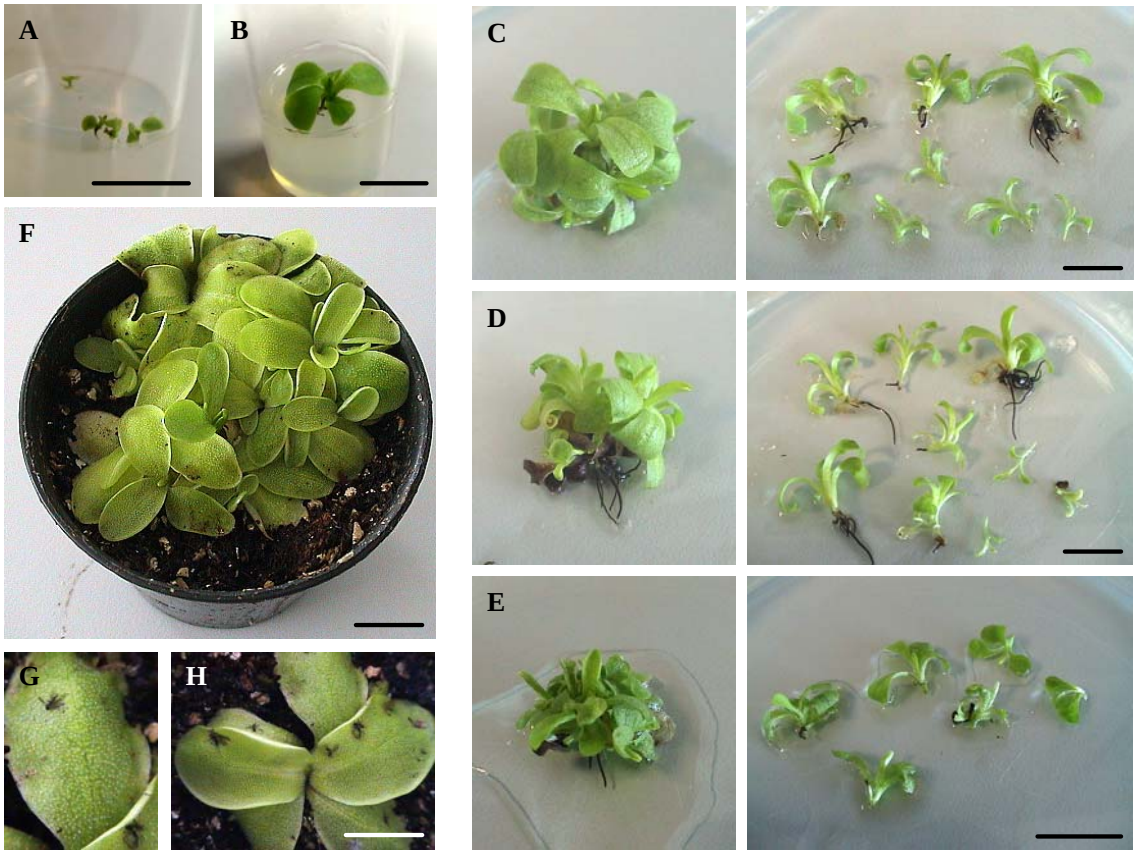


Figure 2.3.4 - Micropropagation of *P. vulgaris*: seedlings 2 weeks after germination (A); seedling explants used in the assays (B); whole culture and individualized shoots at the end of proliferation phase cultured in ¼MS without PGR (C), supplemented with BA at 0.1 mg/L (D) or with Zea at 0.1 mg/L (E); acclimatized plants with 2 months in *ex vitro* conditions (F); leaf detail of acclimatized plant with captured insects (G, H). Bars = 1 cm.

All the explants used in the assays were obtained from cultures grown in ¼MS PGR free medium, and it is therefore likely that if the cultures grown in PGR supplemented medium were to be sub-cultured, the results of the treatments would show great differences, considering that these explants appear to have little viability based on morphological aspects. This is an essential issue considering that it is not only important to produce a large amount of new shoots, but also that these must be able to regenerate new plantlets. As noted before, the results presented in Figure 2.3.2 show that the *P. vulgaris* shoots have a great predisposition for rooting. In fact, initial explants as well as the new produced shoots rooted spontaneously without the addition of auxins. Independently of the growth media, more than 50% of the new shoots were able to produce roots in all cultures. Only the youngest shoots (and therefore smaller shoots) did not produce roots and it seemed that the formation of roots was related with the size of the respective shoot and not with the specific growth medium. This suggests that the

development of a more efficient protocol should be directed to obtain a larger number of shoots without compromising shoot viability, taking into perspective that the rooting phase does not need optimization. Figure 2.3.3 also shows that, as was the case with *P. lusitanica*, a culture medium combining cytokinins (BA or Zea at 0.1 mg/L) with auxins (IBA at 0.01 mg/L) was not effective in inducing proliferation.

The development of micropropagation protocols for the two *Pinguicula* species revealed some interesting differences in response to the *in vitro* environment. As opposed to *P. lusitanica*, *P. vulgaris* did not flower spontaneously. In addition, *P. lusitanica* showed considerably higher proliferation rates, while the shoots produced by *P. vulgaris* where in turn larger in size. A few *P. vulgaris* plantlets with well developed roots were acclimatized successfully and the leaves of the micropropagated plants were functional and able to catch insects (Figure 2.3.4 F, G, H).

All in all, ¼MS PGR-free medium was the best medium for the micropropagation of *P. vulgaris*, as it provided the highest proliferation rate and rooting response. However, alternative strategies should be sought for to enhance the viability of the cultures and biomass production considering that the developed protocol does not ensure sufficient material for subsequent bioassays and chemical analysis. Altering abiotic conditions such as temperature, humidity and lighting might be required to enhance growth efficiency, since *P. vulgaris* inhabits a colder environment than *P. lusitanica*.

2.3.3. *D. intermedia*

In the case of *D. intermedia*, all seeds were successfully sterilized and germination occurred during the second week after inoculation (Figure 2.3.6 A). Higher germination rates were obtained for the control (84.22 ± 3.85 %) than for the cold stratification treatment (65.70 ± 6.71 %), although differences were not significant ($P \geq 0.05$). These results are interesting as many *Drosera* species need a cold treatment to break dormancy and germinate (Jang and Park, 1999; Jang et al., 2003; Jayaram and Prasad, 2006). Seedlings had a high proliferation capacity, producing enough explants for the subsequent experiments (Figure 2.3.6 B).

The effect of basal media strength and addition of Kin at 0.1 mg/L on the growth of *D. intermedia* shoots in *in vitro* conditions was investigated and the results are summarized in Figure 2.3.5. The results show that the cultures had a high proliferation capacity in PGR-free media and in media supplemented with Kin (Figure 2.3.6 C).

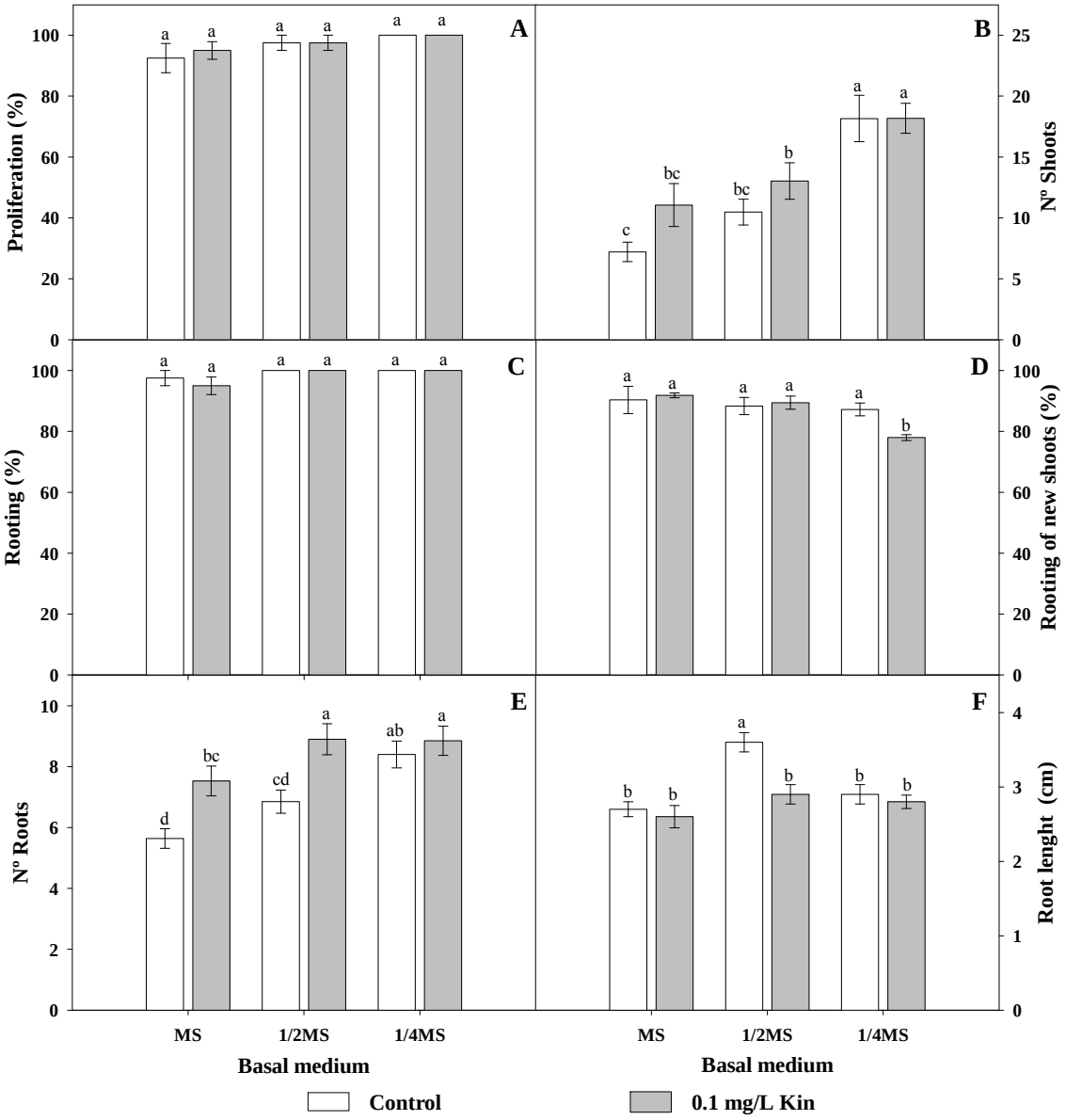


Figure 2.3.5 - Effect of MS medium concentration and addition of Kin (0.1 mg/L) on proliferation and rooting of *D. intermedia* shoots: proliferation frequency (A), mean number of developed shoots per initial explant (B), rooting percentage of initial explant (C), rooting percentage of new produced shoots (D), mean number of developed roots per initial explant (E), longest root length of initial shoot (F). Control: media without growth regulators. Values represent means ± SE of 4 replications with 10 shoots. In each graph columns with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

Concerning the percentage of initial explants with proliferation capacity, no significant differences were observed either among the MS macronutrient concentrations or between the control and Kin supplemented media ($P \geq 0.05$). In all cases multiplication percentages above 90 % were recorded (Figure 2.3.5 A). The highest number of shoots was obtained in PGR-free ¼MS medium (18.16 ± 1.91) and ¼MS medium supplemented with Kin at 0.1 mg/L (18.18 ± 1.23), and no significant differences were observed between the PGR-free and the Kin supplemented media (Figure 2.3.5 B) ($P \geq 0.05$). However, the number of produced shoots was significantly influenced by the basal media strength ($P < 0.05$) and ¼MS was the most effective in inducing proliferation. From these results, we can conclude that decreasing the MS macronutrient concentration significantly enhances the multiplication of *D. intermedia* shoots and that the use of Kin does not affect proliferation. The promotion of shoot proliferation by media with low concentration of macronutrients is coherent with the results obtained for *P. lusitanica* and *P. vulgaris*, and other carnivorous plants.

Rooting frequency of initial explants was not affected by MS macronutrient concentration or by the presence of Kin (Figure 2.3.5 C) ($P \geq 0.05$). High rooting percentages were obtained in PGR-free media and also in media with Kin, reaching 100 % in some cases. Taking into account that rooting percentages close to 100 % were obtained in all tested media, an additional rooting phase on medium with auxins was unnecessary. Noteworthy, the new developed shoots also rooted in very high percentages (Figure 2.3.5 D). Over 80 % of the produced shoots rooted in all tested media, except for shoots cultured in ¼MS medium supplemented with Kin which was statistically the less effective medium ($P < 0.05$). Because the tested media afforded very high proliferation rates and rooting percentages of 100 % in some cases, no further culture media were investigated. These results show that large amounts of *D. intermedia* plantlets can be produced in one single step. On average, one shoot cultured on PGR-free ¼MS medium produces 15.8 plantlets in 8 weeks.

Culture media supplemented with Kin did significantly enhance the number of roots produced ($P < 0.05$), with ¼MS (8.85 ± 0.48) and ½MS (8.90 ± 0.51) being the most effective basal media (Figure 2.3.5 E). The results presented in Figure 2.3.5 E also show

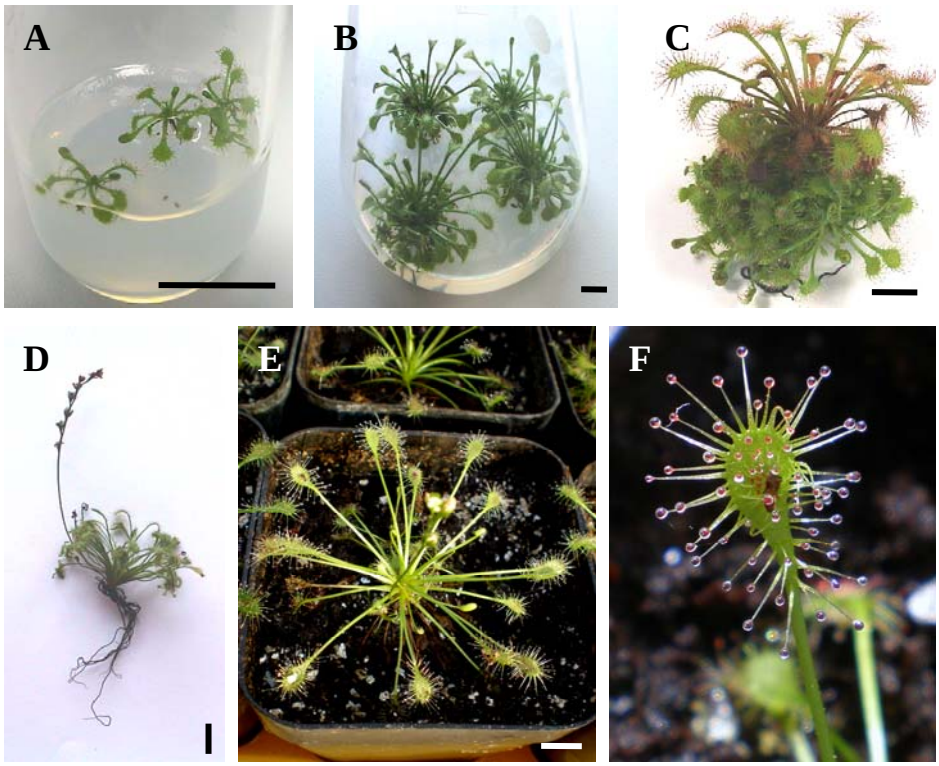


Figure 2.3.6 - Micropropagation of *D. intermedia*: seedlings 2 weeks after germination (A); seedling explants used in the assays (B); shoots at the end of proliferation phase (C); rooted shoot prior to acclimatization step (D); acclimatized plant with 2 months in *ex vitro* conditions (E); leaf detail of acclimatized plant and captured insects (F). Bars = 1 cm.

that increasing the basal media strength has a negative effect on the root formation in *D. intermedia* shoots. The longest roots were obtained in ½MS medium without PGR, with an average length of 3.60 ± 0.13 cm ($P < 0.05$), however, in general this parameter does not seem to be influenced by basal media concentration or addition of Kin (Figure 2.3.5 F). *In vitro* produced plantlets did not show any apparent morphological variation and 100 % of regenerated plantlets with well developed roots (Figure 2.3.6 D) were successfully acclimatized to *ex vitro* conditions. Their leaves were functional and able to catch preys, and their flowers produced capsules and seeds normally (Figure 2.3.6 E, F). These results show that it is possible to produce *D. intermedia* plantlets in a large scale using a simple and efficient micropropagation protocol. Shoots, obtained from seedlings, showed high proliferation and rooting capacity and overall, the medium found to be best was PGR-free ¼MS medium.

2.3.4. *D. rotundifolia*

As opposed to the other species studied in this work, several micropropagation protocols have been developed for *D. rotundifolia* (Anthony, 1992; Jang and Park, 1999; Bobák et al., 2005). Most authors micropropated *D. rotundifolia* by direct shoot organogenesis from isolated leaves and showed that shoot regeneration readily occurs on PGR-free medium. However, these protocols reveal as a major problem that the newly formed shoots were either very small in size, or showed inhibited development on media with high regeneration rates. Therefore it was interesting to see how the micropropagation techniques using meristem cultures employed in this work would compare to the previously developed protocols.

The results obtained by Anthony (1992) in the initiation step of the culture establishment point out the advantages of using seeds as starting material. While no contamination was detected in the seedling cultures (Figure 2.3.8 A,B), about 95 % of the original *D. rotundifolia* cultures were lost due to contamination in the work described by the authors. Due to the prostrate growth habit of *D. rotundifolia* it is likely that leaves in close proximity to the soil are infested with soil-borne organisms, since lower contamination frequencies were obtained for *Drosera capensis* and *Drosera binata* with a more upright growth habit (Anthony, 1992).

As an initial approach *D. rotundifolia* seedlings were cultured in full strength MS medium and ¼MS medium (Figure 2.3.7). The obtained results were however disappointing. Despite the fact that a high percentage of shoots cultured in ¼MS responded (90.00 % ± 4.08; Figure 2.3.7 A) and produced a considerable amount of new shoots (10.68 ± 1.14; Figure 2.3.7 B), these were very small in size and in some cases it was not feasible to count or subculture them (Figure 2.3.8 C). In addition, no root formation was observed whatsoever, which is in great contrast to the response of *D. intermedia* cultures to *in vitro* conditions. Noteworthy, the same culture time in PGR-free ½MS medium was sufficient to produce shoots with extensive root systems by direct organogenesis from *D. rotundifolia* leaves (Anthony, 1992). In turn, Bobák et al. (1995) obtained an average number of 18.3 buds per explant in liquid full strength MS medium, which 38 days after culture initiation formed shoots with three to seven leaflets and rooted spontaneously on the mother leaf explant. Because the previously

developed protocols provided considerably higher yields than the presented results, no further assays were tested to micropropagate *D. rotundifolia*. An optimized protocol for the micropropagation of *D. rotundifolia* should be directed at the improvement of shoot elongation and rooting response, while maintaining the high proliferation rates. Considering that BA and NAA do not seem to enhance shoot elongation (Anthony, 1992; Bobák et al., 2005), it could be worthwhile to supplement the culture medium with gibberellic acid since it has shown to promote shoot elongation in several cases (Sugla et al., 2007; Pandeya et al., 2010; Purkayastha et al., 2010).

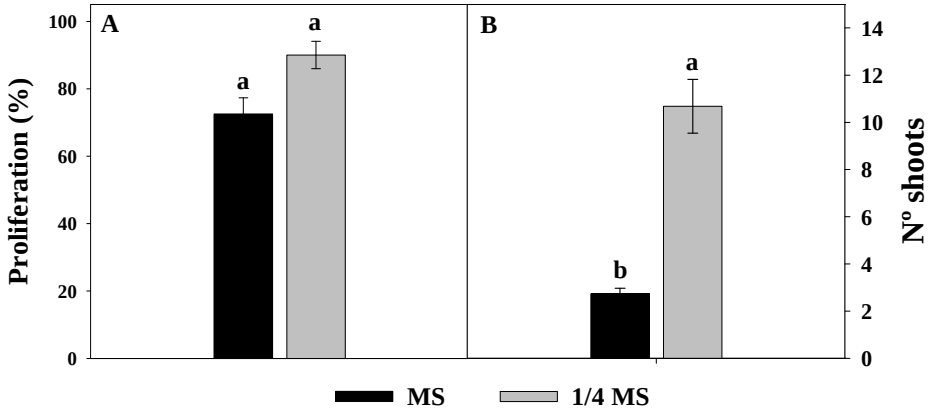


Figure 2.3.7 - Effect of MS medium concentration on proliferation of *D. rotundifolia* shoots: proliferation frequency (A) and mean number of developed shoots per initial explant (B). Values represent means ± SE of 4 replications with 10 shoots. In each graph bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

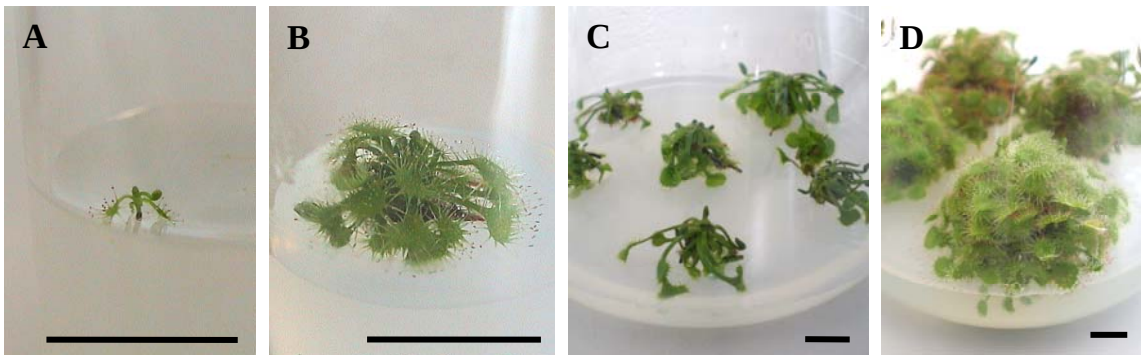


Figure 2.3.8 - Micropropagation of *D. rotundifolia*: seedlings 2 weeks after germination (A); seedling explants used in the assays (B); shoots at the end of proliferation phase (C); shoots obtained after longer periods between subcultures without individualization (D). Bars = 1 cm.

Noteworthy, it was observed that when the subculture periods were delayed and the shoots where left to grow, they produced vigorous cultures which assumed the morphology of field-grown specimens and were comprised by large individual plantlets (Figure 2.3.8 D). The fact that *D. rotundifolia* shoots do not proliferate efficiently when individualized might be related with their growth habit in natural conditions, taking into account that they grow in association with other plants of the same species or *sphagnum* moss which may act as a substratum giving physical support.

Taking this observation into consideration, an alternative method could be devised for evaluating the proliferation of *D. rotundifolia*, using plant biomass as a growth indicator instead of monitoring shoot formation, as the process of plant individualization prior to inoculation seems to compromise regular growth. This method would not be advantageous for evaluating plantlet formation but can be of value when the interest is to compare growth conditions for *in vitro* biomass production.

2.4. CONCLUSIONS

Micropropagation protocols were established for the first time for *P. lusitanica*, *P. vulgaris* and *D. intermedia*. *In vitro* cultures of *D. rotundifolia* were established as well but the growth rates were inferior to the previously developed protocols. Although the micropropogation protocol for *P. vulgaris* generates considerable amounts of plantlets in a short time and can be used to propagate the species efficiently, it is still susceptible to improvement as shoot viability can be an issue.

All species shared the trait of growing more vigorously in media with lower macronutrients concentration, which seems to be characteristic for carnivorous plants. In addition, these species showed to grow well in the absence of PGR, as high proliferation rates were obtained in PGR-free media in most cases. The growth parameters of *P. vulgaris* were either unaffected or negatively influenced by the addition of cytokinins to the culture media and ¼MS PGR-free medium was found to be the best to micropropagate this species. The same was the case for *D. intermedia*, where growth was not positively influenced by the addition of Kin at 0.1 mg/L and ¼MS PGR-free medium provided the best results. On the contrary, *P. lusitanica* shoots were susceptible to supplementation with PGRs, showing higher proliferation and rooting response in ½MS media supplemented with cytokinins at 0.5 mg/L.

In summary, efficient micropropagation protocols were developed for *P. lusitanica* and *D. intermedia* which allow for the regeneration of plantlets which can be used to replenish declining populations in the nature, making a valuable contribution to the conservation of the species, and biomass production for the subsequent chemical analysis and biological assays. Cultures of *P. vulgaris* and *D. rotundifolia* were established *in vitro* but need further optimization.

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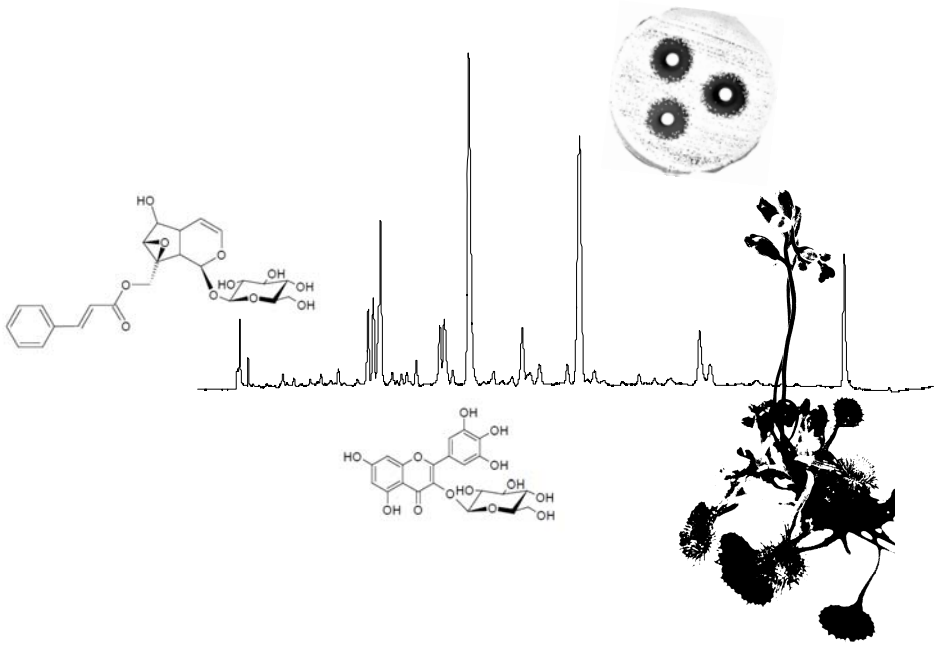
CHAPTER 3

CHEMICAL INVESTIGATION OF *PINGUICULA LUSITANICA* AND *DROSERA INTERMEDIA*



3.1. INTRODUCTION

3.1.1. Phytochemical characterization of extracts

Natural products are usually extracted as complex mixtures containing many constituents present in different concentrations and representing a broad spectrum of physical and chemical properties (Jaroszewski, 2005). Furthermore, the composition of extracts is initially often completely unknown and therefore the isolation and structure elucidation of individual constituents from complex mixtures such as plant extracts can pose a considerable challenge. This chapter will focus on the structure elucidation of the major compounds of the extracts prepared from *in vitro* cultured *P. lusitanica* and *D. intermedia*. These species were selected for analysis because they are the least studied of the four species comprised in this work and biomass could be readily produced.

3.1.2. Phytochemical data

The secondary metabolites produced by *P. lusitanica* and *D. intermedia* are poorly described in literature. Extracts prepared from micropropagated *D. intermedia* have been investigated for their content in naphthoquinones by Budzianowski (1996), but the complete characterization of its main secondary metabolites remains unclear. The apolar fraction of the methanol extract yielded 7-methylhydrojuglone-4-*O*-glucoside, hydroplumbagin-4-*O*-glucoside and their corresponding free quinones (7-methyljuglone and plumbagin, respectively) after treatment with *β*-glucosidase. *P. lusitanica*, on the other hand, has not been studied at all from a phytochemical perspective, possibly due to its scarce existence. Of the known 85 species of the *Pinguicula* genus, only two have been the subject of a biochemical study, namely *P. vulgaris* (Marco, 1985; Damtoft et al., 1985) and *P. moranensis* (Damtoft et al., 1994). Several iridoid glycosides (globularin, globularicisin, scutellarioside-II and 10-*O*-benzoylcatalpol) and a caffeoyl phenylethanoid glycoside, namely acteoside, were identified in these two species.

3.1.3. Collection of plant material and sample preparation

An important requirement in analytical chemistry is that the sample analyzed is representative. This means that samples must be collected, treated and stored in such a

way that their chemical composition is similar to the average composition of the total material. In the analysis of plant material from field specimens the collection of a representative sample is difficult due to variability of individual plants among a species and due to seasonal and geographical variations in the plant's metabolic profile (Romanik et al., 2007). These issues are however obviated when using *in vitro* cultured plant material.

Sample preparation represents the first stage in an analytical procedure and is of great importance. The sample preparation methods and strategies used depend on whether the aim of the extraction is analytical or preparative and whether the plants used for extraction contain known compounds, or unknown molecules, where thermal stability may be important (Nyiredy, 2004). Prior to extraction it is important to reduce particle size and increase interfacial surface area in order to enhance extraction efficiency. Plant material is often dried before being grinded or milled, however the drying process can lead to heat decomposition of metabolites or to increased binding with the plant matrix (Marczak et al., 2005; Rodrigues et al., 2006). When drying is to be avoided, lyophilisation can be used as an alternative process, or, when the secondary metabolites produced by a specific plant are unknown, the fresh plant material can be grinded after adding liquid nitrogen, preventing the loss of the most volatile components.

After obtaining a homogeneous sample, the extraction can be performed with a specific solvent or either a series or mixture of solvents. Several extraction methods can be used depending on the research objective. Maceration, Soxhlet extraction, ultrasound assisted extraction, microwave assisted extraction, supercriticial fluid extraction, accelerated solvent extraction and hydrodistillation have been used to prepare extracts from plant material and some techniques will be discussed in further detail in Chapter 5 (section 5.1.3). When the approach of the analytical task is qualitative, standard maceration is an appropriate method for obtaining a representative sample of the plants major secondary metabolites and preventing possible thermal decomposition of compounds. Furthermore, it is important to use a solvent that will extract the widest range of classes of metabolites when the chemical profile of a plant's extract has not been characterized before (Nyiredy, 2004). Methanol and ethanol are often the selected solvents (Table 3.1.1). An organic solvent is preferred in some cases over water as it halts metabolic processes and denaturing enzymes. This is important because the disruption of the

cellular structures during the extraction process releases enzymatic glucosidases which are other wise compartmentalized. These enzymes hydrolyse glycosylated compounds into their corresponding aglycone. In natural product analysis researchers are interested in obtaining the intact form of the metabolites instead of their aglycones, as great structural diversity can be observed in the sugar moieties.

Table 3.1.1. Classes of natural products extracted by several solvents. Compounds in bold are commonly obtained only by one solvent (adapted from Cowan, 1999).

Water	Ethanol	Methanol	Chloroform	Dichloromethanol	Ether	Acetone
Anthocyanins	Tannins	Anthocyanins	Terpenoids	Terpenoids	Alkaloids	Flavonols
Starches	Polyphenols	Terpenoids	Flavonoids		Terpenoids	
Tannins	**Polyacetylenes**	Saponins			**Coumarins**	
Saponins	Flavonols	Tannins			**Fatty acids**	
Terpenoids	Terpenoids	**Xanthoxyllines**				
Polypeptides	**Sterols**	**Totarol**				
Lectins	Alkaloids	**Quassinoids**				
	Propolis	**Lactones**				
		Flavones				
		Phenones				
		Polyphenols				

A common drawback of classical and modern extraction methods in sample preparation of complex matrices is that additional clean-up procedures are often required before chromatographic analysis. The use of most extraction methods results in non-selective co-extraction of relatively large amounts of undesirable components (lipids, sterols, chlorophylls), which can severely affect the separation and detection performance of subsequent analysis. Solid phase extraction (SPE) is a simple preparation technique based on the principles used in liquid chromatography, in which the solubility and functional group interactions of sample, solvent, and adsorbent are optimized to perform sample fractionation, concentration or clean-up. A wide range of chemically modified adsorbent materials enable separation on the basis of different types of physicochemical interactions (Huie, 2002).

3.1.4. Analytical techniques

The chemical characterization of crude plant extracts can be a technical demanding task. For each compound, the order of atoms and stereochemical orientations have to be

elucidated in a complex manner and the compounds cannot be sequenced as is the case for genes or proteins. Consequently and unlike genomics and proteomics, a single analytical technique that is capable of profiling all secondary metabolites in a plant extract does not exist (Wolfender et al., 2003).

3.1.5. Separation techniques

3.1.5.1. High Performance Liquid Chromatography (HPLC)

The most important separation technique in natural product analysis is HPLC. This chromatography technique is capable of separating water-soluble, thermally-labile and nonvolatile compounds, with speed, precision and high resolution and has the ability, when combined with a detection technique, to identify and quantitate the compounds present in any sample that can be dissolved in a liquid (Marston and Hostettmann, 2009). It was originally named high pressure liquid chromatography due to the increased pressure that was needed to circulate the mobile phase through the columns in comparison to gas chromatography, but "pressure" was replaced by "performance" as particles got smaller and columns became shorter (Marston, 2007). Gas chromatography is another separation technique that provides excellent resolution and is very adequate for the analysis of samples containing volatile compounds such as essential oils. However, less than 20% of organic compounds can be separated by gas chromatography, meaning that derivatisation is often necessary (Marston, 2007).

The basic principle of a HPLC setup is the use of a two-phase system to separate compounds according to specific molecular properties like polarity or hydrogen bond formation. The two phases are the stationary phase, the actual HPLC column, and the mobile phase, the eluent. Chromatography is generally carried out in the reverse-phase mode, on octadecyl carbon chain (C18) bonded silica columns because most natural products contain a substantial apolar part and reversed columns are better suited therefore. In reverse phase chromatography the stationary phase is non-polar and the polarity of the mobile phase is gradually decreased and, as a result, the most polar compounds are eluted first, while the more apolar are bound longer to the column before eluting, resulting in different retention times. Gradient elution is generally performed with binary solvent systems, i.e., with water containing acetate or formate

buffer, and methanol or acetonitrile as organic modifier (de Rijke et al., 2006). Reverse phase chromatography can also be performed in a purely isocratic mode where the solvent conditions are held constant.

3.1.6. Hyphenated techniques

The research in the field of natural products has changed dramatically due to the development of hyphenated techniques. Hyphenated methods are defined as methods combining two or more analytical techniques, usually a separation and a spectroscopic technique, into one integrated technique (Jaroszewski, 2005).

3.1.6.1. HPLC-UV

Ultraviolet (UV) detection is the most simple and most widely used amongst all HPLC detectors. Most natural products absorb UV light in the range of 200–550 nm, including substances having one or more double bonds or that have unshared electrons. Thus, even compounds having weak chromophores can be detected by UV at short wavelengths. In this case, however, mobile-phases that exhibit high UV cut-offs should be avoided because they might blind the detection of natural products with weak chromophores (Wolfender, 2009).

Three types of UV detectors are available: fixed wavelength, multiple wavelength, or diode array (DAD). DAD provides UV spectra directly online and is particularly useful for the detection of natural products with characteristic chromophores. With this type of compounds, DAD-UV spectral libraries can be built and used for dereplication (rapid identification of previously identified compounds), but compounds have to be analysed under the same HPLC conditions, as the composition of the mobile phase might affect the UV bands. With DAD-UV detection all wavelengths are stored during analysis, and thus multiple wavelengths can be monitored at the same time for detection of different classes of compounds which is particularly useful in the provisional sub-group classification of natural products (de Rijke et al., 2006). However, this detection technique suffers from some limitations because not all natural products possess UV chromophores and the amount of structural information derived from UV spectra is limited.

3.1.6.2. HPLC-MS

The coupling of HPLC and mass spectrometry (MS) has resulted in one of the most sensitive analytical methods. MS detection provides important structural information online such as molecular weight, molecular formula, and diagnostic fragments, which are crucial for rapid online characterisation of natural products (Wolfender, 2009).

A MS setup consists of three modules: the ionization source, the mass analyzer and the detector. The molecules are ionized at the ionization source, separated according to their mass to charge ratio (*m/z*) at the mass analyzer and then the detector calculates the abundance of each ion present. The great range of application of HPLC-MS techniques is largely due to atmospheric pressure ionisation interfaces including electrospray ionisation (ESI) and atmospheric pressure chemical ionisation, which surpassed the inherent incompatibilities of a liquid mobile phase and a high vacuum mass analyser by generating ions outside the vacuum part of the mass spectrometer (Marston, 2007). Furthermore, these soft ionization methods add hardly any collision energy to the sample and therefore enable very precise determination of the molecular weight. They provide mainly molecular ion species in the form of either protonated molecules ($[M+H]^+$) or deprotonated molecules ($[M-H]^-$), when operating in positive mode or negative mode, respectively (Wolfender, 2009). Different adducts are also produced, depending on the solutes and the modifiers used. However, the intense molecular ion species which are generated provide only limited structural information. Complementary fragmentation information can be generated by tandem MS or in-source collision induced fragmentation. The generated collision induced spectra are however not comparable to those recorded by electron impact (EI) and this hampers direct use of the standard EI-MS natural products libraries for the rapid identification of metabolites. The Time-Of-Flight (TOF) detector is a widely used mass analyzer and is capable of separating all ions in one measurement, instead of the more conventional quadrupole ion trap, where one *m/z* is selected per measurement by an applied electromagnetic field. In order to get a full mass spectrum of a sample multiple measurements are needed.

The combination of HPLC and MS is an efficient way of rapidly investigating the content of a natural extract. The fragmentation patterns of most natural product classes

are relatively specific and together with the accurate molecular weight, one can get an approximate idea of the compound under analysis. However, the integration of these techniques presents some limitations. Because ionisation is compound dependant in HPLC-MS, the great range of structural diversity of natural products cannot be covered by one type of MS experiment. Different classes of natural products will ionize more or less efficiently according to the applied ionization methods. This can be partly solved by using multiple ionization methods for the same sample. Also, the structural information gained by HPLC-MS is rather limited because although information on the molecular weight and fragmentation pattern is important, the variable ionization efficiency of fragments may confuse the analysis. In addition, MS spectra cannot provide an unequivocal structural determination, particularly in the case of isomers or when no reference material is available (Bieri et al., 2006).

3.1.6.3. HPLC-NMR

NMR spectroscopy is the most powerful and versatile technique for structure elucidation of natural products. An NMR spectrum gives information on the intramolecular distances between atoms or functional groups and on the orientation of substituents about chiral centers, enabling a full assignment of the molecular structure and determination of the stereochemistry (Havsteen, 2002; Jaroszewski, 2005). In addition, it is a highly non-selective detection technique as ^{1}H-NMR spectroscopy will detect any hydrogen-containing compound present in the HPLC eluate in a sufficient amount, regardless of its structure.

The principle of NMR spectroscopy is to measure the energy of a radiofrequency pulse required to alter the direction of the spin of a given type of nucleus. NMR experiments are conducted on nuclei of elemental isotopes that have a non-zero spin. The atomic nuclei commonly studied are the naturally occurring ^{1}H and ^{13}C isotopes (Table 3.1.2). Placing a sample in a strong magnetic field will orientate the spin of all nuclei in the direction of the external magnetic field and by applying radiofrequency pulses the state of spin of a nucleus can be changed. As soon as the radiofrequency wave is shut off, the nuclear spin relaxes to its previous direction and the amount of energy absorbed by the sample as a function of the strength of the magnetic field is measured. The energetic difference between the two spin states of a specific nucleus is called energy of

resonation and the frequency at which the nucleus resonates reflects its chemical environment in the molecule. Electron density locally diminishes the magnetic field and therefore information on the molecular structure such as the presence of electronegative atoms as oxygen and nitrogen, double bonds and aromatic systems can be obtained with NMR by measuring the frequency shift. This frequency shift is converted into a dimensionless value know as chemical shift (δ) (Havsteen, 2002).

Table 3.1.2. NMR data for important nuclei in phytochemistry (Eisenreich and Bacher, 2007).

Isotope	Spin	Natural Abundance %
^{1}H	1/2	99.985
^{2}H	1	0.015
^{3}H	1/2	3×10^{-16}
^{13}C	1/2	1.100
^{14}N	1	99.634
^{15}N	1/2	0.366
^{19}F	1/2	100.000
^{29}Si	1/2	4.670
^{31}P	1/2	100.000
^{77}Se	1/2	7.600

The elucidation of a compounds' structure can be complicated because often there are many protons in the same type of environment, however 2D-NMR methods pose a significant aid in these cases. Although there are a large number of NMR experiments available, a small subset is usually sufficient to determine the structures of low molecular weight metabolites (Bross-Walch et al., 2005). Two-dimensional ^{1}H-^{1}H correlation experiments, such as Double-Quantum-Filtered Correlation Spectroscopy (DQF-COSY) (Piantini et al., 1982) or Total-Correlation Spectroscopy (TOCSY) (Braunschweiler and Ernst, 1983) are used to assign the proton spins via scalar couplings, i.e., the mutual influence that neighbouring nuclei exert on each other via chemical bonds. The spectra from COSY experiments contain mainly cross-peaks due to vicinal couplings while TOCSY spectra additionally contain correlations with other protons in the same spin system, which is very useful for compounds containing many separate spin systems. Another important ^{1}H homonuclear experiment is NOESY (Nuclear-Overhauser-Effect Spectroscopy) (Macura and Ernst, 1980) which yields correlations of dipolar-coupled protons through space and is used for obtaining stereochemical information but also for linking separate spin systems. Furthermore,

proton-detected $^{1}H–^{13}C$ correlation experiments, known also as heteronuclear 2D techniques, such as Heteronuclear Single Quantum Coherence (HSQC; Bodenhausen and Ruben, 1980) or Heteronuclear Multiple Bond Coherence (HMBC; Bax and Summers, 1986) are used to correlate the ^{13}C chemical shifts with protons via one or two/three bonds, respectively. On the basis of these experiments, a set of constraints for the constitution and the configuration of a molecule can be obtained which may provide sufficient information to unambiguously assign the structure of a typical metabolite (Bross-Walch et al., 2005). It is to be noted, however, that these techniques are material limited, and the more powerful the experiment, the greater the amount of material needed to obtain reliable NMR experiments.

There are several methods to deliver the HPLC fraction into the NMR detection coil. The simplest way is to couple the HPLC directly to the NMR spectrometer and to perform on-flow analysis of the chromatographic fractions. The drawback of this on-flow NMR method is that the residence time of the sample in the NMR flow cell is determined by the flow supplied by the HPLC pump, and not by the time necessary to obtain a sufficient S/N (signal to noise ratio) in the NMR spectrum. The stopped-flow cell technique addresses this problem by stopping the flow cell of the HPLC pump as soon as the fraction of interest is inside the NMR flow cell, while keeping the pressure constant in order not to disturb the separation. Chromatography is then resumed under normal flow as soon as the NMR measurement is complete. However, a complete set of NMR experiments can take several hours and this can cause on-column diffusion. An alternative to this method is to collect the fractions in capillary storage loops during the HPLC elution, which can then be transferred into the NMR system independently from the ongoing separation (Wolfender et al., 2001; Bross-Walch et al., 2005).

The major drawback of NMR spectroscopy in structure elucidation is its relative insensitivity which is mainly caused by the small energy difference between ground and excited state of nuclear spins (Eisenreich and Bacher, 2007). This lack of sensitivity renders the on-flow measurements of minor products impossible and hampers the direct observation of ^{13}C-NMR resonances even for the main constituents of a crude plant extract. Information of the ^{13}C-NMR resonances represents an important element for natural product identification but the vast majority of carbon present in natural organic matter is not available for NMR detection because of the low abundance of the ^{13}C

isotope (Table 3.1.2). The difficulty of obtaining ^{13}C information is probably the biggest hindrance to a wider use of HPLC-NMR. As a consequence, many plant metabolites, especially with characteristic ^{1}H signals which are not well separated on the NMR scale, are difficult to identify at present by this technique. Compounds which exhibit very few ^{1}H signals and which have mainly quaternary carbons or hydroxyl substituents in their structure will also yield little structural information in HPLC-^{1}H-NMR (Wolfender et al., 2003).

Another problem is that the whole range of the ^{1}H-NMR signals is not directly observable in classical LC-^{1}H-NMR spectra because of the need for solvent suppression. Specific NMR pulse sequences can suppress the solvent signals, but as a result, the signals of the analytes of interest which reside under the solvent peak, will be suppressed together with the solvent signal. This can be a major drawback when dealing with unknown constituents. One way of overcoming the problem of solvent suppression is to perform the separation in fully deuterated solvents. This, however, cannot be envisaged routinely on standard HPLC columns due to the cost of the deuterated solvents (Wolfender et al., 2001).

3.1.6.4. HPLC-SPE-NMR

The inherent lack of sensitivity of HPLC-NMR has improved over the years by miniaturization of the flow cells, higher magnetic field strengths, and cryogenically cooled NMR probes and preamplifiers. However, all these advancements still rely on analyte concentrations delivered by the HPLC column. Moreover, the range of HPLC eluents used is still restricted to those suitable for NMR analysis. The introduction of on-line solid phase extraction (SPE) in HPLC-NMR has enhanced considerably the sensitivity of this technique. In HPLC-SPE-NMR compounds are detected postcolumn by UV or MS and automatically trapped on SPE cartridges, enabling multiple trapping and subsequent transfer of the analytes to the NMR flow probe using a deuterated solvent. In order to enhance trapping efficiency, the flow in organic solvents eluting from the HPLC is mixed with additional water and as a consequence of the change in polarity, the compounds are trapped on the cartridge. The cartridge is dried with nitrogen gas after which the fraction is eluted with deuterated solvent to the NMR flow cell and can be analyzed at any time (Figure 3.1.1).

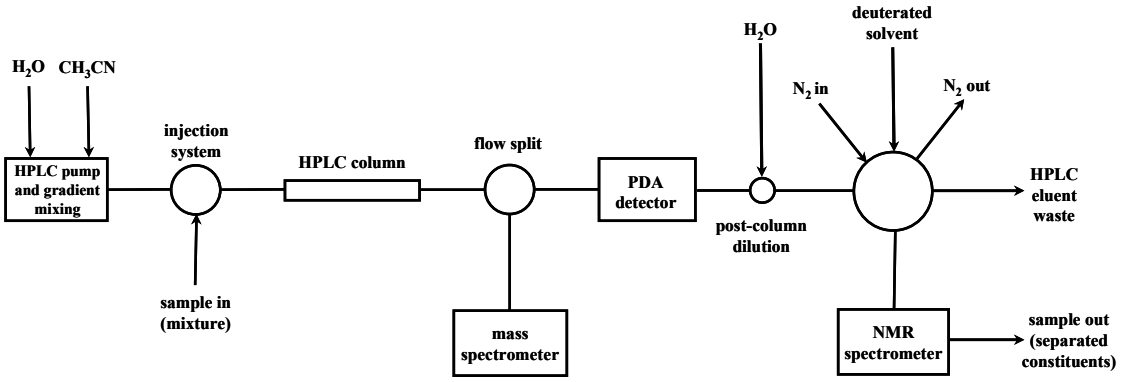


Figure 3.1.1 - Schematic diagram of an HPLC-SPE-NMR system (Jaroszewski, 2005). The mixture components are separated on a HPLC column with non-deuterated solvents, and a chromatogram is recorded using a photodiode array detector. The HPLC column eluate is diluted with water and the chromatographic peaks are trapped on individual SPE cartridges, triggered by signals from the UV or MS detector. The cartridges are dried with a stream of nitrogen gas and the analytes eluted with a deuterated solvent into the NMR flow-probe. The analyzed content of the probe can be discarded or collected for further analysis.

The introduction of the SPE interface between the chromatography and NMR has several advantages. The collected fractions elute in very small solvent volumes into the NMR flowprobe and are therefore highly concentrated and the amount of sample can be increased by multiple trapping on the same cartridge. Moreover, the whole chromatography can be run with protic solvents, and deuterated solvent is only required for elution from the dried cartridge. The interfacing of liquid chromatography with NMR spectroscopy using the integrated SPE device has enhanced the potentialities of NMR in structural determination by improving its sensitivity and facilitating acquisition of 2D NMR data (Clarkson et al., 2005). These improvements allow obtaining structural information directly from crude extracts using minute amounts of sample, avoiding the lengthy and laborious preparative-scale isolation process (Clarkson et al., 2005; Jaroszewski, 2005).

3.1.4. Objectives

P. lusitanica and D. intermedia are two species which are becoming increasingly scarce and little is known about their secondary metabolite production profile. The objectives of this chapter are to:

i) identify the major compounds produced by these species using the hyphenated HPLC-ESI-MS and HPLC-SPE-NMR techniques;

ii) discuss the possible biological and taxonomic importance of the identified secondary metabolites.

3.2. EXPERIMENTAL

3.2.1. Plant material and sample preparation

In vitro cultures of *P. lusitanica* and *D. intermedia* were produced according to the optimized protocols described in Chapter 2 (Sections 2.3.1 and 2.3.3, respectively). Fresh micropropagated plants were powdered in a mortar with liquid nitrogen and extracted twice for 24 h with methanol in the case of *P. lusitanica* and with methanol, water or *n*-hexane in the case of *D. intermedia*. The percentage yields (extract mass/fresh weight mass) for the *P. lusitanica* extract was 3.0% and 3.7%, 2.3% and 0.2% for the methanol, water and *n*-hexane extracts prepared from *D. intermedia*, respectively. The obtained extracts were filtered (Whatman nº1, Springfield Mill, England), concentrated by rotary vacuum evaporation, dissolved in water and lyophilized.

A solid phase extraction column (Supelclean LC-18 Packing; 60 mL; 10 g) was used for sample preparation to remove the most apolar and fatty constituents of the extracts and served as a sample cleanup. The column was activated with 100 mL methanol, washed with 100 mL acetonitrile, and equilibrated with 100 mL of 30% acetonitrile in water solution. The crude methanol extract of *P. lusitanica* was dissolved in 30% acetonitrile in water solution at approximately 40 mg/mL and loaded onto the column. Another 100 mL of 30% acetonitrile in water solution were loaded on the column and collected. The remaining extract was eluted from the column with acetonitrile and discarded. The column was washed successively with 100 mL of acetonitrile, chloroform and methanol before further use. The same procedure was performed for the methanol and *n*-hexane extract prepared from *D. intermedia*, except that the *n*-hexane extract was eluted from the column with 50% acetonitrile in water, because the extract would not elute completely with the 30% aqueous acetonitrile solution. The water extract was not subjected to the SPE cleanup procedure because of the low affinity of its constituents with the column material. Instead, the water extract was dissolved in the analysis solvent and centrifuged at 10000 rpm during 15 min to remove the insoluble fraction of the extract in order not to obstruct the column. The recovered fractions were concentrated under vacuum evaporation, freeze-dried and stored at -20ºC until analysis.

3.2.2. HPLC-MS and HPLC-SPE-NMR measurements

3.2.2.1. General experimental setup

The HPLC-ESI-MS / HPLC–SPE–NMR system consisted of a Agilent 1200 quaternary solvent delivery pump, an Agilent 1200 autosampler, a Bruker Daltonics MicrOTOF ESI mass spectrometer (Figure 3.2.1 A), a BSFU column oven, a Bruker diode array detector, a Knauer K120 pump for postcolumn water delivery, a Spark Prospekt 2 solid phase extraction device (Figure 3.2.1 B), containing HySphere resin SH cartridges (10 × 2 mm, 25-35 μm) for the *P. lusitanica* extract, or Oasis HLB Prospekt 2 Symbiosis cartridges (10 × 2 mm, 96/pkg) for *D. intermedia* extracts, and a Bruker Avance III 600 NMR spectrometer equipped (Figure 3.2.1 C) with a 5 mm (30 μL) inverse cryoflow probe operating at 300 K. Chromatography, peak trapping, and analyte transfer from the SPE unit to the NMR spectrometer were controlled with HyStar 3.2 software, whereas the NMR experiments were controlled and processed with TopSpin 2.0 (Bruker BioSpin).

Figure 3.2.1 - Equipment used for the HPLC-ESI-MS and HPLC-SPE-NMR experiments: Bruker Daltonics micrOTOF (time of flight) ESI mass spectrometer (A), Spark Holland Prospekt 2 SPE unit (B) and Bruker Avance III 600 MHz NMR spectrometer (C).

3.2.2.2. HPLC-MS and HPLC-SPE-NMR experiments

3.2.2.2.1. HPLC-(DAD) gradient optimization

Several trial runs were performed to obtain an optimized gradient for the *P. lusitanica* extract which are shown in Table 3.2.1. These were performed with a smaller diameter analytical column (Alltima C18; 3 μm; 150 × 2.1 mm i.d.) to allow a better separation. The final working gradient optimized for the HPLC-SPE-NMR experiments was carried out on an Alltima HP 3 μm (150 × 4.6 mm i.d.) column using a binary eluent consisting of nondeuterated water containing 0.1% formic acid (v/v) (A) and acetonitrile (B), with a 0.6 mL/min flow rate and the following linear gradient: at 0 min, 10% B; at 5 min, 15% B; at 43 min, 25% B; at 45 min, 95% B; at 47 min 95% B; and at 50 min 10% B, followed by a 5 min conditioning step (Table 3.2.1, gradient 3).

Table 3.2.1. Tested experimental conditions for the optimization of the working gradient.

Gradient 1		**Gradient 2**		**Gradient 3**	
Time (min)	**Acetonitrile (%)**	**Time (min)**	**Acetonitrile (%)**	**Time (min)**	**Acetonitrile (%)**
0	5.0	0	10.0	0	10.0
40.0	95.0	40.0	35.0	5.0	15.0
45.0	95.0	45.0	95.0	43.0	25.0
47.0	5.0	47.0	95.0	45.0	95.0
55.0	5.0	50.0	10.0	47.0	95.0
		55.0	10.0	50.0	10.0
				55.0	10.0
150 × 2.1 mm i.d. column; 0.2 mL/min				150 × 4.6 mm i.d. column; 0.6 mL/min	

In the case of the *D. intermedia* extracts the chromatographic separations were carried out using the same binary solvent and the following optimized gradients: at 0 min, 5.0% B; at 30 min, 95% B; at 31 min, 100% B; at 45 min, 100% B; at 46 min 5.0% B, followed by a 10 min conditioning step, for the *n*-hexane extract; at 0 min, 5.0% B; at 45 min, 35% B; at 47 min, 100% B; at 57 min, 100% B; at 59 min 5.0% B, followed by a 5 min conditioning step, for the water extract; at 0 min, 8.0% B; at 45 min, 30% B; at 47 min, 100% B; at 57 min, 100% B; at 59 min 8.0% B, followed by a 5 min conditioning step, for the methanol extract.

3.2.2.2.2. HPLC-ESI-MS experiments

The extracts were analyzed by ESI-MS in positive and negative mode. Ions were detected in negative mode in the range of *m/z* 100 to 1500 and in both MS experiments working conditions were as follows: nebulizer pressure was 3.0 bar, with a drying gas flow of 8.0 L/min and drying gas temperature of 190 ºC, capillary spray was 4.2 kV. In the case of the methanol extract of *P. lusitanica*, the ESI-MS measurements were performed using gradient 2, by flushing the content of the cartridges after the NMR experiments from the probe into vials. The compounds in the vials dissolved in approximately 100 µL of deuterated solvent, were then diluted with a 50% acetonitrile in water solution before a volume of 20 µL of each peak was injected into the mass spectrometer. In the case of *D. intermedia*, a volume of 5 µL of each extract at 50 mg/mL (50% methanol solution in water) was analyzed by HPLC-ESI-MS, using the optimized gradients and a flow rate of 0.2 mL/min (150 × 2.1 mm i.d. column).

3.2.2.2.3. HPLC-SPE-NMR experiments

The methanol extracts of *P. lusitanica* and *D. intermedia* where analyzed by HPLC-SPE-NMR using the solid phase extraction unit to trap the chromatographic peaks. In the case of the *P. lusitanica* extract the HPLC eluate was monitored by DAD, and absorption thresholds at 220, 280 and 312 nm were defined in order to provide automatic start and stop signals for the SPE trappings. The extract was dissolved at 50 mg/mL (50% methanol solution in water) and a total of 4 cumulative trappings were performed for each peak selected for analysis using an injection volume of 20 µL. For the *D. intermedia* extract peaks were trapped manually using the MS instrument as detector. A total of 3 cumulative trappings were performed for each peak selected for analysis after injecting 40 µL of extract prepared at 300 mg/mL (75% methanol solution in water) using the optimized gradient (150 × 4.6 mm i.d. column; 1.0 mL/min flow rate). Prior to the trappings, the SPE cartridges were conditioned with 500 µL of deuterated methanol and equilibrated with 500 µL of water. After the chromatographic separation, water was added to the eluent with the makeup pump at a flow rate of 1 mL/min to lower the elution strength and provide proper retention of the peaks under study on the SPE cartridges. The cartridges were dried with nitrogen gas for 59 min to remove the residual solvents and subsequently, methanol-*d*4 was used for elution and

transfer of the analytes to the NMR flow probe. ^{1}H-NMR spectra were recorded at 600 MHz using standard pulse sequences. The methanol signal at 3.31 ppm was used for chemical shift calibration. 1D and 2D-DQF (double quantum filtered) COSY, TOCSY, ROESY, HSQC and HMBC spectra were acquired using state-of-the-art pulse sequences.

3.2.2.2.4. Direct NMR analysis

The *n*-hexane and methanol extracts were analyzed directly by NMR. The *n*-hexane extract (5 mg) was dissolved in 500 µL of deuterated chloroform and the methanol extract (10 mg) in 1 mL of deuterated methanol and transferred to a 5 mm NMR tube to be analyzed. The spectra were acquired using the standard pulse sequences.

3.3. RESULTS AND DISCUSSION

3.3.1. *P. lusitanica*

3.3.1.1. HPLC gradient optimization

It is necessary to obtain a good separation of the chromatographic peaks before any further work can be undertaken. This is achieved by optimizing the gradient, namely the gradual change of the content of organic solvent in the mobile phase. As previously stated, in the field of natural products research, reverse phase columns are preferred. The stationary phase is non-polar, binding the analytes through hydrophobic interactions, which result from repulsive forces between a polar eluent. By increasing the gradient of the mobile phase the analytes are eluted sequentially based on their polarity. Retention time is longer for molecules which are more apolar, while polar molecules elute more readily. As there was no information available on the biochemical data of *P. lusitanica*, as a first approach a gradient covering the range of 5-95% of acetonitrile (gradient 1, Table 3.2.1) was used. The chromatogram in Figure 3.3.1.1 shows that the separation of the peaks is not satisfactory and that all major peaks are relatively polar. The absorbance was recorded at several wavelengths but 280 nm gave the highest intensity for most of the compounds.

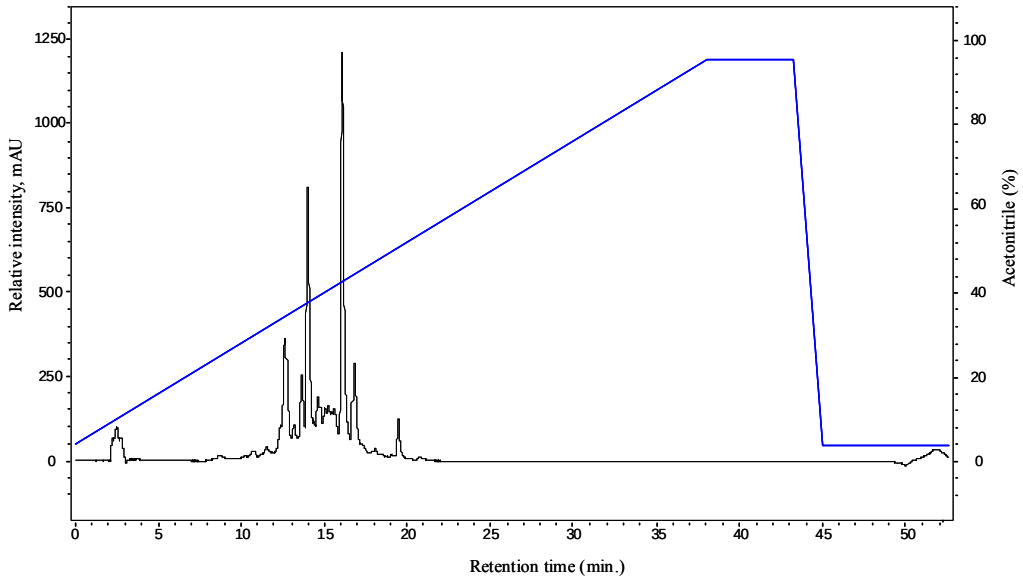


Figure 3.3.1.1 - HPLC chromatogram monitored at 280 nm of *P. lusitanica* methanol extract (150 × 2.1 mm i.d. column). Gradient elution profile is shown as percent of acetonitrile in water (gradient 1, Table 3.2.1).

In an attempt to enhance the chromatographic separation, a second gradient was tested based on the previous chromatographic run. The new gradient covered the range of 10-35% acetonitrile (gradient 2, Table 3.2.1), and a better resolution of the region of interest was achieved, as can be seen in Figure 3.3.1.2.

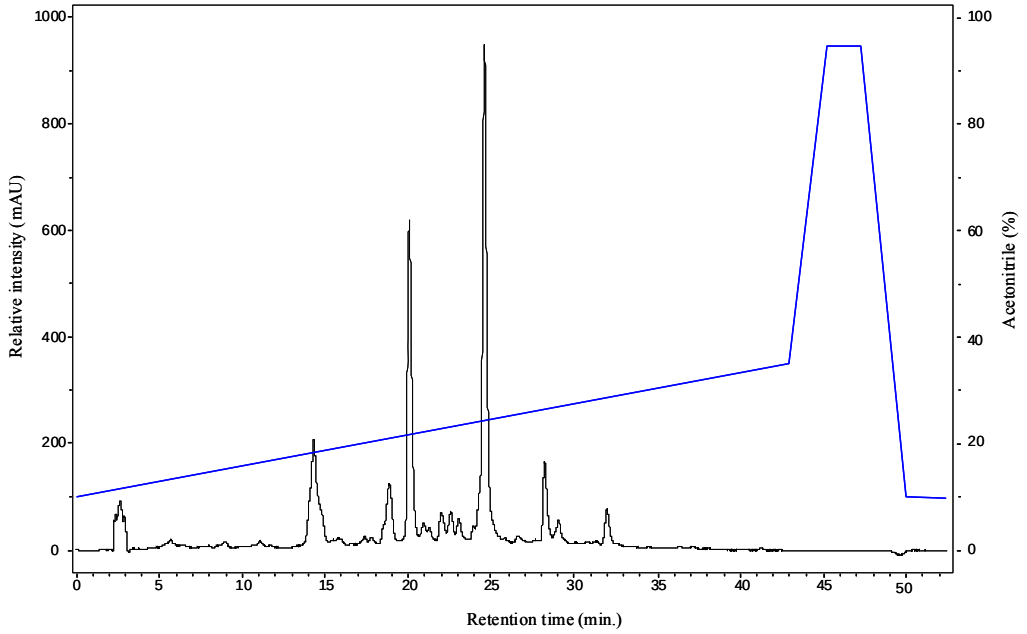


Figure 3.3.1.2 - HPLC chromatogram monitored at 280 nm of *P. lusitanica* methanol extract (150 × 2.1 mm i.d. column). Gradient elution profile is shown as percent of acetonitrile in water (gradient 2, Table 3.2.1).

The final working conditions were based on the former gradient, with some minor adjustments because a column with a larger inner diameter was used (gradient 3, Table 3.2.1). This column is more appropriate for the subsequent experiments, because to obtain good quality NMR spectra it is important to submit the largest amount possible to chromatography in order to enhance the SPE efficiency. Against expectations, the column with larger diameter provided a better resolution, as depicted in Figure 3.3.1.3. The gradient provided satisfactory separation to perform the SPE trappings and the nine most intense peaks were selected for analysis and labeled 1-9. Peak 1 has nearly no UV absorbance at 280 nm, but was selected for analysis because of its strong absorbance at 220 nm.

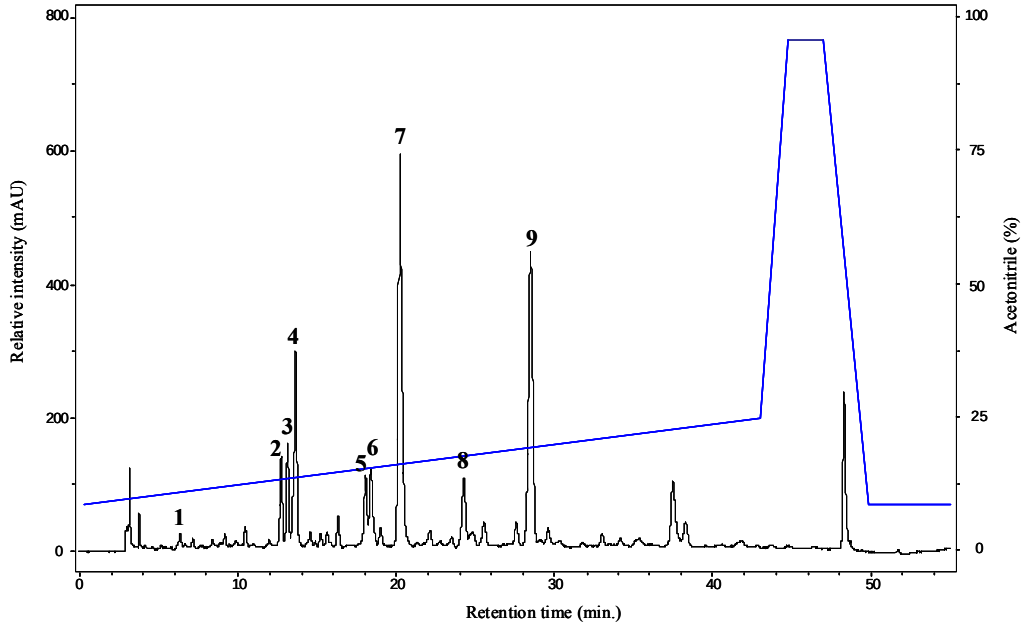


Figure 3.3.1.3 - HPLC chromatogram monitored at 280 nm of *P. lusitanica* methanol extract (150 × 4.6 mm i.d. column). Gradient elution profile is shown as percent of acetonitrile in water (gradient 3, Table 3.2.1). The peaks selected for the HPLC-SPE-NMR experiments are labelled 1-9.

3.3.1.2. HPLC-ESI-MS

The *P. lusitanica* extract was subjected to HPLC-ESI-MS measurements in both positive and negative mode using gradient 2. Table 3.3.1.1 shows the DAD-UV and ESI-MS data and the deduced molecular formulas obtained for the selected peaks for analysis. Because a different gradient was used for the NMR analysis, and no splitter was available when SPE trappings were performed, the molecular weights of some trapped compounds were not known. For that reason, after NMR analysis the trapped peaks were flushed out of the flow cell and recovered in vials. The vials were then analyzed individually by ESI-MS conducted in negative mode for molecular weight determination and therefore some peaks were not analyzed in positive mode. In most cases the negative mode ESI-MS spectra showed base peaks corresponding to the molecular ion with few other fragment ions, whereas the spectra obtained in positive mode showed fragmentation patterns with some structural information.

Table 3.3.1.1. DAD-UV and ESI-MS spectral data of selected peaks of *P. lusitanica* chromatogram for analysis.

Peak	t_R (min)	UV λ_{max} (nm)	MS positive mode		MS negative mode	Molecular Formula
			$[M+H]^+$ (*m/z*, amu)	Fragments (*m/z*, amu)	$[M-H]^-$ (*m/z*, amu)	
1	5.49	236	377.1	215.1, 197.1, 179.1	375.1	$C_{16}H_{24}O_{10}$
2	12.75	320, 300 (sh), 290 (sh), 247	641.2	623.2, 471.1, 325.1, 163.0	639.2	$C_{29}H_{36}O_{16}$
3	13.15	320, 300 (sh), 290 (sh), 247	641.2	623.2, 471.1, 325.1, 163.0	639.2	$C_{29}H_{36}O_{16}$
4	13.66	312, 299 (sh), 228	ND		507.2	$C_{24}H_{28}O_{12}$
4'	13.66	312, 299 (sh), 228	ND		523.2	$C_{24}H_{28}O_{13}$
5	18.12	320, 300 (sh), 290 (sh), 247	ND		653.2	$C_{30}H_{38}O_{16}$
6	18.46	320, 300 (sh), 290 (sh), 247	ND		653.2	$C_{29}H_{36}O_{16}$
7	20.26	320, 300 (sh), 290 (sh), 247	625.2	479.1, 471.1, 325.1, 163.0	623.2	$C_{29}H_{36}O_{15}$
8	24.37	320, 300 (sh), 290 (sh), 247	ND		653.2	$C_{30}H_{38}O_{16}$
9	28.55	280, 221, 217	493.2	331.1, 131.0	537.2*	$C_{24}H_{28}O_{11}$

* Formic acid adduct of peak 9 ($[C_{24}H_{28}O_{11}+HCOO]^-$)

3.3.1.3. HPLC-SPE-NMR

3.3.1.3.1. SPE trapping procedure

From the chromatogram obtained in Figure 3.3.1.3, the nine most intense peaks were selected for trapping with SPE. The trapping was triggered by UV absorbance threshold at three different wavelengths. Peaks 2-8 were trapped using 312 nm, peak 9 using 280 nm and peak 1 using 220 nm (Figure 3.3.1.4). After the first trapping procedure and a preliminary NMR analysis it was decided that four successive trappings would be sufficient to obtain good quality spectra.

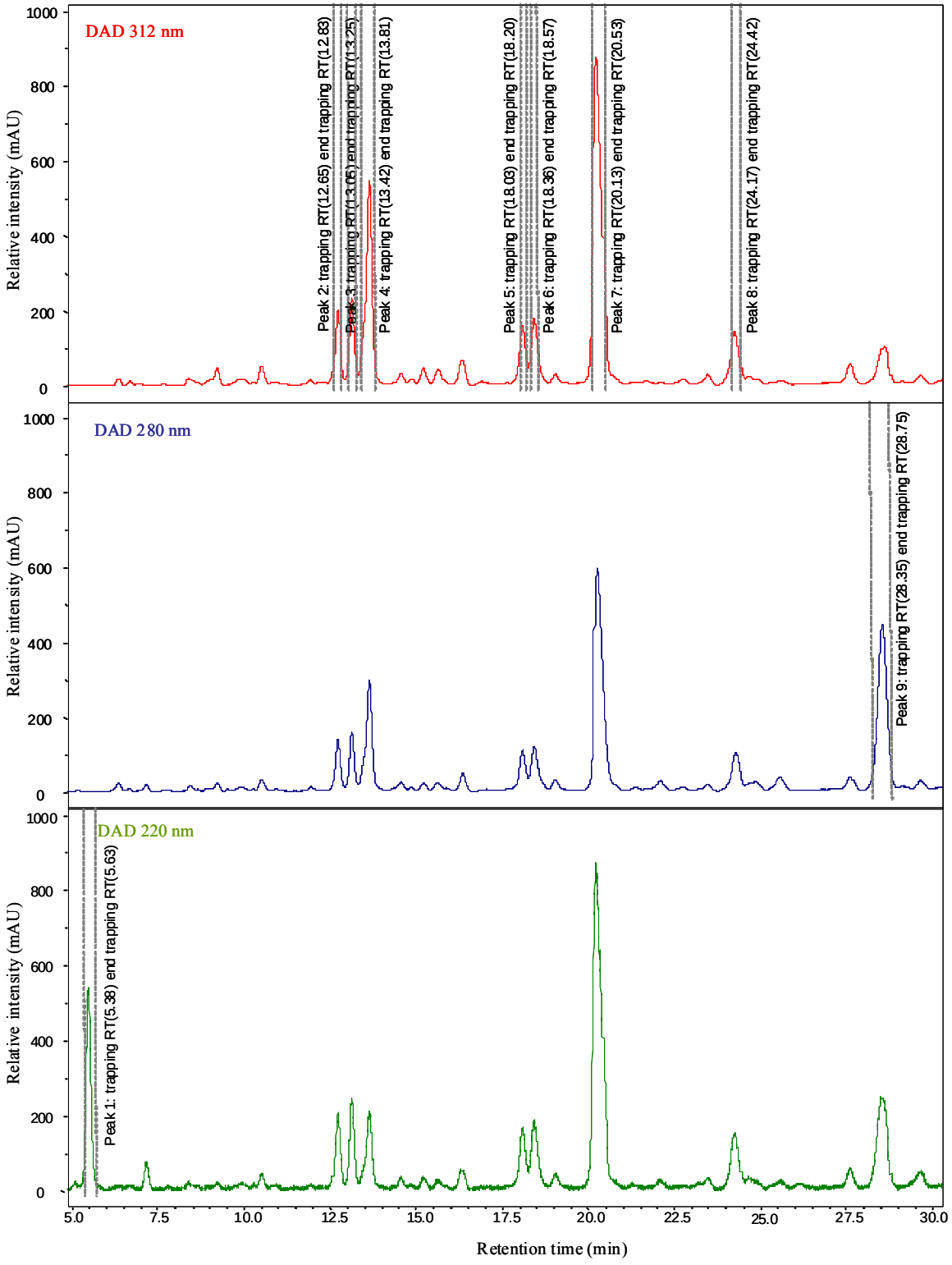


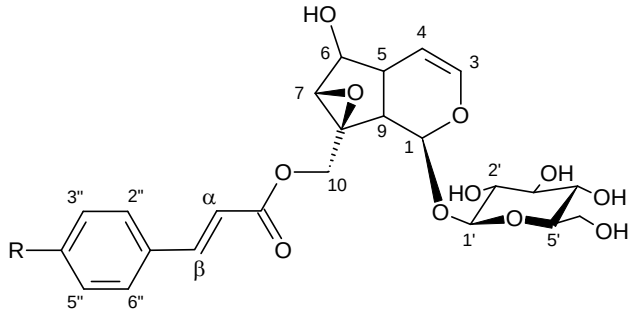
Figure 3.3.1.4 - Chromatogram of *P. lusitanica* methanol extract indicating SPE trappings for peaks 1 (220 nm), 2-8 (312 nm) and 9 (280 nm), and respective retention times.

3.3.1.3.2. HPLC-SPE-NMR

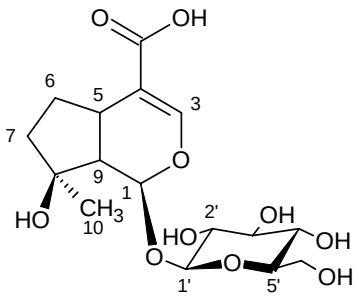
The NMR spectra that were obtained from the trapped peaks were overall of satisfactory quality. Except for peak 8, all ^{1}H spectra were of sufficient quality for the most important protons to be assigned. The NMR spectrum of peak 8 had a too low S/N for any conclusions to be drawn, so no further investigations were pursued. In the case of peaks 1, 4, 7 and 9 the S/N was good enough for 2D spectra to be measured, namely COSY, ROESY, TOCSY, HSQC, and in the case of peak 7 an HMBC spectrum was measured as well. Results are discussed per peak, which are grouped by the family of identified compounds, namely iridoid glucosides and phenylethanoid glycosides.

3.3.1.3.2.1. Iridoid glucosides

The methanol extract of *P. lusitanica* contains three iridoid glucosides, namely mussaenosidic acid (peak 1), scutellarioside II (peak 4) and globularin (peak 9). In addition, another iridoid glucoside was co-eluted with peak 4 (peak 4') and its structure was not conclusively elucidated, but the obtained data suggest that peak 4' might be the 5-OH derivative of scutellarioside II. The structures of the identified compounds are depicted in Figure 3.3.1.5 and the NMR data that allowed the assignment of these compounds is shown in Table 3.3.1.2.



Globularin (peak 9): R=H
Scutellarioside II (peak 4): R=OH



Musseanosidic acid (peak 1)

Figure 3.3.1.5 – Structures of the iridoid glucosides from *P. lusitanica*.

Table 3.3.1.2. ^{1}H and ^{13}C NMR data of the iridoid glucosides obtained by HPLC-SPE-NMR from the methanol extract of *P. lusitanica*.

	1		**4**		**4'**		**9**	
Position	δ_H*	δ_C**	δ_H*	δ_C**	δ_H*	δ_C**	δ_H*	δ_C**
Aglycone								
1	5.46 (1H, d, 4.2)	95.0	5.08 (1H, d, 10.0)	95.5	5.35 (1H, d, 9.0)	95.5	5.08 (1H, d, 9.8)	95.3
3	7.41 (1H, s)	151.8	6.36 (1H, d, 6.0)	141.4	6.39 (1H, d, 6.0)	142.4	6.36 (1H, dd, 1.7, 6.0)	141.5
4			5.07***	103.6	4.96***	107.4	5.08***	103.4
5	3.15 (1H, m)	31.7	2.29 m	38.9			2.30 (1H, m)	38.7
6a	2.30 (1H, td, 16, 14, 7.5)	30.5	3.95 (1H, d, 8.6)	78.9	4.13 (1H, br s)	78.5	3.96 (1H, dd, 8.2, 1.0)	79.2
6b	1.47 (1H, td, 14, 14, 7.5)							
7	1.72 (2H, t, 7.5)	40.4	3.49 (1H, s)	62.7	3.61 (1H, br s)	63.2	3.50 (1H, d, 1.0)	62.5
9	2.22 (1H, dd, 9.2, 4.1)	52.0	2.65 (1H, dd, 9.4, 8.2)	43.4	2.66 (1H, d, 9.0)	51.1	2.66 (1H, dd, 9.7, 7.7)	43.3
10a	1.33 (3H, s)	24.4	4.27 (1H, d, 12.6)	64.0	4.23 (1H, d, 12.8)	63.5	4.26 (1H, d, 12.6)	64.3
10b			4.98 (1H, d, 12.5)		4.95 (1H, d, 12.7)		5.03 (1H, d, 12.6)	
Glucosyl								
1'	4.68 (1H, d, 7.9)	99.5	4.74 (1H, d, 7.9)	100.0	4.71 (1H, d, 7.8)	100.0	4.76 (1H, d, 7.9)	100.0
2'	3.19 (1H, t, 8.6)	74.4	3.19 (1H, t, 8.3)	74.6	3.19***	74.6	3.18 (1H, t, 9.2)	74.4
3'	3.36 (1H, t, 9.0)	77.7	3.35 (1H, t, 9.1)	77.5	3.35***	77.5	3.35 (1H, t, 9.1)	77.7
4'	3.25 (1H, t, 9.0)	71.6	3.26 (1H, t, 9.2)	71.1	3.26***	71.1	3.26 (1H, t, 9.1)	71.3
5'	3.30****	77.9	3.32****	78.0	3.32****	78.0	3.30****	78.2
6a'	3.64 (1H, dd, 11.9, 6.4)	62.7	3.92 (1H, dd, 1.4, 11.9)	62.7	3.95 (1H, dd, 11.4, 1.8)	62.7	3.66 (1H, dd, 11.9, 6.4)	62.7
6b'	3.90 (1H, dd, 11.9, 1.8,)		3.66 (1H, dd, 6.0, 12.0)		3.68***		3.92 (1H, dd, 11.9, 1.9)	
Coumaroyl /Cinnamoyl								
2"			7.49 (1H, d, 8.5)	131.0	7.49***	131.0	7.63 (1H, dd, 6.7, 2.9)	129.2
3"			6.80 (1H, d, 8.5)	116.5	6.80***	116.5	7.41 m	129.7
4"							7.41 m	129.7
5"			6.80 (1H, d, 8.5)	116.5	6.80***	116.5	7.41 m	129.7
6"			7.49 (1H, d, 8.5)	131.0	7.49***	131.0	7.63 (1H, dd, 6.7, 2.9)	129.2
α			6.37 (1H, d, 15.9)	114.8	6.37***	114.8	6.57 (1H, d, 16.0)	118.3
β			7.65 (1H, d, 15.9)	146.7	7.65***	146.7	7.73 (1H, d, 16.0)	146.5

*Relative to the residual methanol signal set to δ 3.31, δ values given in ppm; number of protons, multiplicity of signals (s, singlet; br s, broad singlet; d, doublet; dd, double doublet; t, triplet; td triple doublet; m, multiplet) and coupling constants (apparent splittings) given as numerical values in Hz are shown in parenthesis. **^{13}C NMR chemical shifts obtained from HSQC experiments, relative to the resonance of the solvent set to δ 49.05. ***Signal unclear due to overlapping signals. **** Supressed due to overlapping with solvent signal, chemical shift determined by HSQC.

The assignments of the identified compounds based on the obtained MS and NMR data will be discussed in further detail for each peak in the following section. The results of the ESI-MS experiments of peak 9 show that the molecular formulas that can be deduced from the deprotonated $[M-H]^-$ (*m/z* 537) and protonated $[M+H]^+$ (*m/z* 493) base peak ions obtained do not match (Table 3.3.1.1). The difference is due to the fact that in the ESI-MS experiments conducted in negative mode, the measured ion corresponds to the formic acid adduct of the molecular ion ($[M+HCOO]^-$). Taking this into consideration, the molecular weight of compound 9 corresponds to 492 for which a molecular formula of $C_{24}H_{28}O_{11}$ can be deduced. This result shows the importance of conducting MS experiments in both negative and positive mode, as different information can be obtained from each kind of experiment.

The ^{1}H-NMR spectrum of peak 9 showed signals of an anomeric proton at δ 4.76 (d, *J*= 7.9 Hz) with a coupling constant characteristic of a sugar moiety with a β configuration. From the anomeric proton, it was possible to assign every proton and carbon of the sugar moiety by detailed analysis of ^{1}H-^{1}H COSY and HSQC spectra. The signal of the H-5' proton was located under the solvent signal, and its splitting pattern could therefore not be determined, but its chemical shift could be deduced from the HSQC spectrum. The splitting patterns and coupling constants of the protons made it possible to assign the fragment as a β-D-glucopyranosyl unit (Bross-Walch et al., 2005; Feng et al., 2006). The presence of a sugar unit can also be confirmed by the ESI-MS results conducted in positive mode, due to the appearance of a peak at *m/z* 331 (Table 3.3.1.1) resulting from the loss of an anhydrohexose unit (*m/z* 331= 493-162). In addition, in the downfield region of the ^{1}H-NMR spectrum, five aromatic protons at δ 7.63 (H-2", H-6") and δ 7.41 (H-3", H4", H5") and two olefinic doublets resonating at δ 6.57 (H-α) and δ 7.73 (H-β) were consistent with a cinnamoyl moiety. The large coupling constant (*J*= 16.0 Hz) of the olefinic protons suggests a *trans* stereochemistry (*E* conformation) at the double bond of the cinnamoyl moiety. Despite the fact that the aromatic protons of the cinnamoyl moiety are superimposed, it is possible to determine the number of protons corresponding to each signal by analyzing their respective integrals. The integral of the signals at δ 7.63 (2H) and δ 7.41 (3H) is approximately two and three times higher, respectively, than the signal at δ 7.73 corresponding to one single proton (Figure 3.3.1.6).

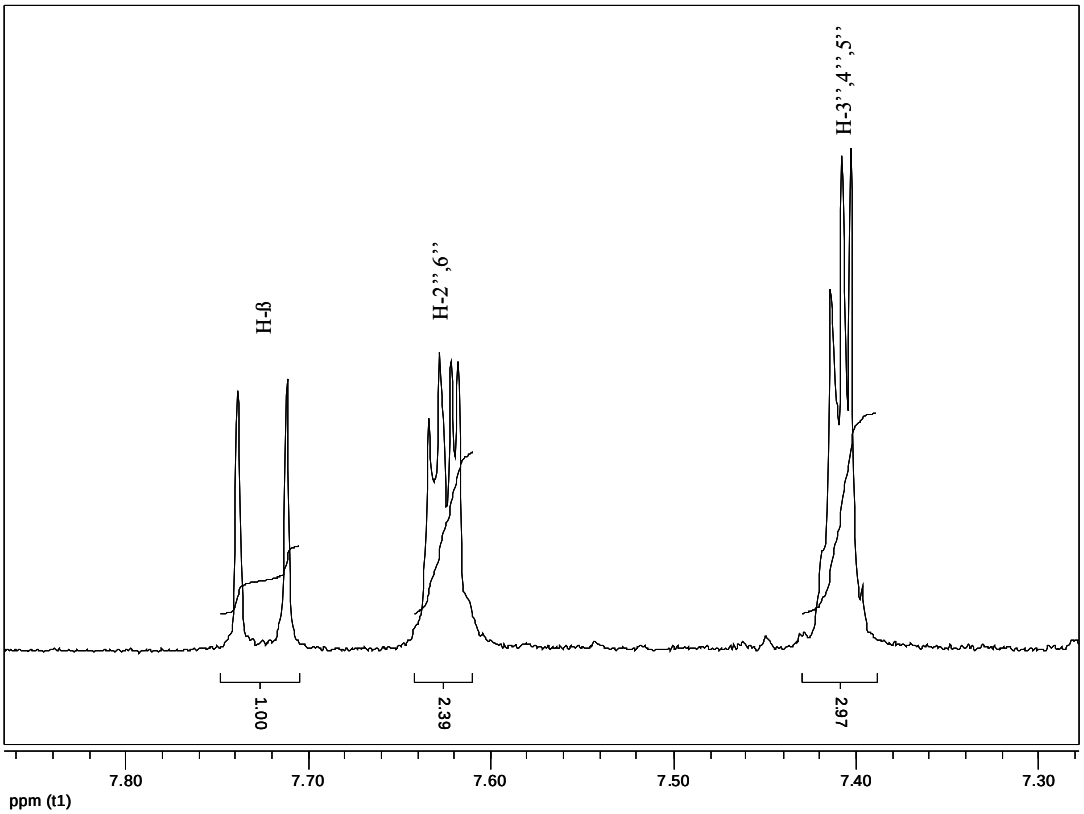


Figure 3.3.1.6 - ^{1}H-NMR spectrum between δ 7.85 and 7.25 of peak 9, showing integrals of the signals corresponding to H-2",6" and H-3",4",5" in relation to H-β.

Of the compounds' 24 C atoms, six were attributed to a β-D-glucopyranosyl unit and nine ascribed to an (*E*)-cinnamoyl moiety. The remaining C-resonances that could be deduced from the HSQC spectrum indicated that compound 9 has an iridoid skeleton (cyclopentapyran ring system) with nine C-atoms, one of which is a quaternary carbon with no protons attached. For the analysis of the iridoid skeleton, the starting point was the resonance at δ 6.36 (dd, *J*= 6.0, 1.7 Hz) which was assigned to H-3. The COSY spectrum (Figure 3.3.1.7) showed correlations with the signals located at δ 5.08 (m) and δ 2.30 (m), which were therefore assigned to H-4 and H-5, respectively. The H-5 signal exhibited correlations with protons located at δ 2.66 (dd, *J*= 9.7, 7.7) and δ 3.96 (dd, *J*= 8.2, 1.0) which were then assigned to H-9 and H-6, respectively. The signal located at δ 3.50 (d, *J*= 1.0) was assigned to H-7 through its correlation with the signal located at δ 3.94, previously attributed to H-6, and the last signal located at δ 5.08 (d, *J*= 9.8 Hz) was attributed to H-1 through its correlation with the H-9 proton. The H-10 protons were easily assigned to the AB spin system located at δ 4.26 and δ 5.03 with a large coupling constant (*J* =12.6 Hz).

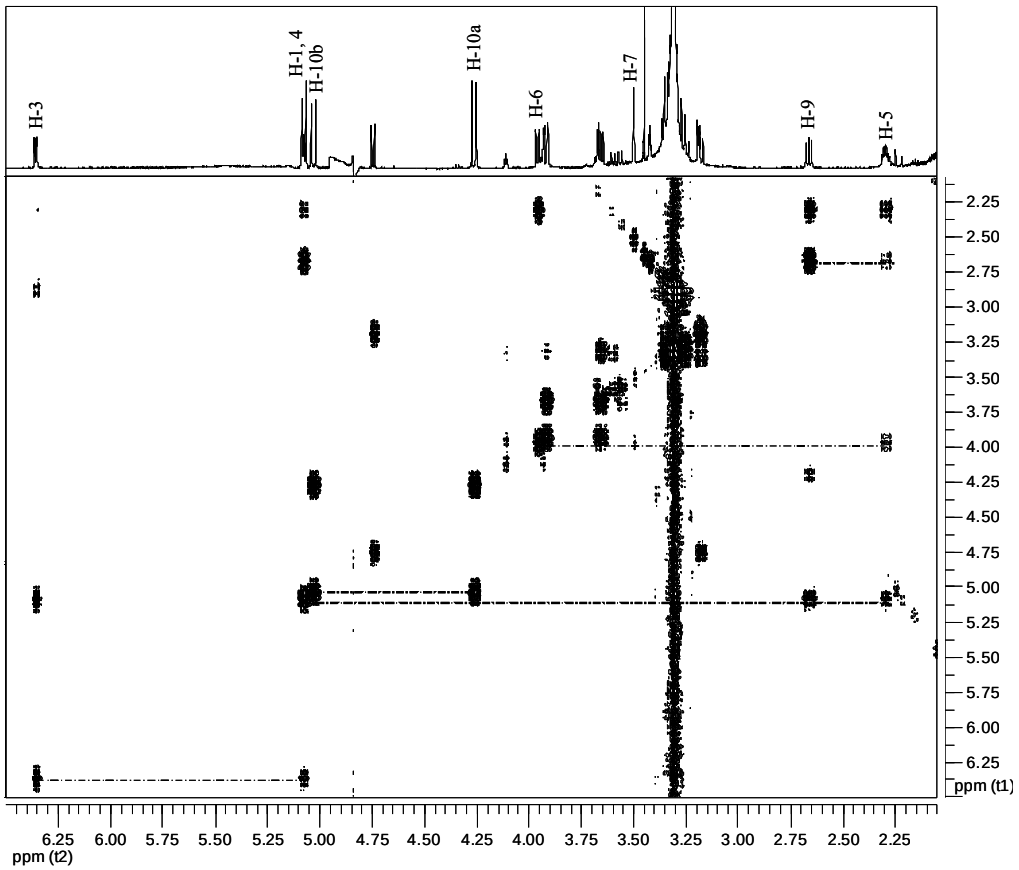


Figure 3.3.1.7 - Assignment of the protons of the iridoid skeleton of peak 1 with aid of the COSY spectrum.

The described unit comprising the iridoid core and the glucopyranose moiety is commonly designated catalpol, and the site of esterification of the cinnamoyl moiety was determined to be at the C-10 position of the iridoid glucoside due to the relatively downfield shifts of the H-10 protons, compared to the regular catalpol unit (Çaliş et al., 1993). The compound was therefore assigned as 10-*O*-(*E*)-cinnamoyl-catalpol, scutelarioside I or globularin. The resonances of the protonated C-atoms of peak 9 deduced from the HSQC spectrum (Figure 3.3.1.8) together with the data obtained from the ^{1}H-NMR spectrum were in good agreement with the previously published ^{13}C and ^{1}H-NMR data (Faure et al., 1987; Boros and Stermitz, 1990).

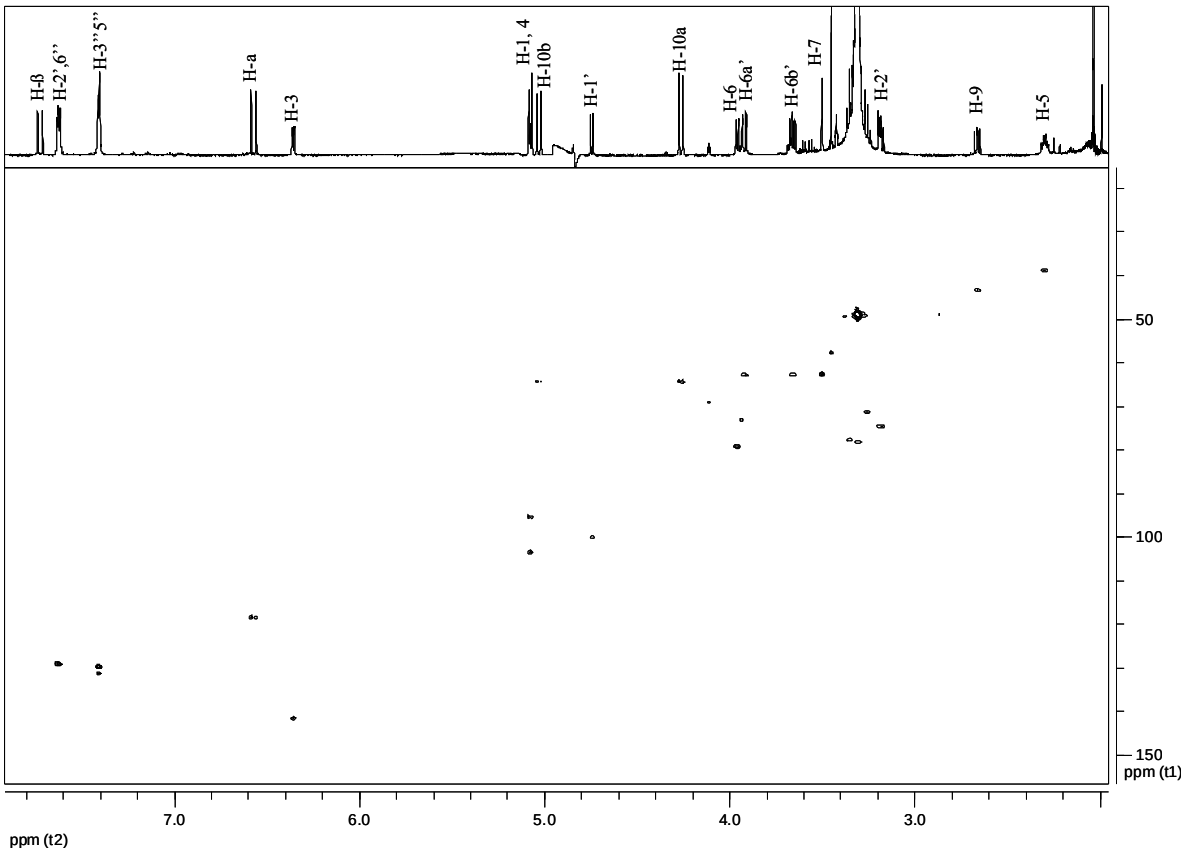


Figure 3.3.1.8 - HSQC spectrum obtained in HPLC-SPE-NMR experiment for peak 9.

From the ESI-MS experiments it could be deduced that peak 4 is a mixture of two co-eluting compounds. The spectrum shows one major peak at *m/z* 507 (compound 4) and one minor peak at *m/z* 523 (compound 4') (Table 3.3.1.1). Because the two compounds were trapped in different amounts their signals in the ^{1}H-NMR spectrum can be distinguished based on their relative intensities. The structure of compound 4 will be discussed first because its signals are more prominent making its assignment easier. The ^{1}H-NMR data of compound 4 quickly reveals that it bears great similarities with compound 9, showing only differences in the aromatic region (Table 3.3.1.2). The two signals at δ 7.63 and 7.41 belonging to the cinnamoyl moiety of compound 9 were replaced by two signals at δ 6.80 (d, J= 8.5 Hz) and δ 7.49 (d, J= 8.5 Hz), corresponding to two protons each. These signals showed an AA'XX' spin system consistent with a 1,4-disubstituted aromatic ring, which together with the olefinic protons at δ 6.37 (d, J= 15.9 Hz) and δ 7.65 (d, J= 15.9 Hz) are indicative of a 4-coumaroyl group. The ESI-MS experiments conducted in negative mode revealed a peak at *m/z* 507, from which a molecular formula of $C_{24}H_{28}O_{12}$ could be deduced, suggesting that it has one extra hydroxyl group than compound 9, corroborating the results obtained from the NMR

experiments. Therefore, compound 4 could be assigned as the coumaroyl analogue of compound 9, 10-*O*-(*E*)-coumaroyl-catalpol or scutelarioside II (Figure 3.3.1.5). The HSQC spectrum provided the ^{13}C chemical shifts of all protonated carbons of the molecule, and comparison with literature data confirmed its structure (Çaliş et al., 1993).

The difference of *m/z* between the molecular ions of compounds 4 and 4' suggests that compound 4' is substituted by one extra hydroxyl group (*Δ* +16 amu). Most of the signals of compound 4' are overlapped by the signals of compound 4 but some signals show slight deviations. By superimposing the HSQC spectra of compound 4 and 9, which are practically identical except for the substituting group at the C-10 position, it was possible to identify the C atoms belonging to compound 4' (Figure 3.3.1.9).

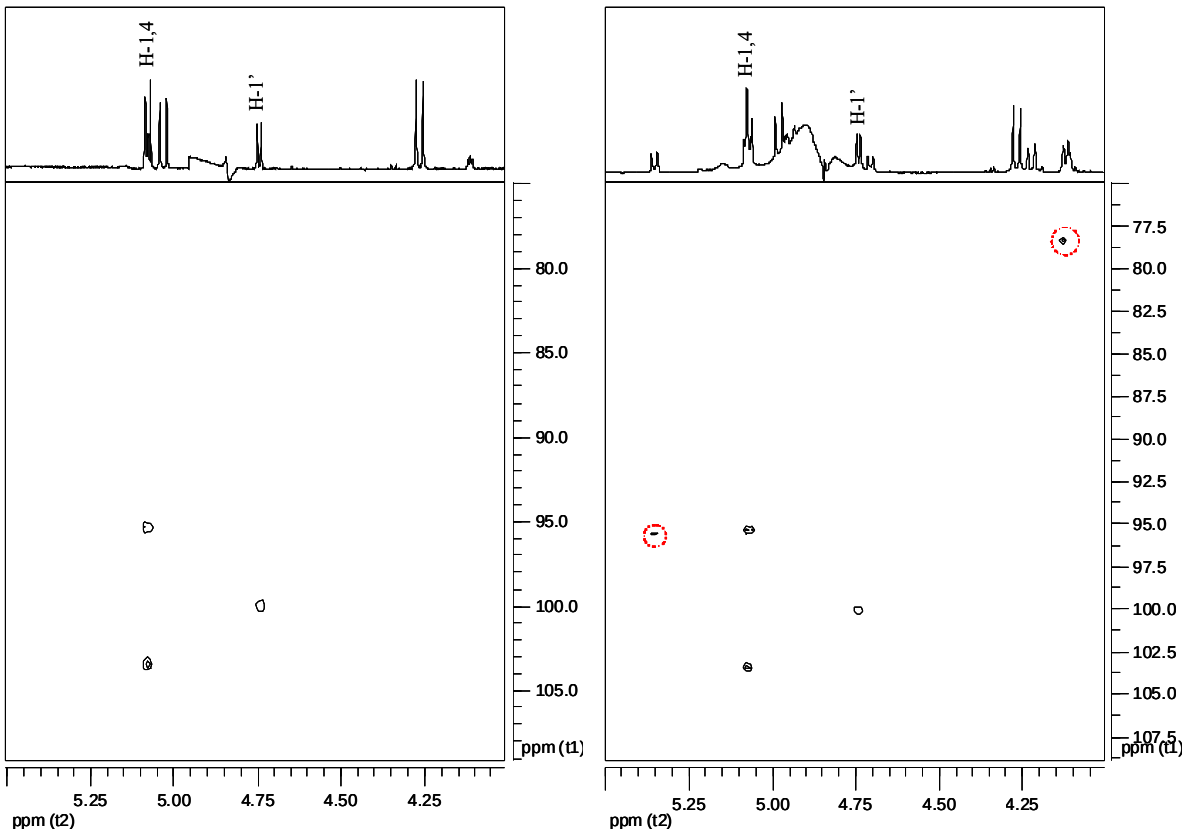


Figure 3.3.1.9 - HSQC spectra of compound 9 (left) and compound 4 (right). The highlighted signals show the additional protonated carbons corresponding to compound 4'.

Using this approach, it could be seen that the differences between compound 4 and 4' resided in the iridoid skeleton, as the coumaroyl and the glucopyranose moiety remained unchanged, except for the anomeric proton of compound 4' which showed a

slight deviation (Table 3.3.1.2). The H-1 proton of compound 4' on the other hand, shifted somewhat downfield ($\Delta\delta$ +0.27) and could be assigned due to its characteristic splitting pattern (d, J= 9.0 Hz) and due to the corresponding C-ressonance (δ 95.5) which is identical to that of compound 4' (Table 3.3.1.2). From the H-1 proton it was possible to assign H-9 at δ 2.66 (m) through a strong cross peak in the COSY spectrum. The large downfield shift of C-9 ($\Delta\delta$ +7.7) of compound 4' in comparison to compound 4 suggests that the substitution with the extra hydroxyl group is at one of the adjacent positions. The HSQC spectrum showed another distinct signal at δ_C 78.5 (Figure 3.3.1.9), connected to a broad singlet at δ_H 4.13. The ROESY spectrum shows that this proton is coupled to another broad singlet at δ_H 3.61, which was in turn connected to a carbon atom at δ_C 63.5. The ROESY spectrum also shows that these protons are coupled in the same way as the H-6 and H-7 in compound 4 and 9. Therefore, the peaks in the ^{1}H-NMR spectrum of compound 4' at δ 4.13 and δ 3.61 were assigned to H-6 and H-7, respectively. The same connectivity could not be observed in the COSY spectrum, possibly due to its lower resolution.

The ^{1}H-NMR spectrum of peak 4 showed an additional signal at δ 6.39 (d, J= 6.0 Hz) that did not belong to the structure of the major compound and was assigned as H-3 of compound 4', due to its similar C-resonances and identical splitting pattern in comparison with H-3 of compound 4 (Table 3.3.1.2). The proton assigned as H-3 was correlated with a peak resonating at δ 4.96, which was assigned as H-4. The H-4 of compound 4' could not be correlated with any other proton besides H-3, which does not happen for compound 4. This observation, together with the fact that the H-6 of compound 4' lost its large coupling constant and the large downfield shifts of C-4 ($\Delta\delta$ -3.8) and C-9 ($\Delta\delta$ -7.7), suggests that compound 4' is the 5-OH derivative of compound 4. The proposed structure has not been previously identified and therefore must be carefully assigned using complete ^{1}H and ^{13}C NMR spectra, especially as these 5-OH derivatives have not been found in this plant family. To assign the structure unambiguously the compound will have to be isolated using preparative techniques, which will be a difficult task as compounds 4 and 4' have exactly the same retention time.

With aid of the HSQC spectrum all the protons and carbons of the sugar unit of peak 1 could be assigned (Table 3.3.1.2), indicating the presence of a glucopyranosyl moiety in

compound 1. The coupling constant of the anomeric proton at δ 4.68 (J = 7.9 Hz) indicated the β-configuration of the glucopyranose unit. The ^{1}H-NMR spectrum also showed a doublet located at δ 5.46 (d, J= 4.2 Hz) which is a characteristic signal for H-1 of an iridoid molecule (Boros and Stermitz, 1990; Feng et al., 2006). The COSY spectrum showed that the latter was coupled to a proton at δ 2.22 (dd, J= 9.2, 4.1 Hz), assigned as H-9, which in turn was coupled to a multiplet resonating at δ 3.15, assigned as H-5. In the TOCSY spectrum, a very weak signal could be seen between H-5 and H-9 and a very characteristic singlet resonating at δ 7.41, which was assigned as H-3. The downfield position of this proton suggests that it is located between the oxygen at position 2 of the iridoid skeleton and another electronegative group at C-4, causing a deshielding effect. From the H-5 proton it was possible to assign the remaining signals of the protons connected to the iridoid skeleton with aid of the TOCSY spectrum. The signals at δ 2.30 (td, J= 16, 14, 7.5 Hz) and 1.47 (td, J= 14, 14, 7.5 Hz) were assigned to H6a and H6b, respectively, and the signal at δ 1.72 (t, J= 7.5 Hz) to the two H-7 protons. From the splitting patterns of H-9 and H-7 it could be deduced that C-8 was a unprotonated carbon, and therefore the strong signal located at δ 1.33 (s) belonging to a methyl group, was assigned to the C-10 position. From the molecular formula deduced by the ESI-MS experiments ($C_{16}H_{24}O_5$, Table 3.3.1.1), six C and 5 O atoms were assigned to the glucopyranose moiety and nine C and two O atoms to the iridoid core structure. The remaining C and three O atoms belong to a hydroxyl (OH) and carboxyl (COOH) substituent groups, which are connected to the two unprotonated C atoms (C-4 and C-8) of peak 1. The carboxyl group was assigned to the C-4 position, due to the downfield resonance of H-3 and the hydroxyl group to the C-8 position. From the information deduced from the NMR and MS data it was possible to identify peak 1 as mussaenosidic acid (Damtoft et al., 1985; Boros and Stermitz, 1990).

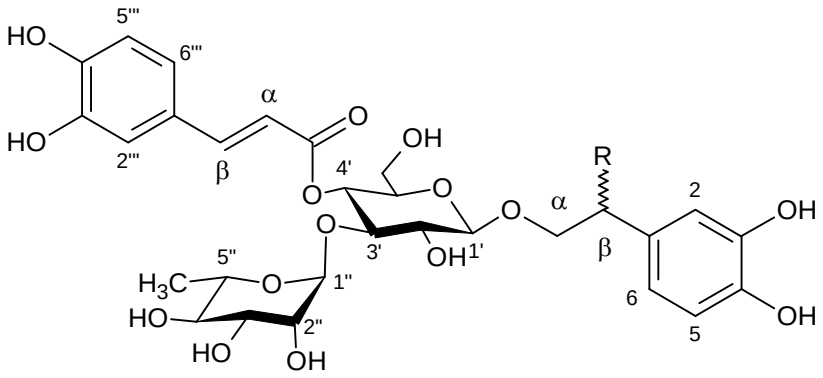
3.3.1.3.2.2. Phenylethanoid glycosides

From the obtained UV spectra it was possible to conclude that peaks 2, 3, 5, 6 and 7 (Table 3.3.1.1) were structurally related and analysis of NMR data (Table 3.3.1.3) revealed that they belong to the family of the phenylethanoid glycosides. The methanol extract of *P. lusitanica* comprised acteoside (peak 7) and the two pairs of enantiomers R and S campneoside I (peaks 2, 3), and R and S campneoside II (peaks 5, 6). The chemical structures of the identified compounds are shown in Figure 3.3.1.10.

Table 3.3.1.3 - ^{1}H and ^{13}C NMR data of the phenylethanoid glycosides obtained by HPLC-SPE-NMR from the methanol extract of *P. lusitanica*.

	2	**3**	**5**	**6**	**7**	
Position	δ_H*	δ_H*	δ_H*	δ_H*	δ_H*	δ_C**
Aglycone						
1						131.4
2	6.88 (1H, d, 1.5)	6.84 (1H, d, 1.5)	6.79 (1H, d, 2.0)	***	6.70 (1H, d, 1.9)	116.9
3						146.1
4						144.3
5	6.80 (1H, d, 8.0)	6.74 (1H, d, 8.3)	6.76 (1H, d, 8.1)	6.76 (1H, d, 8.2)	6.68 (1H, d, 8.0)	116.1
6	***	6.71 (1H, dd, 8.0, 1.5)	6.68 (1H, dd, 8.1, 2.0)	6.66 (1H, dd, 7.8, 1.5)	6.57 (1H, dd, 8.0 1.9)	121.0
*α*a	3.84 (1H, dd , 9.4. 2.8)	3.98 (1H, dd, 10.2, 2.5)	4.02 (1H, dd, 11.0, 8.2)	3.88 (1H, dd, 10.9, 2.8)	4.05 (1H, dt, 8.1, 6.8)	72.0
*α*b	3.70 (1H, dd, 9.4, 3.1)	3.68 (1H, dd, 9.4, 2.9)	3.68 (1H, dd, 9.5, 3.1)	3.68 (1H, dd, 9.5, 3.2)	3.74 (1H, dt, 8.6, 7.2)	
β	4.76 ***	4.75 (1H, dd, 9.1, 2.0)	4.35 (1H, m)	4.37 (1H, m)	2.80 (2H, m)	36.4
OCH_3			3.25 (3H, s)	3.25 (3H, s)		
Glucosyl						
1'	4.40 (1H, d, 7.9)	4.41 (1H, d, 7.8)	4.39 (1H, d, 7.9)	4.42 (1H, d, 7.9)	4.39 (1H, d, 7.9)	104.0
2'	***	***	***	***	3.39 (1H, dd, 9.2, 7.9)	76.0
3'	3.86 (1H, t, 9.4)	3.85 (1H, t, 9.2)	3.82 (1H, t, 9.2)	3.83 (1H, t, 9.1)	3.82 (1H, t, 9.2)	81.4
4'	4.96 (1H, t, 9.3)	4.94 (1H, t, 9.2)	4.92 (1H, t, 9.4)	4.92 (1H, t, 9.1)	4.92 (1H, t, 9.5)	70.2
5'	***	***	3.53***	3.53***	3.52***	76.0
6'	***	***	3.62***	3.62***	3.62***	62.0
			3.52***	3.52***	3.53***	
Rhamnosyl						
1"	5.22 (1H, d, 1.0)	5.22 (1H, d, 1.2)	5.20 (1H, d, 1.6)	5.21 (1H, d, 1.2)	5.19 (1H, d, 1.2)	102.7
2"	***	3.90 (1H, dd, 12.9, 1.4)	3.92 (1H, dd, 3.3, 1.6)	3.92***	3.92 (1H, dd 2.8, 1.9)	71.7
3"	***	***	***	***	3.58***	71.7
4"	***	***	***	***	3.29 (1H, t, 9.5)	73.7
5"	***	***	3.54***	3.54***	3.56***	70.2
6"	1.02 (3H, d, 6.6)	1.10 (3H, d, 6.0)	1.09 (3H, d, 6.2)	1.09 (3H, d, 6.2)	1.09 (3H, d, 6.2)	18.2
Caffeoyl						
1"'						127.6
2"'	7.08 (1H, d, 1.5)	7.05 (1H, d, 1.6)	7.05 (1H, d, 2.0)	7.05 (1H, d, 1.6)	7.05 (1H, d, 1.9)	115.1
3"'						146.8
4"'						149.6
5"'	6.80 (1H, d, 8.4)	6.78 (1H, d, 8.3)	6.78 (1H, d, 8.2)	6.78 (1H, d, 8.2)	6.78 (1H, d, 8.2)	116.4
6"'	6.98 (1H, dd, 8.2, 2.0)	6.96 (1H, dd, 8.3, 1.2)	6.96 (1H, dd, 8.3, 2.0)	6.96 (1H, dd, 8.2, 1.5)	6.96 (1H, dd, 8.2 1.9)	123.0
α"'	6.30 (1H, d, 15.9)	6.27 (1H, d, 15.9)	6.27 (1H, d, 15.9)	6.27 (1H, d, 15.9)	6.27 (1H, d, 15.9)	114.6
β"'	7.62 (1H, d, 15.9)	7.60 (1H, d, 15.9)	7.59 (1H, d, 15.9)	7.59 (1H, d, 15.8)	7.59 (1H, d, 15.9)	147.6
CO						167.9

*Relative to the residual methanol signal set to *δ* 3.31, *δ* values given in ppm; number of protons, multiplicity of signals (s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet) and coupling constants (apparent splittings) given as numerical values in Hz are shown in parenthesis. **^{13}C NMR chemical shifts obtained from HMBC experiments, relative to the resonance of the solvent set to *δ* 49.05. ***Signal/splitting pattern unclear due to overlapping signals.



Acteoside (peak 7): R=H; Campneoside I (peak 2, 3): R=OH; Campneoside II (peak 5,6): R=OCH_3

Figure 3.3.1.10 - Structure of the phenylethanoid glycosides from *P. lusitanica*.

The molecular formula of $C_{29}H_{36}O_{15}$ of peak 7 was deduced from its molecular ion peak in the negative mode ESI-MS spectrum (*m/z* 623; Table 3.3.1.1). The ^{1}H-NMR spectrum of peak 7 showed signals of two aromatic rings, both with coupling patterns corresponding to a 1,2,4-trisubstituted benzene: δ 6.70 (d, *J*= 1.9, H-2), 6.68 (d, *J*= 8.0 Hz, H-5), 6.57 (dd, *J*= 8.0, 1.9 Hz, H-6) and δ 7.05 (d, *J*=1.9 Hz, H-2'''), 6.78 (d, *J*= 8.2 Hz, H-5'''), 6.96 (d, *J*= 8.2, 1.9 Hz, H-6'''). A *trans*-coupled olefinic pair of doublets was observed at δ 6.27 and 7.59 (d, *J*= 15.9 Hz), which together with the aromatic proton resonances indicated the presence of a caffeoyl moiety. In addition, the ^{1}H-NMR spectrum showed signals of two sugar moieties. Characteristic resonances of an anomeric proton appearing at δ 5.19 (d, *J*= 1.2 Hz, H-1") together with a methyl group at δ 1.09 (d, *J*= 6.2, H6") suggested the presence of a rhamnopyranosyl unit, and a second doublet corresponding to an anomeric proton of a glucopyranosyl residue was observed at δ 4.39 (*J*= 7.9 Hz, H-1'). The assignment of all resonances of the rhamnopyranose and the glucopyranose residues (Table 3.3.1.3) was possible from a COSY spectrum. In the upfield region of the ^{1}H-NMR spectrum, characteristic signals of an $-CH_2-CH_2-$ group were observed at δ 2.80 (m, 2H), 3.74 (ddd *J*= 17.0, 8.0, 2.0 Hz) and 4.05 (ddd, *J*= 17.0, 8.0, 2.0 Hz), assigned as H-β, H-αb and H-αa, respectively, which together with the second aromatic spin system form a hydroxytyrosyl unit.

From the amount trapped of peak 7 it was possible to obtain a HMBC spectrum which is extremely helpful as it yields correlations between H and C-atoms through long range couplings, allowing to link fragments of a molecule (Fig. 3.3.1.11). This way, it could

be seen that H-1' of the glucopyranose moiety was correlated with C-*α* of the hydroxytyrosyl group. The HMBC spectrum of peak 7 also indicated the attachment point of the rhamnosyl and the caffeoyl residues to the glucopyranose unit, through the correlations between H-3' and C-1" and H-4' and C-*α*", respectively. Peak 7 was therefore assigned as acteoside (*β*-[3',4'-dihydroxyphenyl]-ethyl-*O*-*α*-L-rhamnopyranosyl[1→3]-*β*-D-[4-*O*-caffeoyl]-glucopyranoside), commonly known as verbascoside. The HMBC also allows to derive chemical shifts of quaternary C-atoms, thus it was possible to assign all ^{13}C and ^{1}H-NMR resonances, which were in agreement with the published data (Wu et al., 2004).

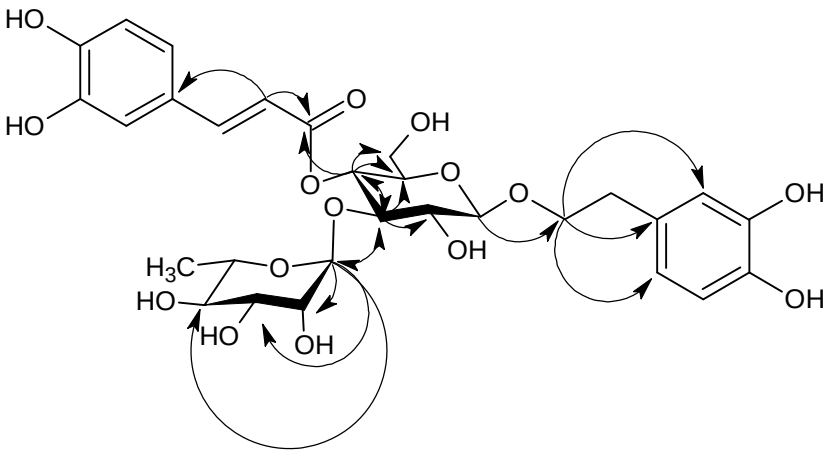


Figure 3.3.1.11 - Selected correlations observed in the HMBC spectrum of peak 7 (H→C).

Peaks 2 and 3 are isomers with a molecular formula of $C_{29}H_{36}O_{16}$, as deduced from the ESI-MS experiments (Table 3.3.1.1). The ^{1}H-NMR spectra show that these two peaks are related to peak 7, showing only differences at the H-*β* and H-*α* positions of the aglycone (hydroxtyrosol unit). Furthermore, the deduced molecular formula suggests that the isomers are substituted by one extra hydroxyl group in comparison to acteoside. The signals belonging to H-*α* and H-*β* of acteoside were replaced by signals at *δ* 4.76, 3.70, 3.84 (peak 2) and *δ* 4.75, 3.98, 3.68 (peak 3) indicating the approximate site of hydroxylation. The S/N obtained for the ^{1}H-NMR spectra of peaks 2 was relatively low and it was not possible to assign all the protons of the molecules, but by comparing the obtained diagnostic information with data in literature it was possible to assign peaks 2 and 3 as R/S *β*-hydroxyacteoside, or R/S campneoside II. Although both isomers were separated in chromatography, it was not possible to distinguish them. It is interesting to

see that campneoside II follows the same fragmentation pattern as acteoside after the loss of the hydroxyl residue (18 amu) giving the precursor ion at *m/z* 623 (Table 3.3.1.1).

The ^{1}H-NMR spectra showed that peaks 5 and 6 are also isomers and related to 7. From the ESI-MS experiments, a molecular formula of $C_{30}H_{38}O_{16}$ was deduced, suggesting that they are substituted by a methoxy group (OCH_3) in comparison to acteoside. This is supported by the strong singlet appearing at δ 3.35 (3H) in the ^{1}H-NMR spectra of peak 5 and 6. From the obtained diagnostic ^{1}H-NMR information it was possible to assign peaks 5 and 6 as R/S β-methoxyacteoside or R/S campneoside I. Once more, it was not possible to distinguish both isomers. However, this might be achieved if larger amounts of material are trapped and better NMR spectra are obtained, because the two pairs of isomers were separated in chromatography using the optimized gradient, which was not the case in a previous work (Wu et al., 2004).

3.3.1.4. Biological and taxonomical relevance

^{1}H-NMR spectroscopy is a very non-selective technique, as it detects any proton in a sample regardless of its structure, thus the relative amounts of the components of an extract can be deduced to a good approximation by comparing the intensity of a specific signal. Table 3.3.1.4 shows the S/N of the identified compounds in the methanol extract of *P. lusitanica* compounds. Assuming that the SPE efficiency was the same for all peaks, it is possible to infer that acteoside (S/N = 226.2) is the most abundant in the *P. lusitanica* extract, followed closely by mussaenosidic acid (S/N = 211.5). The relative amount of these two compounds is similar, suggesting that the pathways for these two types of compounds in *P. lusitanica* are of equal importance. It has been suggested that the function of phenylethanoid glycosides in plants is resistance to, or protection from, fungal or viral attacks (Jiménez and Riguera, 1994).

The benefit of iridoid glucosides for the plant is not clear, although the bitterness of many of these compounds is considered to be a deterrent for herbivores. Konno et al. (1999) have also demonstrated that the iridoid aucubin is a strong protein denaturant when hydrolyzed by the plant's enzymes. The authors suggest that the glycosilated form of the iridoid is compartmentalized in the cells of the intact plant, but when the tissue is

Table 3.3.1.4. S/N of the secondary metabolites of the *P. lusitanica* methanol extract obtained in HPLC-SPE-NMR experiments.

Compound	S/N*
Iridoid glucosides	
Mussaenosidic acid (peak 1)	211.5
Globularin (peak 9)	79.6
Unknown (peak 4')	16.0
Scutelarioside II (peak 4)	47.3
Phenylethanoid glycosides	
Acteoside (peak 7)	226.2
R or S Campneoside I (peak 5)	14.4
R or S Campneoside I (peak 6)	16.8
R or S Campneoside II (peak 2)	3.4
R or S Campneoside II (peak 3)	11.9

* Signals determined for the respective anomeric protons under identical acquisition conditions, obtained for 4 cumulative trappings and injection volume of 20 μL (50 mg/mL).

damaged by herbivores, the compound is hydrolyzed and reacts irreversibly with the proteins and renders these more or less indigestible, contributing to the plants defence mechanism. This suggests that iridoid glucosides may play an important role in the defense mechanism against herbivores and that further investigation is needed to better understand the biological function of these compounds. The biosynthetic pathway of catalpol, the iridoid center core of globularin and scutelarioside, has been previously determined by experiments using deuterium-labeled precursors (Damtoft et al., 1994). It was shown that mussaenosidic acid is a precursor of catalpol, therefore it can be assumed that globularin and scutelarioside II are also derived from mussaenosidic acid.

Table 3.3.1.4 also shows that the concentration of both isomers of campneoside I is similar (S/N = 14.4 and 16.8). It is interesting to observe that the isomer of campneoside II that is eluted first (peak 2) has a lower S/N (3.4) than the other isomer (11.9). However, it is assumable that the isomers are present in similar amounts because the UV intensities of both isomers of campneoside II are similar and they have the same chromophores. This could mean that peak 2 was extracted less efficiently in the SPE cartridge. It is likely that these isomers are derived from acteoside after hydroxylation, to give campneoside II and posterior methylation, to give campneoside I. The fact that both pairs of isomers were identified in *P. lusitanica* in similar amounts suggests that

the reactions involved in the hydroxylation and methylation are chemical processes, or reactions without a stereochemical preference, in case we are dealing with enzymatic processes.

Phenylethanoid glycosides and iridoid glucosides occur simultaneously in several plant families (Bignoniaceae, Labitaeae, Oleaceae) and are of taxonomic and genetic importance. The genera *Digitalis* (Taskova et al., 2005), *Veronica* (Jensen et al., 2005; Pedersen et al., 2007) and *Plantago* (Rønsted et al., 2000; 2003) of the Plantaginaceae family, and *Rehmannia* (Albach et al., 2007) of the Scrophulariaceae family, have been extensively studied and important taxonomical information has been inferred from their content in iridoid and phenylethanoid glycosides. Globularin and scutellarioside II were found in *P. vulgaris* and mussaenosidic acid in *Utricularia australis* (Damtoft et al., 1985), both members of the Lentibulariaceae family, which is in good agreement with the metabolites found in *P. lusitanica*. Within the *Pinguicula* genus, acteoside has only been identified in *P. moranensis* (Damtoft et al., 1994), possibly due to the use of less sensitive analytical techniques. *P. vulgaris* and *P. moranensis* belong to the subgenus Pinguicula, while *P. lusitanica* was included in the Isoloba subgenus, according to the classification of Casper (1966). Also, an analysis of the *trn*K intron (Cieslak et al., 2005) within species of the *Pinguicula* genus confirmed that *P. lusitanica* is genetically considerably distinct from *P. vulgaris* and *P. moranensis* (Figure 3.3.1.12). The fact that these species have the same compounds in their constitution suggests that the two main chemical pathways of iridoid and phenylethanoid synthesis are rather conserved in the *Pinguicula* genus.

3.3.2. *D. intermedia*

3.3.2.1. HPLC gradient optimization and SPE trappings

In vitro produced plant material of *D. intermedia* was extracted with *n*-hexane, water and methanol. The prepared extracts vary considerably in composition and therefore a specific gradient was optimized for each one. The obtained HPLC-ESI-MS chromatograms and the respective gradients are depicted in Fig. 3.3.2.1. The chromatogram of the *n*-hexane extract shows that it is composed by one single major

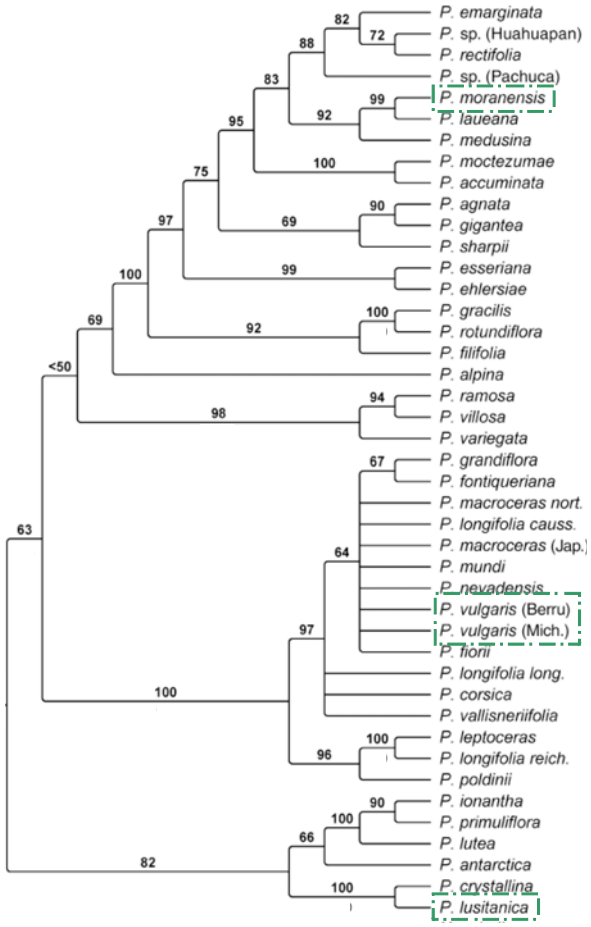


Figure 3.3.1.12 - Phylogenetic tree of the *Pinguicula* genus, based on the analysis of the *trnK* intron [adapted from Cieslak et al., (2005)]. The highlighted species have been previously analyzed for their secondary metabolite profile.

compound and therefore it was not necessary to perform SPE trappings. The compound has a long retention time meaning it is relatively apolar and was dissolved in deuterated chloroform and analyzed directly in a NMR tube. The HPLC chromatogram of the methanol extract showed a large amount of metabolites with a satisfactory separation. The ten most intense peaks were selected for SPE trapping and the obtained MS data is displayed in Table 3.3.2.1.

The chromatogram of the water extract showed a low content in secondary metabolites with 3 major peaks. The most intense peak at t_R= 22.4 min was also extracted in the methanol extract and selected for SPE-NMR analysis and was therefore not further investigated. The peak at t_R = 18.8 min showed a pseudo-molecular ion peak $[M-H]^-$ at

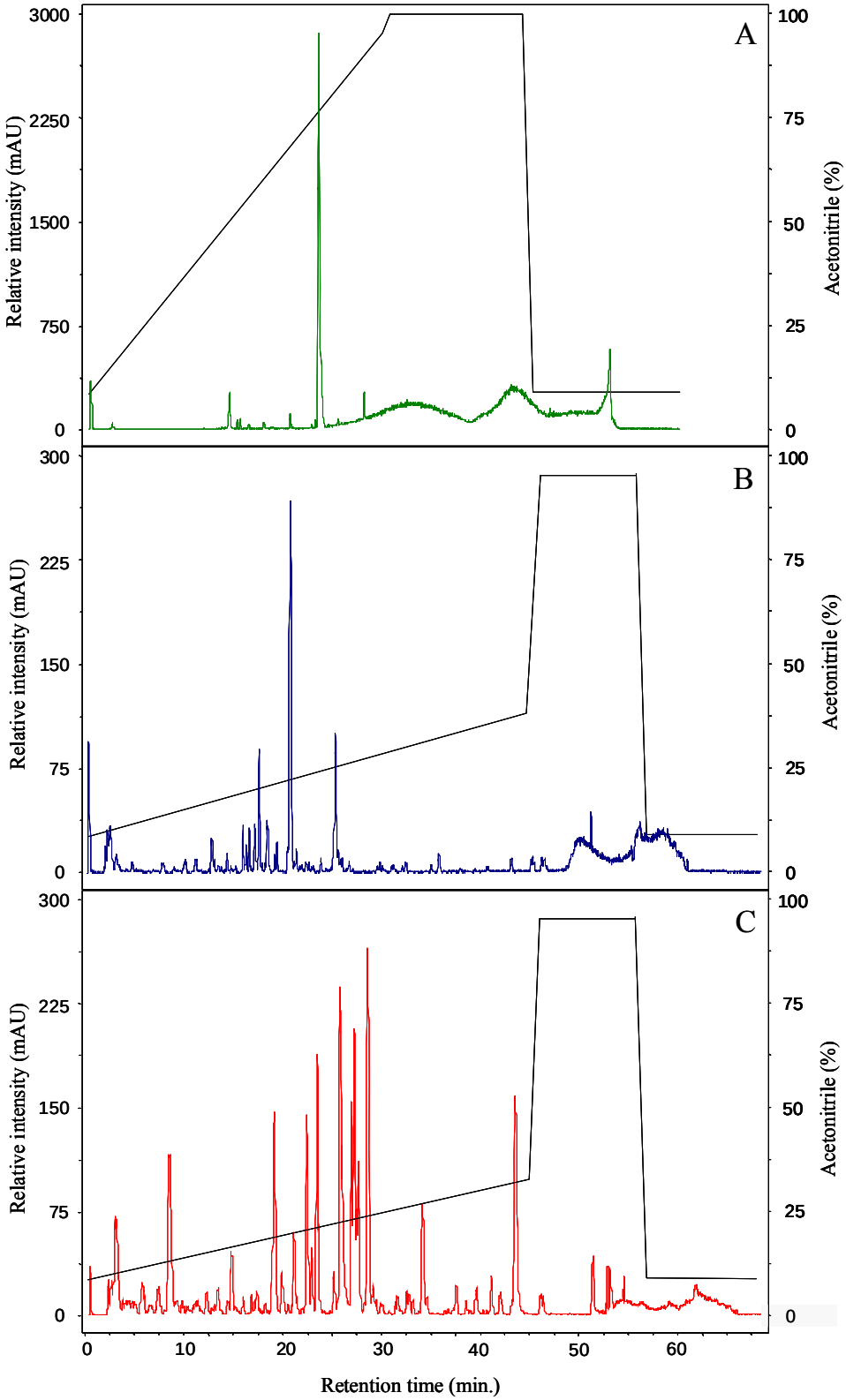


Fig. 3.3.2.1 - HPLC-ESI-MS chromatograms measured in negative mode of the *n*-hexane (A), water (B) and methanol (C) extracts of *D. intermedia* (150 × 2.1 mm i.d. C18 column). Gradient elution profile is shown as percent of acetonitrile in H_2O.

Table 3.3.2.1 - ESI-MS data of the major peaks of the *n*-hexane, water and methanol extracts of *D. intermedia*.

Peak	t_R (min)	MS negative mode		MS positive mode $[M+H]^+$ (*m/z*, amu)	Possible Molecular Formula
		$[M-H]^-$ (*m/z*, mau)	Fragments (*m/z*, amu)		
***n*-hexane**					
1	23.4	187.0	175.0, 159.0, 131.0	189.1	$C_{11}H_8O_3$
water					
1	18.8	385.2	223.1, 205.1, 153.1	387.2	$C_{19}H_{30}O_8$
2	22.4	371.1	249.1	373.1	$C_{16}H_{20}O_{10}$
3	27.3	401.2	301.0	403.2	$C_{19}H_{30}O_9$
methanol					
1	8.3	513.2	351.1, 188.0	515.2	$C_{23}H30O_{13}$
2	18.9	371.1	249.1	373.1	$C_{16}H_{20}O_{10}$
3	22.2	479.1	316.0	481.1	$C_{21}H_{20}O_{13}$
4	23.3	631.1	317.0	633.1	$C_{28}H_{24}O_{17}$
5	25.6	301.0	257.0, 229.0, 173.1	303.0	$C_{14}H_6O_8$
6	26.8	463.1	301.0	465.1	$C_{21}H_{20}O_{12}$
7	27.1	491.1	476.1, 328.0, 313.0	493.1	$C_{22}H_{20}O_{13}$
7'	27.1	463.1	301.0	465.1	$C_{23}H_{24}O_{12}$
8	28.5	615.1	301.0	617.1	$C_{28}H_{23}O_{16}$
9	34.0	315.0	300.0	317.0	$C_{15}H_8O_8$
10	43.5	329.0	314.0, 299.0, 271.0	331.0	$C_{16}H_{10}O_8$

m/z 385 in the negative ion ESI-MS spectrum, for which a possible molecular formula of $C_{19}H_{30}O_8$ was deduced. A search in natural product libraries (DNP, 2007) gave several hits for glycosylated dihydroxymegastigmadiene isomers (Figure 3.3.2.2). This possibility is supported to some extent by the obtained fragmentation in the ESI-MS spectra, which showed fragments at *m/z* 223 and 205 suggesting the successive loss of one hexosyl moiety ($[(M-H)-162]^-$) and a hydroxyl unit ($[(M-H)-162-18]^-$), respectively. The possible molecular formula ($C_{19}H_{30}O_9$) deduced for peak 3 (t_R = 27.3 min) of the water extract also gave hits for a megastigmadiene derivative, substituted with one extra hydroxyl group in comparison to peak 1, enhancing the probability that these compounds might be produced by *D. intermedia*. However, these results are very inconclusive and taking into account that they have not been previously identified in plants of the Droseraceae family, further investigation is needed to confirm the presence

of these compounds in the metabolome of *D. intermedia*. Due to the low levels of these metabolites in the water extract it was not possible to analyze them by SPE-NMR.

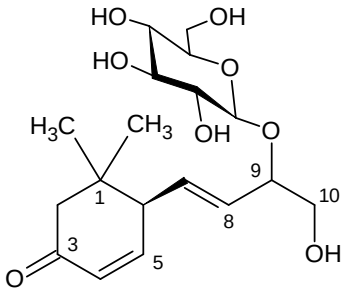


Figure 3.3.2.2 - Example of dihydroxymegastigamadiene derivative: (6R)-9,10-dihydroxy-4,7-megastigmadien-3-one-9-*O*-*β*-D-glucopyranoside (Saleem et al., 2006).

3.3.2.2. Direct NMR analysis of the *n*-hexane extract

The negative-ion ESI-MS spectrum showed a pseudo-molecular ion peak $[M-H]^-$ at *m/z* 187 corresponding to the molecular formula $C_{11}H_8O_3$. Because the extract was very concentrated and relatively clean a ^{13}C-NMR spectrum could be obtained. The spectrum exhibited 8 carbon signals in the range δ_C 115.1 - 161.2 and two carbon signals resonating further downfield at δ_C 161.2 and δ_C 190.3 ascribable to a naphthalenedione ring and an additional signal resonating upfield at δ_C 16.6 consistent with a methyl group (Table 3.3.2.2). The carbons of the naphthalenedione ring bore four directly attached hydrogens according to the obtained HSQC. Also a characteristic signal of a phenolic hydroxyl group could be seen at δ 11.97 in the ^{1}H-NMR spectrum, leaving two oxygen atoms belonging to two carbonyl groups to be assigned. The described structure is consistent with plumbagin (5-hydroxy-2-methyl-1, 4-naphthalenedione or 5-hydroxy-2-methyl-1,4-naphthoquinone; Figure 3.3.2.3) (Sankaram et al., 1986) which is considered a taxonomic marker for the family Droseraceae (Culham and Gornall, 1994). The connectivities shown in the HMBC spectrum made it possible to assign the entire molecule (Table 3.3.2.2; Figure 3.3.2.4).

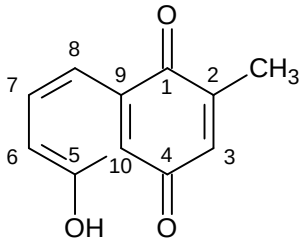


Figure 3.3.2.3 - Structure of plumbagin.

The coupling constants of the protons belonging to the methyl group at δ 2.19 (3H, d, *J*= 1.5 Hz) and the proton at δ 6.80 (*J*= 1.5 Hz) indicated that they are in the same spin system and therefore the aromatic proton could be assigned as H-3. From the H-3 proton connectivities could be seen with signals at δ_C 16.6, 115.1, 149.6, 161.2 and 184.8 in the HMBC, which could be assigned as CH_3, C-10, C-2, C-5 and C-1, respectively. From the ^{1}H-NMR spectrum it could be seen that the remaining aromatic protons at δ 7.25 (dd, *J*= 8.2, 1.4 Hz), δ 7.60 (dd, *J*= 8.2, 7.5 Hz) and δ 7.63 (dd, *J*= 7.5, 1.4 Hz) are coupled forming an ABC spin system. The signal at δ 7.60 was assigned as H-7, the signal at δ 7.63 as H-8 and the signal at 7.25 as H-6 due to its relative upfield position.

Table 3.3.2.2 - ^{1}H and ^{13}C NMR data of *D. intermedia n*-hexane extract in (in $CDCl_3$).

Position	δ_H*	δ_C**	**HMBC***
1		184.8	
2		149.6	
3	6.80 (1H, d, 1.5)	135.4	C-1, C-2, C-5, C-10, CH_3
4		190.3	
5		161.2	
6	7.25 (1H, dd, 8.2, 1.4)	124.2	C-4, C-5, C-8, C-9, C-10
7	7.60 (1H, dd, 8.2, 7.5)	136.0	C-5, C-6, C-8, C-9
8	7.63 (1H, dd, 7.5, 1.4)	119.3	C-1, C-4, C-6, C-7, C-10
9		132.1	
10		115.1	
CH_3	2.19 (3H, d, 1.5)	16.6	C-1, C-2, C-3, C-4

*Relative to the residual methanol signal set to δ 3.31, δ values given in ppm; number of protons, multiplicity of signals (d, doublet; dd, double doublet) and coupling constants (apparent splittings) given as numerical values in Hz are shown in parenthesis. **^{13}C NMR chemical shifts obtained from HMBC experiments, relative to the resonance of the solvent set to δ 77.00. ***Correlations observed in the HMBC spectrum (H→C).

These assignments were supported by the HMBC spectrum which showed connectivities between H-6 and C-5, C-10 and signals at δ_C 119.3, 132.1 and 190.3 corresponding to C-8, C-9, and C-4, respectively; and between H-8 and C-1, C-4 and C-10 and signals at δ_C 124.2 and 136.0, corresponding to C-6 and C-7, respectively.

The ^{13}C and ^{1}H NMR spectra depicted in Fig. 3.3.2.4 also show that no other peaks belonging to contaminating compounds can be seen, meaning that the SPE procedure is

a very effective and simple method to purify plumbagin from *n*-hexane extracts of *D. intermedia*.

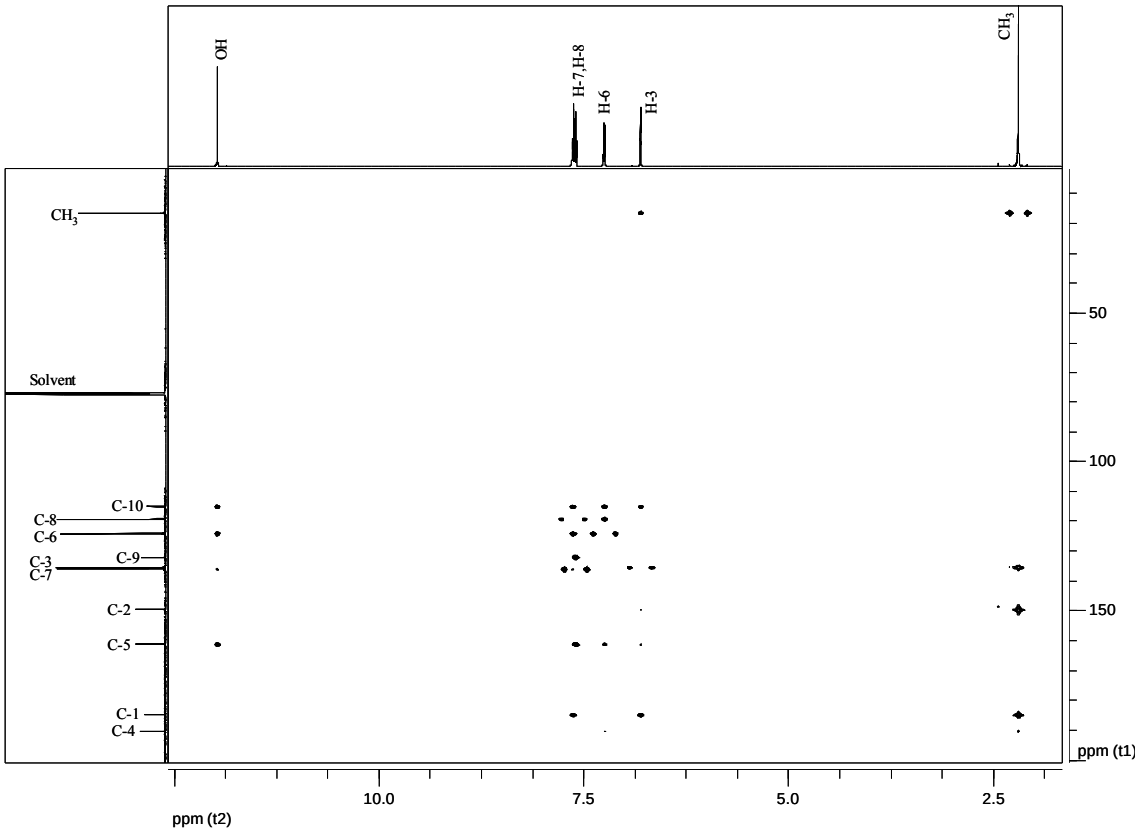


Figure 3.3.2.4 - HMBC spectrum of *n*-hexane extract of *D. intermedia*.

These results also show that *n*-hexane is a very selective extractant for plumbagin as none of the compounds extracted with water or methanol were extracted in significant amounts with *n*-hexane. This can be explained by the fact that plumbagin is relatively apolar and therefore has a higher affinity towards *n*-hexane than the more polar solvents such as water or methanol. Because plumbagin is a compound with a broad range of biological activities and commercial value, a method to extract the naphthoquinone from *D. intermedia* plants is developed and discussed in Chapter 5, as well as its biological and ecological aspects (section 5.1.2.2).

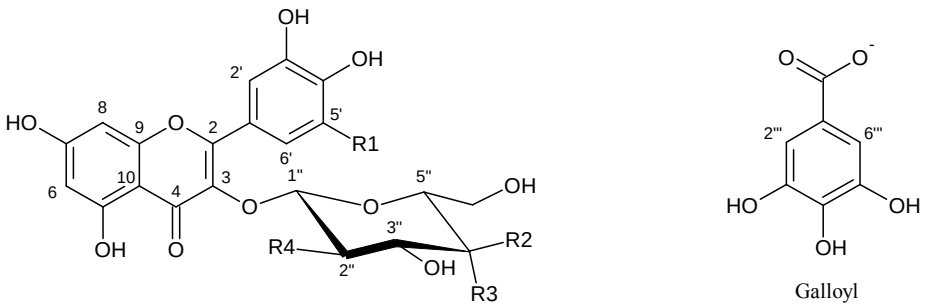
3.3.2.3. HPLC-SPE-NMR analysis of the methanol extract of *D. intermedia*

A preliminary analysis of the NMR spectra of the trapped peaks showed that the methanol extract comprised at least three groups of secondary metabolites. Peaks 3, 4, 6, 9 and the minor compound of peak 7 showed characteristic signals of flavonoid

moieties in the aromatic region of the respective ^{1}H-NMR spectra and will be discussed in further detail in the next section. The ^{1}H-NMR spectra of peaks 5, 9, 10 and the major compound of peak 7 were very characteristic showing few ^{1}H-NMR signals and were assigned as ellagic acid derivatives. For peak 1 it was only possible to obtain 1D ^{1}H-NMR spectra but the diagnostic signals suggest that the compound bears a naphthalene ring and belongs to a different class of natural products. Peak 2 was lost during transfer from the SPE cartridge to the NMR probe, due to an incorrect transfer volume. Because peak 2 was one of the minor peaks of the methanol extract it was not further investigated.

3.3.2.3.1. Flavonoid glucosides

The methanol extract comprised 6 flavonoid glycosides, namely quercetin-3-*O*-galactoside (peak 6), quercetin-3-*O*-glucoside (peak 7'), quercetin-3-*O*-(2"-*O*-galloylgalactoside) (peak 8), myricetin-3-*O*-galactoside (peak 3), myricetin-3-*O*-glucoside (peak 3') and myricetin-3-*O*-(2"-*O*-galloyl)-galactoside (peak 4). The structures of the identified compounds are depicted in Figure 3.3.2.5 and the NMR data that allowed the assignment of these compounds is shown in Table 3.3.2.3.



Peak	R1	R2	R3	R4
3	OH	H	OH	OH
3'	OH	OH	H	OH
4	OH	H	OH	galloyl
6	H	H	OH	OH
7'	H	OH	H	OH
8	H	H	OH	galloyl

Figure 3.3.2.5 - Structures of the flavonoid glucosides of *D. intermedia*.

Table 3.3.2.3 - ^{1}H and ^{13}C NMR data of the flavonoid glucosides obtained by HPLC-SPE-NMR from the methanol extract of *D. intermedia*.

Position	**3**		**3'**		**4**	**6**		**7'**	**8**
	δ_H*	δ_C**	δ_H*	δ_C**	δ_H*	δ_H*	δ_C**	δ_H*	δ_H*
Aglycone									
2		158.6					150.0		
3		135.8					135.8		
4		ND					ND		
5		163.0					163.1		
6	6.21 (1H, d, 2.1)	99.6	6.21***	99.6	6.16 (1H, d, 2.0)	6.21 (1H, d, 2.0)	100.0	6.21 (1H, d, 2.0)	6.17 (1H, d, 2.0)
7		166.0					166.2		
8	6.40 (1H, d, 2.1)	94.3	6.39 (1H, d, 2.1)	94.3	6.32 (1H, d, 2.0)	6.41 (1H, d, 2.0)	95.0	6.40 (1H, d, 2.0)	6.34 (1H, d, 2.0)
9		158.4					158.5		
10		105.6					105.6		
1'		121.6					123.1		
2'	7.38 (1H, s)	109.7	7.30 (1H, s)	109.7	7.27 (1H, s)	7.84 (1H, d, 2.1)	117.9	7.71 (1H, d, 2.0)	7.65 (1H, d, 2.2)
3'		146.5					145.8		
4'		137.8					159.1		
5'		146.5				6.87 (1H, d, 8.5)	116.1	6.87 (1H, d, 8.5)	6.79 (1H, d, 8.5)
6'	7.38 (1H, s)	109.7	7.30 (1H, s)	109.7	7.27 (1H, s)	7.60 (1H, dd, 8.5, 2.1)	123.1	7.60 (1H, dd, ***, 2.0)	7.50 (1H, d, 8.5, 2.2)
Sugar unit									
1"	5.20 (1H, d, 7.8)	105.2	5.26 (1H, d, 7.8)	104.2	5.79 (1H, d, 8.0)	5.17 (1H, d, 7.8)	105.7	5.26 (1H, d, 7.6)	5.69 (1H, d, 8.0)
2"	3.83 (1H, dd, 9.7, 7.9)	73.0	3.52***	75.5	5.45 (1H, dd, 9.7, 8.1)	3.82 (1H, dd, 9.6, 8.0)	73.3	3.45 (1H, t, 7.7)	5.44 (1H, dd, 9.9, 8.0)
3"	3.57 (1H, dd, 8.0, 1.6)	74.9	3.43***	77.8	3.85 (1H, dd, 9.8, 3.2)	3.54 (1H, dd, 6.3, 5.0)	75.1	3.35 (1H, t, 9.5)	3.82 (1H, dd, 9.9 3.3)
4"	3.87 (1H, dd, 3.2, 0.5)	69.9	3.38***	70.8	3.96 (1H, d, 3.2)	3.85 (1H, d, 3.0)	70.0	3.42 (1H, t, 9.0)	3.93 (1H, d, 3.2)
5"	3.49 (1H, td, 6.2, 6.0, 0.5)	77.0	3.23***	78.2	3.42***	3.48 (1H, td, 6.0, 5.4, 0,8)	77.3	3.22 (1H, dd, 5.3, 2.5)	3.42 (1H, dt, 2.8, 2.6, 1.5)
6a"	3.65 (1H, dd, 11.2, 6.0)	61.7	3.90***	62.3	3.88 (1H, dd, 11.9, 2.2)	3.64 (1H, dd, 11.2, 6.0)	62.0	3.71 (1H, dd, 11.8, 2.3)	3.68 (1H, dd, 11.8, 6.0)
6b"	3.58 (1H, dd, 9.3, 6.2)		3.71***		3.70 (1H, dd, 5.9, 2.2)	3.56 (1H, dd, 6.4, 3.2)		3.57 (1H, dd, 11.2, 5.8)	3.59 (1H, dd, 6.5, 6.0)
Galloyl									
2'"/6'"					7.15 (2H, s)				7.13 (2H, s)

*Relative to the residual methanol signal set to δ 3.31, δ values given in ppm; number of protons, multiplicity of signals (s, singlet; d, doublet; dd, double doublet; dt, double triplet), coupling constants (apparent splittings) given as numerical values in Hz are shown in parentheses. **^{13}C NMR chemical shifts obtained from HMBC experiments, relative to the resonance of the solvent set to δ 49.05. ***Signal unclear due to overlapping signals.

These metabolites can be subgrouped in the class of the flavonols (3-hydroxyflavones) and their general structure is depicted in Figure 3.3.2.6. The assignment of each peak is discussed in further detail in the next section.

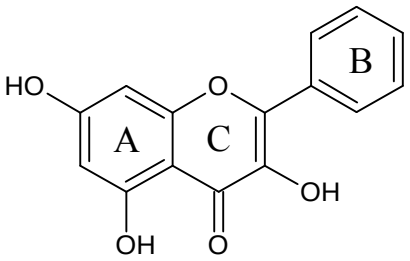


Figure 3.3.2.6 - Flavonol structure and ring nomenclature (Middleton et al., 2000).

The negative-ion ESI-MS spectrum of peak 6 showed a pseudomolecular ion $[M-H]^-$ peak at *m/z* 463 suggesting a molecular formula of $C_{21}H_{20}O_{12}$, together with ion fragments at *m/z* 301 coherent with the loss of one hexosyl unit ($[(M-H)-162]^-$). The ^{1}H-NMR spectrum (Table 3.3.2.3) showed five aromatic proton resonances, belonging to two different spin systems determined by a TOCSY experiment. One pair of doublets at δ 6.41 (d, *J*= 2.0 Hz) and 6.21 (d, *J*= 2.0 Hz), suggest one tetra-substituted aromatic ring and three doublets at δ 7.84 (d, *J*= 2.1 Hz), 7.60 (dd, *J*= 8.5, 2.1 Hz) and 6.87 (d, *J*= 8.5 Hz), form a separate spin system indicating the presence of an 1,2,4-tri-substituted aromatic ring. These signals are characteristic of a flavonol aglycone (Figure 3.3.2.6) and analysis of the HMBC spectrum with aid of reference data gave the full assignment of the C-resonances of the aglycone of peak 6, which was confirmed to be quercetin (Tatsis et al., 2007). The signals at δ 6.41 and 6.21 could be assigned as H-8 and H-6, respectively, of the A ring of the flavonol and the signals at δ 7.84, 7.60 and 6.87 as H-2', H-6' and H-5', respectively, of the B ring of the flavonol. ^{1}H-^{1}H COSY connectivities were used to assign the protons of the sugar unit. The multiplicities and coupling constants for the proton resonances in the ^{1}H-NMR spectrum indicated the presence of one O-linked galactopyranosyl moiety and the anomeric proton signal at δ 5.17 (d, *J* = 7.8 Hz) indicated the β-configuration of the unit. Therefore, the compound was assigned as quercetin-3-*O*-β-D-galactopyranoside, also known by the trivial name hyperoside (Tatsis et al., 2007).

The negative ion ESI-MS spectrum of peak 3 showed a pseudo-molecular ion peak at *m/z* 479 for which a molecular formula of $C_{21}H_{20}O_{13}$ could be deduced, suggesting that

it is a hydroxylated derivative of hyperoside. The ^{1}H-NMR spectrum shows that the proton resonances of the A ring of the aglycone and of the sugar moiety are identical to that of hyperoside. However, instead of three proton resonances at the B ring of the aglicone, only one singlet can be seen, suggesting that the phenyl group is substituted at the C-5 position with one extra hydroxyl group and that the aglycone is myricetin. This substitution explains the appearance of one singlet with a higher intensity at δ 7.38 (2H, s) which corresponds to the overlapping signals of the symmetrical H-2' and H-6' protons of the B ring. Therefore peak 3 was assigned as myricetin-3-*O*-β-D-galactopyranoside. The ^{1}H-NMR spectrum of peak 3 showed signals belonging to a second myricetin moiety, namely a singlet belonging to the H-2' and H-6' protons of the B ring of the flavonoid at δ 7.30 (2H, s) and a doublet belonging to H-8 at δ 6.39 (d, J= 2.1 Hz). The signal belonging to H-6 of the minor compound was overlapped by the same proton signal of myricetin-galactoside. Also, the signal corresponding to a second anomeric proton could be seen at δ 5.26 (d, J= 7.8 Hz). It was not possible to determine the multiplicities and coupling constants of the protons comprising the sugar moiety, however by analyzing the COSY and HSQC spectra it was possible to determine the chemical shift of each H and C-atom, allowing to assign it as a glucopyranosyl unit, by comparison with literature data (Slimestad et al., 1995). The minor compound of peak 3 was therefore assigned as myricetin-3-*O*-β-D-glucopyranoside.

Peak 8 showed a pseudo-molecular ion peak $[M-H]^-$ at *m/z* 615 consistent with a molecular formula of $C_{28}H_{24}O_{16}$. The aromatic region of the ^{1}H-NMR spectrum of peak 8 is very similar to that of peak 6 indicating that it is another quercetin glycoside derivative. The proton resonances of the sugar unit were assigned by analyzing their multiplicities and coupling constants which suggested that it is a β-galactopyranosyl unit. From the obtained molecular formula for peak 8 it can be deduced that after the assignment of the quercetin unit ($C_{15}H_9O_7$) and the hexosyl unit ($C_6H_{10}O_5$) a fragment of $C_7H_5O_4$ is left to be assigned. The molecular formula of the fragment suggests that peak 9 is substituted with a galloyl group, which is consistent with the NMR data as one extra aromatic singlet can be seen at δ 7.13 corresponding to the two symmetrical H-2''' and H-6''' protons of the galloyl unit (Akdemir et al., 2001). The H-2'' proton showed a great downfield shift ($\Delta\delta$ + 1.61) in comparison to the same proton of peak 3 (δ 3.83), suggesting that the substituent group is *O*-linked at the C-2'' position of the sugar

moiety. Peak 8 was therefore assigned as quercetin-3-*O*-*β*-(2"-galloylgalactoside) and confirmed with literature data (Pakulski and Budzianowski, 1996a).

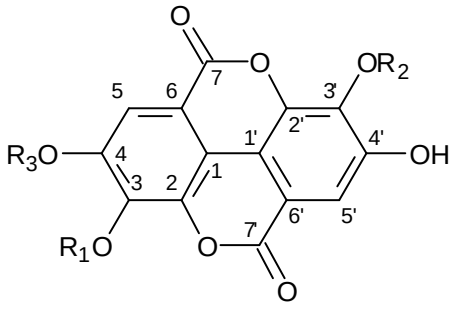
Peak 7 was comprised of two co-eluting compounds. The ESI-MS spectrum suggested that the minor compound was an isomer peak 6, showing a pseudo-molecular ion $[M-H]^-$ peak at *m/z* 463. The aromatic region of the ^{1}H-NMR spectrum of the minor compound of peak 7 minor has the characteristic set of signals of a quercetin moiety, which is almost identical for peak 6 (Table 3.3.2.3), indicating that the difference resides in the sugar unit. Discrimination between quercetin glycosides can be based on the differences of the glycoside protons chemical shifts, especially that one of the anomeric proton (Hansen et al., 1999; Lommen et al., 2000). The minor compound of peak 7 could therefore be assigned as quercetin-3-*O*-*β*-D-glucopyranoside or isoquercitrin based on the characteristic signal of the anomeric proton at *δ* 5.26 (d, *J*= 7.6 Hz), which resonates slightly further downfield and has a smaller coupling constant in comparison to the anomeric proton of hyperoside (*δ* 5.17, d, *J*= 7.8 Hz), as has been previously reported (Tatsis et al., 2007).

The negative ion ESI-MS spectrum of peak 4 showed a pseudo-molecular ion peak at *m/z* 631 suggesting a molecular formula of $C_{28}H_{24}O_{17}$. The intensities of the peaks that could be seen in the ^{1}H-NMR spectrum were low, but faint signals of a *meta* coupled pair of doublets at *δ* 6.16 (d, 2.0) and *δ* 6.32 (d, 2.0) and an intense singlet at *δ* 7.27 (2H, s) suggested that peak 4 is a myricetin derivative. Also an anomeric proton resonating at *δ* 5.79 (d, *J*= 8.0 Hz) and the signals between *δ* 5.45-3.42 indicated the presence of a sugar moiety, which was identified as a galactopyranosyl unit by comparison of the multiplicities and coupling constants of the sugar moiety of hyperoside. The singlet at *δ* 7.15 and the downfield shift of the H-2" proton of the sugar moiety, as verified for peak 8 (Table 3.3.2.3), suggested the linkage position of a galloyl group. This way, peak 4 could be assigned as myricetin 3-*O*-(2"-*O*-galloyl)-*β*-D-galactopyranoside and confirmed with literature data (Akdemir et al., 2001).

3.3.2.3.2. Ellagic acid derivatives

The second largest group of secondary metabolites identified in *D. intermedia* comprised ellagic acid (peak 5) and their derivatives 3-*O*-methylellagic acid (peak 9),

3,3'-di-*O*-methylellagic acid (peak 10) and 3,3'-di-*O*-methylellagic acid 4-*O*-*β*-D-glucoside (peak 7). The chemical structures of the identified compounds are shown in Figure 3.3.2.7 and the NMR data that led to their assignment in Table 3.3.2.4.



Peak	R_1	R_2	R_3
5	H	H	H
7	CH_3	CH_3	Glucopyranosyl
9	CH_3	H	H
10	CH_3	CH_3	H

Figure 3.3.2.7 - Structures of the ellagic acid derivatives of *D. intermedia*.

The assignment of peak 5 was a complicated task due to the few signals that could be seen in the ^{1}H-NMR spectrum. In fact, in the entire spectrum only one signal could be seen, namely a singlet at δ 7.59 (coupled to δ_C 111.5 in HSQC) indicating that the compound is highly functionalized and has very few protonated carbons (Table 3.3.2.4). The negative ion ESI-MS spectrum showed a pseudo-molecular $[M-H]^-$ at *m/z* 301 consistent with a molecular formula of $C_{14}H_6O_8$. A search in a natural products database (DNP, 2007) based on the molecular formula indicated ellagic acid as a candidate. Ellagic acid is a compound bearing only two symmetrical protonated carbons which resonate at δ 7.47 (in DMSO-*d*6) according to literature data (Li et al., 1999). Assigning a compound based on a single resonance is risky and undesirable and, therefore it is important to gather more information to support the assignment. An additional hazard in assigning ellagic acid derivatives comes from the fact that both quercetin and ellagic acid have a molecular weight of 302, making them difficult to distinguish in MS experiments. However, negative ion-mode HPLC-MS^n experiments have pointed out that when the *m/z* 301 ion is further fragmented the quercetin fragments produce ions at *m/z* 179 and 151, while the equivalent ellagic acid ion yields fragments at *m/z* 257 and 229. This data supports the assignment of peak 5 as ellagic acid, because in-source fragmentation of peak 5 yielded the corresponding characteristic fragments (Figure 3.3.2.8). In addition, ellagic acid has been identified in extracts of other *Drosera* species (Paper et al., 2005).

Table 3.3.2.4 - ^{1}H and ^{13}C NMR data of the ellagic acid derivatives obtained by HPLC-SPE-NMR from the methanol extract of *D. intermedia*.

	5		**7**	**9**	**10**
Position	δ_H*	δ_C**	δ_H*	δ_H*	δ_H*
Aglycone					
1		112.1			
2		ND			
3		140.7			
4		152.3			
5	7.59 (2H, s)	111.5	7.93 (1H, s)	7.56 (1H, s)	7.59 (2H, s)
6		ND			
7		159.2			
3-OCH_3			4.20 (3H, s)	4.18 (3H, s)	4.18 (6H, s)
1'					
2'					
3'					
4'					
5'			7.58 (1H, s)	7.59 (1H, s)	
6'					
7'					
3'-OCH_3			4.19 (3H, s)		
Sugar unit					
1"			5.15 (1H, d, 7.8)		
2"			3.59 (1H, dd, 8.9, 7.8)		
3"			3.53 (1H, dd, 9.1, 8.7)		
4"			3.48 (1H, dd, 9.2, 7.5)		
5"			3.55***		
6"			3.92 (1H, dd, 12.2, 1.9)		
			3.75 (1H, dd, 12.2, 5.3)		

*Relative to the residual methanol signal set to δ 3.31, δ values given in ppm; Number of protons, multiplicity of signals (s, singlet; d, doublet) and coupling constants (apparent splittings) given as numerical values in Hz are shown in parenthesis. **^{13}C-NMR chemical shifts obtained from HMBC experiments of total methanol extract, relative to the resonance of the solvent set to δ 49.05. ***Signal unclear due to overlapping signals.

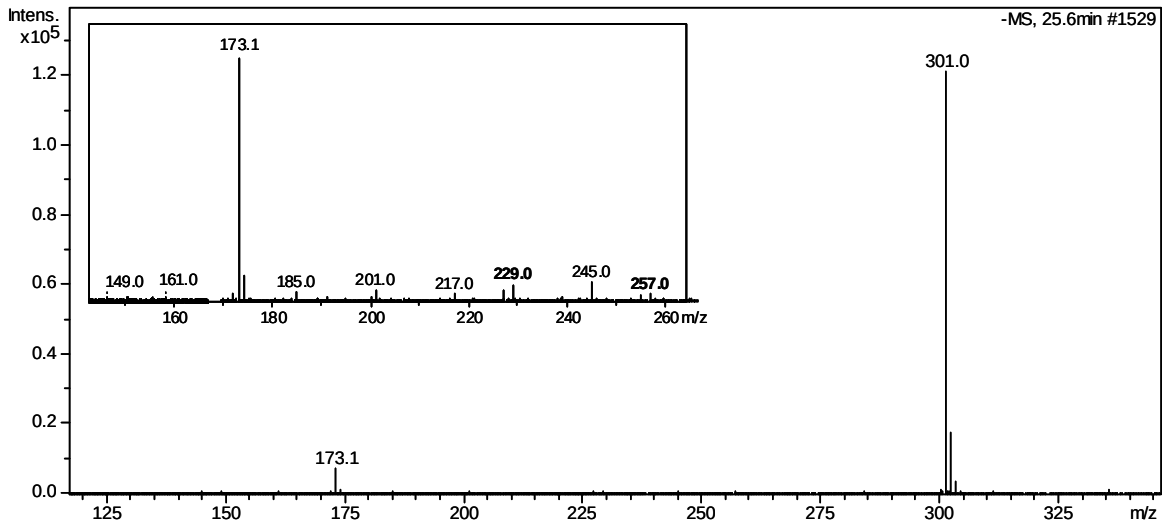


Figure 3.3.2.8 - Negative-ion ESI-MS spectrum of peak 5 of *D. intermedia* methanol extract.

Ellagic acid derivatives, bearing 12 non-protonated and only two protonated aromatic carbons, are very difficult to assign using regular NMR spectroscopy experiments, because ^{1}H-^{1}H experiments such as COSY, TOCSY or NOESY would give no information and even a good quality HSQC spectrum indicates only one single C-resonance of the core structure. Perhaps the most indicated experiment to assign similar compounds is the Incredible Natural Abundance Double Quantum Transfer Spectroscopy (INADEQUATE) which is a specially designed COSY experiment that yields ^{13}C-^{13}C correlations, however, the drawback of this experiment is its extremely low sensitivity. A different approach has been described to achieve the complete assignment of ellagic acid derivatives using HMBC experiments with different delay times in order to optimize the detection of long range couplings (Li et al., 1999).

In this case, the trapped peak was not subjected to further NMR experiments due to the low amount of trapped material, which did not provide enough signal to obtain a good quality ^{13}C spectrum. Instead a different approach was used, which involved dissolving the entire methanol extract in deuterated methanol and analyzing it directly by NMR in order to obtain some C-resonances through HMBC experiments. The HMBC spectrum showed that the H-5 proton was correlated with signals at 112.2, 140.7, 159.2 and a faint signal at 152.3 which were in good agreement with the resonances referred in literature for C-1, C-3, C-7 and C-4, respectively, confirming the assignment of peak 5.

The negative ion ESI-MS spectrum of the major compound of peak 7 showed a $[M-H]^-$ at *m/z* 491 suggesting a molecular formula of $C_{22}H_{20}O_{13}$. The ^{1}H-NMR spectrum also showed very few signals in the aromatic region, namely two singlets appearing at δ 7.93 and 7.58 and a doublet resonating at δ 5.15 (d, J= 7.8 Hz) suggesting that peak 7 was a glucosylated ellagic acid derivative. The chemical shifts and the multiplicities of the protons of the sugar moiety determined by analyzing the COSY and TOCSY spectra allowed to assign it as a glucopyranosyl unit and the coupling constant of the anomeric proton (J= 7.7 Hz) indicated its β-configuration. The ^{1}H-NMR spectrum also showed a signal appearing at δ 4.20 which at first resembled a doublet but were actually two intense singlets belonging to two methyl groups. This result is in agreement with the ESI-MS experiments which indicated an aglycone of *m/z* 329, suggesting a structure based on an ellagic acid skeleton substituted by two methyl groups. The 328 *m/z* fragment obtained in the negative-ion spectrum corresponds to the radical ion of the

aglycone ($[M-H]^{\bullet-}$-162). With aid of reference data the major compound of peak 7 was assigned as 3,3'-di-*O*-methylellagic acid 4-*O*-*β*-D-glucopyranoside (Papulski and Budzianowski, 1996b).

The molecular formula deduced for peak 10 ($C_{16}H_{10}O_8$) from the ESI-MS experiments suggests that is the deglycosilated analogue of the major compound of peak 7. This is supported by the ^{1}H-NMR experiments which showed only two signals at *δ* 7.59 (2H, s) and *δ* 4.18 (6H, s), consistent with the signals of two symmetrical aromatic protons (H-5, H-5') and two symmetrical methyl groups. By comparison with reference data peak 10 was assigned as 3,3'-di-*O*-methylellagic acid (Hillis and Yazaki, 1973; Papulski and Budzianowski, 1996b). It is interesting to see that the in-source fragmentation reported in Table 3.3.2.1 shows the successive losses of the methyl groups (*m/z* 314 = 329-15 and *m/z* 299 = 314-15). The same analysis quickly showed that peak 9 was the mono-methylated derivative of peak 10. The ^{1}H-NMR spectra showed two signals at *δ* 7.56 (1H, s) and *δ* 7.59 (1H, s), assigned as H-5 and H-5', respectively, and a singlet at *δ* 4.18 (3H, s) belonging to a methyl group, making it possible to assign peak 9 as 3-*O*-methylellagic acid (Papulski and Budzianowski, 1996b).

3.3.2.3.3. Naphthoquinone glycosides

The pseudo-molecular ion at *m/z* 513 obtained in the negative ion ESI-MS spectrum suggested a molecular formula of $C_{23}H_{30}O_{13}$ for peak 1 and search in a natural product database (DNP, 2007) gave a hit for plicataloside, a diglycosylated methylnaphthalene-triol. This structure was in good agreement with results obtained in the ESI-MS experiments, showing fragments at *m/z* 351 (*m/z* 513-162) and 189 (*m/z* 351-162), corresponding to the loss of two hexosyl units. The fragment obtained at *m/z* 189 corresponding to the aglycone is consistent with a naphthalene ring substituted with one methyl and three hydroxyl groups. Furthermore, the ^{1}H-NMR spectra shows 4 signals in the aromatic region belonging to different spin systems and two anomeric protons resonating at *δ* 4.78 (d, *J*= 7.8 Hz) and 5.07 (d, *J*= 7.8 Hz) (Table 3.3.2.5). One singlet can be seen at *δ* 7.21 and three protons forming a ABC spin system at *δ* 6.76 (d, *J*= 7.6 Hz), 7.30 (dd, *J*= 8.1, 8.0 Hz) and 7.95 (d, *J*= 8.4 Hz), seem to be coherent with the structure of plicataloside (Figure 3.3.2.9).

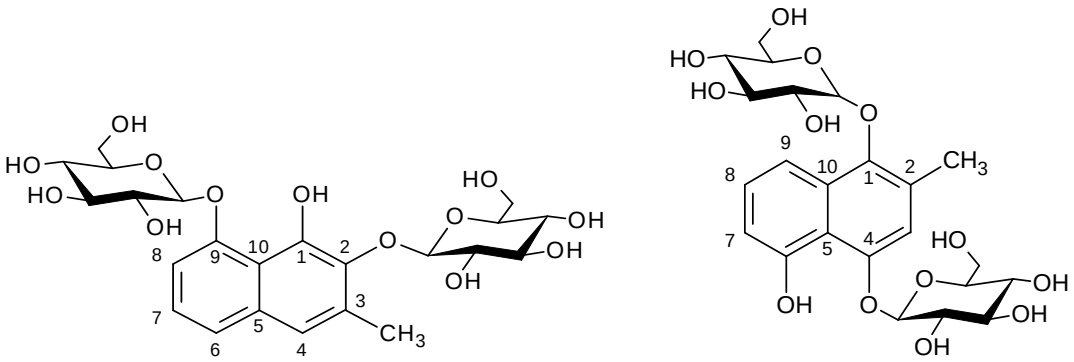


Figure 3.3.2.9 - The structure at the left corresponds to plicataloside (3-methyl-1,2,8-naphthalenetriol 2,8-di-*O*-*β*-D-glucopyranoside) and the structure at the right to hydroplumbagin 1,4-di-*O*-*β*-D-glucopyranoside.

However, when comparing the obtained chemical shifts with literature data for plicataloside (Wessels et al., 1996) the results are discrepant. On the other hand, the chemical shifts obtained for the aromatic protons of the aglycone are very similar to those reported for a 4-glycosylated hydroplumbagin aglycone (Kreher et al., 1990), which is also coherent based on biogenetic factors, considering that this compound has been previously identified in *D. intermedia* (Budzianowski, 1996). The signal appearing at δ 7.21 was assigned as H-3 and the signals belonging to the second aromatic ring at δ 6.76, 7.30 and 7.95 were assigned as H-6, H-7 and H-8, respectively. Peak 1 has got one extra sugar moiety attached but unfortunately it was not possible to obtain 2D experiments, such as NOESY which would indicate the site of glycosilation. However, bearing in mind that the chemical shifts of the aromatic protons are similar to those reported for hydroplumbagin 4-*O*-*β*-D-glucopyranoside, it is assumable that the site of glycosilation is at the C-1 instead of the C-5 position of the aglycone. Peak 1 was therefore tentatively assigned as hydroplumbagin di-1,4-*O*-*β*-D-glucopyranoside, but further experiments will have to confirm this assignment, as this structure has not been reported before.

3.3.3. Biological and taxonomical importance

Flavonoids are one of the largest groups of secondary metabolites and are widespread in the plant kingdom (de Rijke et al., 2006). Plant flavonoids play an important role in plants as defence and signalling compounds in reproduction, pathogenesis and symbiosis (Maxwell and Philips, 1990). It has been demonstrated that this group of

secondary metabolites is involved in response mechanisms against stress, as caused by elevated UV-B radiation (Middleton and Teramura, 1993; Olsson et al., 1998), infection by microorganisms or herbivore attack (de Rijke et al., 2006). In addition, they are pigment sources for flower colouring compounds (Goto and Kondo, 1991) and play an important role in interactions with insects (Biggs and Lanea, 1978).

Table 3.3.2.5 - ^{1}H NMR data of the naphthoquinone glycosides obtained by HPLC-SPE-NMR from the methanol extract of *D. intermedia*.

	Peak 1
Position	δ_H *
Aglycone	
1	
2	
3	7.21 (1H, s)
4	
5	
6	6.76 (1H, d, 7.6)
7	7.30 (1H, dd, 8.4, 7.6)
8	7.95 (1H, d, 8.4)
9	
10	
CH_3	2.49 (3H, s)
Glucosyl 1	
1'	5.07 (1H, d, 7.8)
2'	**
3'	**
4'	**
5'	**
6'	3.95 (1H, dd, 12.1, 1.9)
	3.74 (1H, dd, 11.3, 6.9)
Glucosyl 2	
1"	4.78 (1H, d, 7.8)
2"	**
3"	**
4"	**
5"	**
6"	3.73 (1H, dd, 11.5, 1.9)
	3.63 (1H, dd, 11.6, 5.2)

*Relative to the residual methanol signal set to δ 3.31, δ values given in ppm; number of protons, multiplicity of signals (s, singlet; d, doublet; dd, double doublet) and coupling constants (apparent splittings) given as numerical values in Hz are shown in parenthesis. **Signal unclear due to overlapping signals.

Ellagic acid and its derivatives are included in the family of the polyphenols and are also widely distributed, having therefore little taxonomical value. In plants they are often bound to polyol carbohydrates such as glucose forming the known ellagitannins, but that seems not to be the case in *D. intermedia*. The naphthoquinone plumbagin,

however, together with its isomer 7-methyljuglone are the major compounds in the Droseraceae family and are of taxonomic significance. The distribution of these two naphthoquinones within the *Drosera* genus has been used to support taxonomical classifications (Culham and Gornall, 1994). At the family level the presence of naphthoquinones has few taxonomic significance due to its scatered but widespread distribution within flowering plants and other organisms (for further details see section 5.1.2.2). Within the *Drosera* genus however, species can be divided into three main groups considering the relative frequencies of plumbagin and 7-methyljuglone. In the first group 7-methyljuglone is commonly present, often in association with plumbagin; in the second group plumbagin is predominant and 7-methyljuglone rare or absent; and the third group apparently lacks naphthoquinones altogether, which support previous classifications based on morphological characteristics (Culham and Gornall, 1994). Interestingly, 7-methyljuglone is reported to be produced by *D. intermedia* (Budzianowski, 1996), but could not be identified in this work, possibly due to a different extraction protocol.

The classes of natural products identified in *D. intermedia* are structurally distinct and it is difficult to determine the relative amount of each compound by comparing the intensities of the signals in their respective ^{1}H-NMR spectra because they do not have protons in a similar chemical environment. However, by identifying the signals corresponding to the most characteristic protons of each compound in the ^{1}H-NMR spectrum recorded from the total methanol extract and comparing their intensities, it was possible to deduce by approximation that ellagic acid and 3,3'-di-*O*-methylellagic acid are the major compounds, followed by 3,3'-di-*O*-methylellagic acid 4-*O*-*β*-D-glucopyranoside, hyperoside and myricetin-3-*O*-*β*-D-glucopyranoside. The relative quantification of the extract components directly from the total extract has the advantage that the trapping efficiency of each compound does not interfere with the quantification.

The occurrence of plumbagin in other unrelated carnivorous genera, such as *Nepenthes* (Nepenthaceae) (Rischer et al., 2002) and *Triphyophyllum* (Dioncophyllum) (Bringmann et al., 2000) is interesting, indicating that a taxonomic link to the Droseraceae is possible and that the carnivorous habit might have a similar origin. Within the Droseraceae family this theory was supported by a more recent work where

combined *rbcL* and 18S rDNA sequence data were used to infer phylogenetic relationships among *Drosera*, *Dionaea*, and *Aldrovanda*, and revealed that all *Drosera* species form a clade, sister to a clade including *Dionaea* and *Aldrovanda* (Rivadavia et al., 2003). However, outside the Droseraceae family it is more likely that naphthoquinone evolution has followed a parallel or convergent course in these carnivorous plant families and that it bears no relation with the carnivorous habit, considering that no naphthoquinones have been found in other carnivorous species such as *Cephalotus follicularis* (Cephalotaceae), *Byblis gigantea* (Byblidaceae), *Roridula gorgonias* (Roridulaceae), *Sarracenia flava*, *Heliamphora nutans* (Sarraceniaceae), *Utricularia Iongifolia* (Lentibulariaceae) (Zenk et al., 1969; Culham and Gornall, 1994) or the the other species studied in this work, *P. lusitanica*. It is believed that naphthoquinones are produced by carnivorous plants as antimicrobial compounds to protect the prey from decomposition during digestion (Culham and Gornall, 1994). Other possible roles for these compounds are as allelopathic (Spencer et al., 1986; Gonçalves et al., 2009), insecticidal, or antifeedant chemicals (Villavicencio and Perez-Escandon, 1994; Gonçalves et al., 2008).

This work provided a detailed insight on the compounds produced by *D. intermedia*, which had not been done so far for any member of the *Drosera* genus because most studies concentrated specifically on one class of natural products. When comparing the secondary metabolite profile of *D. intermedia* and *Dionae muscipla* the similarities are remarkable, both species produce plumbagin and more interestingly, the same 3-*O*-flavonols and ellagic acid derivatives (Pakulski and Budzianowski, 1996a; 1996b), indicating a taxonomical proximity between these two genera, which is in agreement with molecular biology studies (Rivadavia et al., 2003).

3.4. CONCLUSIONS

This chapter dealt with the identification of the major secondary metabolites produced by *P. lusitanica* and *D. intermedia* using the hyphenated techniques HPLC-ESI-MS and HPLC-SPE-NMR. The secondary metabolites identified in *P. lusitanica* were grouped into two classes of natural products. The iridoid glucosides mussaenosidic acid, globularin and sucutellarioside II and the caffeoyl phenylethanoid glycosides acteoside, R/S campneoside I and R/S campeneoside II were the major secondary metabolites identified from the *P. lusitanica* methanol extract. By comparing the intensities of the anomeric protons in the respective ^{1}H-NMR spectra it was deduced to an approximate extent that the major components of the methanol extract were acteoside and mussaenosidic acid. The methanol extract also yielded a compound with an iridoid structure which was not conclusively assigned and might not have been reported before.

From the methanol extract of *D. intermedia* several flavonol glycosides (quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-(2"-*O*-galloylgalactoside), myricetin-3-*O*-glucoside, myricetin-3-*O*-galactoside and myricetin-3-*O*-(2"-*O*-galloyl)-galactoside) and ellagic acid derivatives (ellagic acid, 3-*O*-methylellagic acid, 3,3'-di-*O*-methylellagic acid and 3,3'-di-*O*-methylellagic acid 4-*O*-*β*-glucoside) could be identified, together with a diglycosylated naphthoquinone, tentatively assigned as hydroplumbagin di-1,4-*O*-*β*-glucoside. Further experiments will be needed to confirm the structure of this compound. The water extract was not investigated by HPLC-SPE-NMR due to its low content in metabolites. The *n*-hexane extract was composed by mainly one single compound which was analyzed directly by NMR and identified as the naphthoquinone plumbagin. The obtained ^{1}H and ^{13}C spectra showed that the sample was nearly pure, meaning that extraction of *D. intermedia* with *n*-hexane followed by SPE is an effective method for obtaining high purity plumbagin samples.

P. lusitanica had never been investigated for its secondary metabolites and great part of the compounds identified in *D. intermedia* were reported for the first time in this species, meaning that this work is a contribution to the better understanding of their biochemistry. In addition, the secondary metabolites identified in this chapter for

P. lusitanica and *D. intermedia* were in agreement with previous chemical studies performed in species of the Lentibulariaceae and Droseraceae families, respectively.

The techniques employed in this chapter allowed for the rapid identification of the major components of each extract without having to recur to preparative scale procedures. To date, the majority of the HPLC-NMR work in the area of natural products has been done with fractions or extracts that have been enriched for particular classes of compounds. In the present work, the initial preparative SPE step was used only to remove the most highly hydrophobic components, and thus, essentially crude extracts were subjected to the HPLC-SPE-NMR analysis. The sensitivity gain was illustrated by the ability to acquire HSQC and HMBC spectra, from which ^{13}C chemical shift data, necessary for structure elucidation of complex natural products, could be obtained. The sensitivity gain is achieved in part by concentrating the analytes present in the chromatographic eluate in a highly sensitive NMR flow probe, and in part by accumulation of the analyte by multiple SPE trapping steps from repeated injections. It is worth mentioning that by using HPLC-SPE-NMR it was possible to obtain NMR spectra with sufficient quality to identify the above mentioned compounds using merely 4 and 36 mg of crude extract of *P. lusitanica* and *D. intermedia*, respectively. By analogy, the amount of plant material needed to prepare enough extract for these experiments is also minute, in contrast to conventional preparative techniques where several hundreds grams of plant material can be used. This can be of utmost importance when dealing with endangered plant species and available material is limited. In summary, the HPLC-SPE-NMR together with HPLC-MS proved to be very effective techniques for the rapid identification of the major secondary metabolites of *P. lusitanica* and *D. intermedia*.

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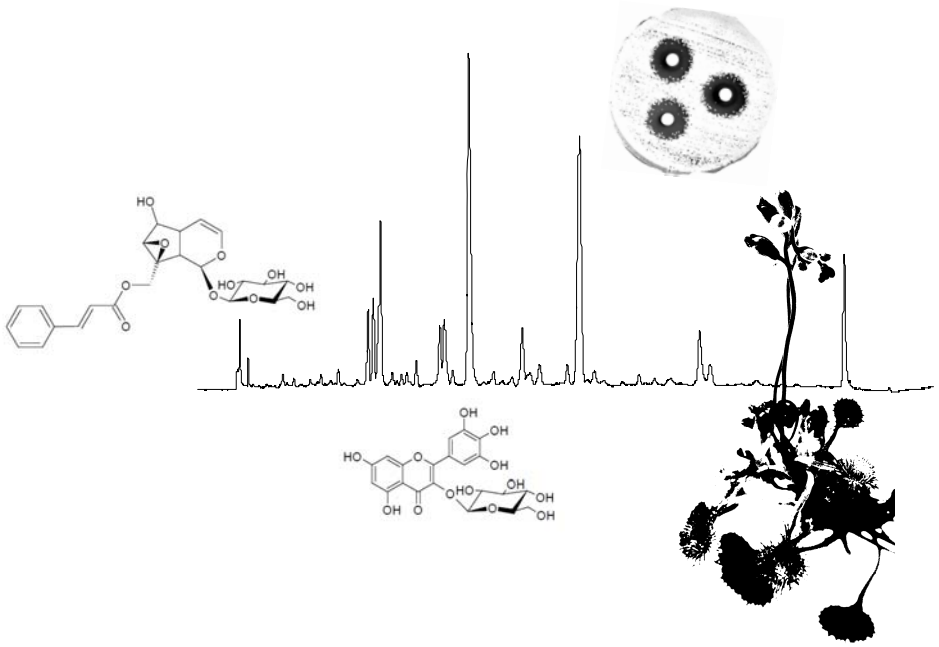
CHAPTER 4

BIOLOGICAL ASSAYS FOR PRELIMINARY SCREENING



4.1. INTRODUCTION

4.1.1. Antioxidant activity

4.1.1.1. Reactive oxygen species and their biological importance

Free radicals are highly reactive and unstable chemical species which can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals (Valko et al., 2007). Radicals derived from oxygen or, more generally, reactive oxygen species (ROS) represent the most important class of radical species generated in living systems (Miller et al., 1990). The term ROS collectively denotes oxygen-centered radicals such as the superoxide anion ($O_2^{\bullet -}$), hydroxyl ($^{\bullet}OH$) and peroxyl ($ROO^{\bullet}$) as well as non-radical species derived from oxygen, such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hypochlorous acid (HOCl) (Middleton et al., 2000). The generation of ROS is a ubiquitous biological phenomenon in cellular metabolism and plays a dual role in living organisms. Beneficial effects of ROS occur at low to moderate concentrations and involve physiological roles in cellular responses to noxia, as for example in defence against infectious agents, in the function of a number of cellular signalling pathways, and the induction of a mitogenic response (Valko et al., 2007). The harmful effect of free radicals causing potential biological damage is termed oxidative stress and occurs in biological systems when there is an overproduction of ROS on one side and a deficiency of enzymatic and non-enzymatic antioxidants on the other (Kovacic and Jacintho, 2001). In other words, oxidative stress results from the metabolic reactions that use oxygen and represents a disturbance in the equilibrium status of prooxidant/antioxidant reactions in living organisms. The excess ROS can damage cellular lipids, proteins or DNA, inhibiting their normal function. Because of this, oxidative stress has been implicated in a number of human diseases such as cancer, atherosclerosis, hypertension, diabetes *mellitus* and neurodegenerative disorders (Alzheimer's disease and Parkinson's disease) as well as in the ageing process (Valko et al., 2007).

Cellular organisms have defence mechanisms to prevent free radical-induced oxidative stress which involve enzymatic antioxidants (such as superoxide dismutase, glutathione peroxidase, catalase) and non-enzymatic antioxidants represented by ascorbic acid

(Vitamin C), α-tocopherol (Vitamin E), glutathione, carotenoids, flavonoids, and other small molecule antioxidants. Non-enzimatic antioxidants are a special group of compounds that neutralize or quench free radicals and break down radical chain reactions, contributing to maintain the redox homeostasis of the cell. The assessment of the antioxidant activity of a pure compound or mixture of compounds is therefore an important preliminary assay in the context of drug discovery, considering that their antioxidative properties often underlie other biological activities.

4.1.1.2. Methods for the determination of antioxidant capacity

Phytochemicals have been extensively studied for their antioxidant capacity (AOC) and there is an increasing interest in natural antioxidants present in plants that might help prevent oxidative stress (Halliwell, 1999; Gardner et al., 2000; Huang et al., 2010). Antioxidants can deactivate radicals by two major mechanisms, hydrogen atom transfer (HAT) and single electron transfer (SET). The end result is the same, regardless of the mechanism, but kinetics and potential for side reactions differ. SET and HAT reactions may occur in parallel, and the mechanism dominating in a given system will be determined by antioxidant structure and properties, solubility and partition coefficient, and system solvent. SET-based methods detect the ability of a potential antioxidant to transfer one electron to reduce any compound while HAT-based methods measure the ability of an antioxidant to quench free radicals by hydrogen donation, which is more relevant to the radical chain-breaking capacity (Huang et al., 2005). Therefore, it is believed that these are the most relevant reactions where antioxidants typically act.

Various methods to measure antioxidant activity have been developed over the years. However, none of the methods accurately reflects the total antioxidant activity of a sample, because there are diverse antioxidants and free radical and oxidant sources with different chemical and physical characteristics involved in a complex system. Furthermore, the vast amount of methods and variations in methods to measure antioxidants in plant-derived samples that have been proposed make the study of the AOC a complicated issue. Too many analytical methods result in inconsistent results, inappropriate application and interpretation of assays, and improper specification of AOC. The antioxidant capacity needs to reflect both lipophilic and hydrophilic capacity and for physiological activity it needs to reflect and differentiate both radical quenching

(HAT) and radical reduction (SET). Therefore, the antioxidant efficacy of a plant extract is best evaluated based on results obtained by commonly accepted and standardized assays, taking into account different oxidative conditions, system compositions, and antioxidant mechanisms (Prior et al., 2005). Accordingly, AOC was evaluated using the oxygen radical absorbance capacity (ORAC), trolox equivalent antioxidant capacity (TEAC) and Folin-Ciocalteu (F-C) assays, adapted for microplate readings for high throughput. The ORAC assay follows a HAT mechanism while the other two an SET mechanism (Huang et al., 2005; Prior et al., 2005).

4.1.1.2.1. ORAC assay

The ORAC assay is based upon the early work of Glazer (1990) and Ghiselli et al. (1995), and developed further by Ou et al. (2001). The ORAC assay is composed by a synthetic free radical generator [2,2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH)], an oxidizable molecular probe (fluorescein) and an antioxidant. The capacity of the antioxidant to scavenge peroxyl radicals generated by spontaneous decomposition of AAPH is estimated through the change in fluorescent intensity of the probe. The radical-induced oxidation reaction of fluorescein leads to a loss of fluorescence and the AOC of a sample is determined by a decreased rate and amount of non-fluorescent product formed over time (Figure 4.1.1). The reaction is driven to completion and the AOC of the sample is measured by determining the area under the fluorescence decay curve of the sample (AUC_{sample}) compared to that of a control sample (AUC_{blank}). ORAC values are usually reported as Trolox (water soluble analogue of Vitamin E) equivalents using a standard curve generated by plotting standard concentrations of Trolox against their respective net AUC (AUC_{sample} - AUC_{blank}). This assay is unique in that it is run to completion and the dynamic change in fluorescence of the probe over time is accounted for by calculating the AUC, considering both inhibition degree and inhibition time (Prior et al., 2005). In addition, the fluorescence measurement applied in the assay causes less interference by coloured compounds (Prior and Cao, 2000) and it can be adapted to assay lipophilic antioxidant components by using methylated β-cyclodextrin as water solubility enhancer (Huang et al., 2002). Mechanistic studies have determined that the reaction of peroxyl radical neutralization follows a HAT mechanism (Ou et al., 2001). However, the main drawback of the ORAC method is the assumption that the autooxidative mechanism and protection of the fluorescent probe can mimic

critical biological substrates (Frankel and Meyer, 2000). The ORAC assay has been widely used in the nutritional field, but a recent protocol was developed for the measurement of AOC of plant samples (Gillespie et al., 2007).

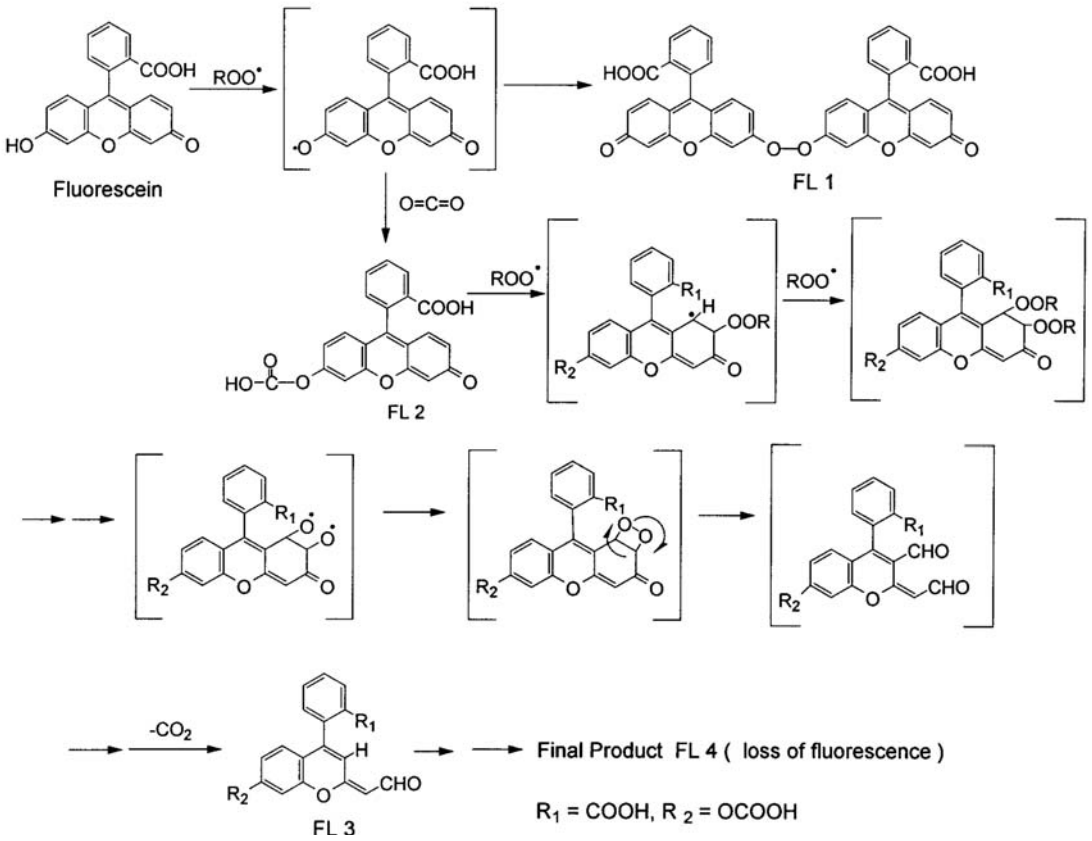


Figure 4.1.1. Proposed fluorescein oxidation pathway in the presence of AAPH (Ou et al., 2001).

4.1.1.2.2. TEAC assay

The TEAC assay was first reported by Miller et al. (1993) and is based on the ability of antioxidants to scavenge the long-life radical cation $ABTS^{\bullet+}$. In this assay, ABTS [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); Figure 4.1.2] is oxidized by peroxyl radicals or other oxidants to its radical cation, $ABTS^{\bullet+}$, which is intensely coloured. The AOC is measured as the ability of test compounds to decrease the colour reacting directly with the $ABTS^{\bullet+}$ radical, reducing it back to ABTS. In an improved method, the $ABTS^{\bullet+}$ radical cation was generated by reaction with potassium persulfate (Re et al., 1999). Chemical generation of the radical requires a long time, therefore a mixture of ABTS and potassium persulfate is generally allowed to stand overnight at room temperature in dark to form $ABTS^{\bullet+}$.

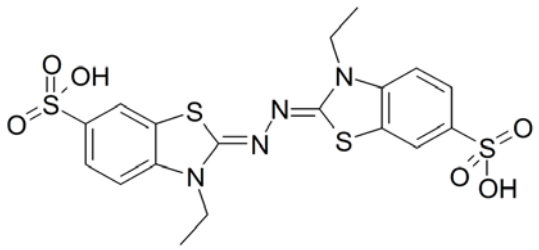


Figure 4.1.2 - Structure of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid).

The absorption maxima of $ABTS^{\bullet+}$ were shown to be at wavelengths of 415, 645, 734 and 815 nm. Among them, 415 and 734 nm are adopted by most researchers to spectrophotometrically monitor the reaction between the antioxidants and $ABTS^{\bullet+}$ (Prior et al., 2005). The AOC is quantified by measuring the absorbance decrease of $ABTS^{\bullet+}$ in the presence of a testing sample or Trolox standards at a fixed time point. The concentration of antioxidants giving the same percentage change of $ABTS^{\bullet+}$ absorbance as that of 1 mM Trolox is regarded as TEAC. Thus, the percentage inhibition of the $ABTS^{\bullet+}$ radical cation, measured by the extent of decolourization, is determined as a function of concentration and time and calculated relative to the reactivity of Trolox as a standard, under the same conditions (Re et al., 1999).

The TEAC assay is operationally simple and can be automated and adapted to microplates (Chen et al., 2004; Erel, 2004). The radical $ABTS^{\bullet+}$ is soluble in both aqueous and organic solvents and is not affected by ionic strength, meaning that it can be used in multiple media to determine both hydrophilic and lipophilic antioxidant capacities of extracts. Also, the assay has the advantage of being able to eliminate colour interference of extract components by measuring $ABTS^{\bullet+}$ at 734 nm (Awika et al., 2003). However, the ABTS radical used in TEAC assays is not found in mammalian biology and thus represents a nonphysiological radical source (Prior et al., 2005).

4.1.1.2.3. F-C assay

The F-C assay has for many years been used as a measure of total phenolics in natural products, but the basic mechanism is an oxidation/reduction reaction and, as such, can be considered as another antioxidant activity method (Huang et al., 2005). The F-C reagent (Folin and Ciocalteu, 1927) was initially intended for the analysis of proteins taking advantage of the reagent's activity towards protein tyrosine (containing a phenol

group) residue. Singleton and co-workers extended this assay to the analysis of total phenols in wine and since then the assay has found many applications (Singleton et al., 1999). The exact chemical nature of the F-C reagent is not known, but it is believed to contain a mixture of the heteropoly acids, phosphomolybdic and phoshotungstic acids, in which the molybdenum and tungsten atoms are in the 6^+ state, giving it an intense yellow colour (Ikawa et al., 2003). In highly basic medium, dissociation of a phenolic proton leads to a phenolate anion which is capable of reducing the F-C reagent, forming blue molybdenum and tungsten complexes that are determined spectroscopically at approximately 760 nm (Huang et al., 2005). The activity according to the F-C assay is often calculated as gallic acid equivalents.

The F-C assay is commonly used to determine the total phenolics content of a sample, however, because the oxidation/reduction reaction mechanism of the F-C reagent is nonspecific to phenolic compounds, other oxidation substracts besides phenols may interfere (Prior et al., 2005). Despite the undefined chemical nature of the F-C reagent, it is a convenient, simple and reproducible assay to determine the reducing capacity of a sample and a specific protocol adapted for microplate readings has been developed for plant samples (Ainsworth and Gillespie, 2007).

4.1.2. Antimicrobial activity

4.1.2.1. The issue of antibiotic resistance

Infectious diseases were the leading cause of death worldwide at the beginning of the twentieth century, but the introduction of penicillin in the 1940s into clinical use and subsequent development of streptomycin, chloramphenicol, tetracycline, erythromycin, rifamycin, and vancomycin between 1940 and 1960 had a striking impact on the treatment of infectious diseases and decreased mortality dramatically. However, new emerging infections, re-emerging infections experienced previously appearing in more virulent forms and antimicrobial-resistant bacterial strains, caused by the indiscriminate use of antibiotics, have shattered the optimism felt in the so-called “Era of antibiotics” that infectious diseases could be controlled and prevented (Cohen, 2000). Today, infectious diseases are the second-leading cause of death worldwide and the third-leading cause of death in the develop countries (Fauci, 2001).

The occurrence of antibiotic resistance is inevitable considering that the widespread use of antibiotics imposes strong selection pressure for the development of resistance and as the frequency of antibiotic use increases, the speed of resistance development also increases (Yoneyama and Katsumata, 2006). Clinically significant resistance usually appears after months to years whenever a new antibiotic is introduced. Penicillin resistance was detected a few years after its clinical debut in 1942 (Travis, 1994) and streptomycin resistance a year after its discovery in 1944 (Davies, 1994). The major concerns today are the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA), which exhibits multiantibiotic resistance against all penicillins (methicillin, dicloxacillin, nafcillin) and many structurally unrelated antibiotics (Neu, 1992; Cushnie and Lamb, 2005), including vancomycin, which was regarded as the antibiotic of last resort (Sievert et al., 2002). Recently, a new class of antibiotic oxazolidinones (linezolid) and cyclic lipopeptides (daptomycin) were introduced for clinical use. Although these new antibiotics are effective against vancomycin-resistant MRSA, it is clear that the problematic pathogens will eventually develop resistance to these compounds too if they are used indiscriminately (Yoneyama and Katsumata, 2006). In fact, clinical isolates of *S. aureus* showing linezolid resistance were reported a year after launch (Tsiodras et al., 2001).

Resistance to antimicrobial agents can be due to an innate property of the bacterium, or acquired as a consequence of mutation or gene transfer. An example of innate intrinsic resistance is *Pseudomonas aeruginosa*, whose low membrane permeability is likely to be a main reason for its innate resistance to many antibiotics (Nakae, 1995). Resistance often arises in the whole bacterial community and the longer that suboptimal levels of antimicrobial agent are in contact with the bacteria, the more likely the emergence of resistance (Coates et al., 2002). The main mechanisms of genetic resistance are inactivation of the antibiotic either by hydrolysis or by modification; modification of the site of action (enzyme, ribosome, cell wall precursor), reducing the affinity for antibiotics; modification of the permeability of the cell wall; overproduction of the target enzyme; and the bypass of the inhibited steps (Yoneyama and Katsumata, 2006).

Antibiotics are usually classified on the basis of their chemical structure and mode of action and members of each class share a common core structure. Although a large number of antibiotics are used clinically, the variety of targets that they inhibit is

limited. Antimicrobial agents inhibit essential components of bacterial metabolism which include four classical targets: cell wall biosynthesis, protein biosynthesis, DNA and RNA biosynthesis and folate byosynthesis (Yoneyama and Katsumata, 2006). Since bacteria are only distantly related to humans, unique and specific targets should be abundant, and novel antibiotics with low toxicity should be relatively easy to find. However, this has not been the case considering that almost 40 years were required for the development of a new structural class of synthetic antibiotics of broad spectrum (oxazolidinones), since the introduction of nalidixic acid in 1962 and that the vast majority of the antibiotics used today are derived from core structures of antibiotics discovered half a century ago (Fichbach and Walch, 2009). The oxazolidinone linelozid has a unique mode of action, inhibiting protein synthesis at the initiation stage (Swaney, 1998).

The main strategy of the pharmaceutical industry for new therapeutics has been modification of existing antibiotics using processes of synthetic tailoring where the core of the antibiotic is left intact, preserving its activity, and the chemical groups at its periphery are modified to improve the drug's properties. Although this approach is effective, it has turned out to be increasingly difficult to launch new drugs to meet the clinical needs, underlining the importance of finding not only new antibiotics but new classes of antibiotics as well (Yoneyama and Katsumata, 2006; Fichbach and Walch, 2009). The great chemical diversity provided by natural products might contribute in finding new lead structures considering that combinatorial chemistry practiced by nature is much more sophisticated than combinatorial chemistry in the laboratory, yielding exotic structures rich in stereochemistry, concatenated rings, and reactive functional groups (Verdine, 1996; Demain, 2009). Though no plant-derived compound has been found to compete with clinically used antibiotics to date, the great structural variety found in plants makes them attractive as a source of novel lead compounds (Cowan, 1999). In fact, secondary metabolites produced by higher plants frequently exhibit significant potency against human bacterial and fungal pathogens (Alviano and Alviano, 2009).

4.1.2.2. Antibacterial and antifungal assays

Antimicrobial activity of pure compounds and plant extracts can be detected by observing the growth response of various microorganisms to samples that are placed in contact with them. Several methods for detecting activity are available, but since they are not equally sensitive or not based upon the same principle, results will be profoundly influenced by the method (Cos et al., 2006). The currently available screening methods for the detection of antimicrobial activity of natural products fall into three groups, including bioautographic, diffusion, and dilution methods. The bioautographic and diffusion methods are known as qualitative techniques since these methods will only give an idea of the presence or absence of substances with antimicrobial activity. On the other hand, dilution methods are considered quantitative assays once they determine the minimum inhibitory concentration (MIC). Bio-autographic methods localize antimicrobial activity directly on a chromatogram and can therefore also indicate the active fraction of an extract, supporting a quick search for antimicrobial agents through bioassay-guided isolation (Valgas et al., 2007). Despite its advantages, this technique was not included in this work. The agar diffusion and dilution methods account for the majority of the assays performed in the search for antimicrobial active sources, possibly due to their inherent simplicity.

4.1.2.2.1. Agar diffusion methods

In the diffusion method, a reservoir containing the sample at a known concentration is brought into contact with an inoculated medium. If there are active compounds present, the growth of the microorganism is either slowed or stopped, resulting in the deformation of the colony and the diameter of the clear zone around the reservoir (inhibition diameter) is measured at the end of the incubation period (Cole, 1994). Two principal assay techniques are commonly employed according to the type of reservoir used: the paper disc diffusion technique, in which the sample is loaded onto a paper disc of fixed dimensions; and the agar well technique, in which holes are punched in the medium and the sample is directly transferred. In general, the relative antimicrobial potency of different samples may not always be compared, giving merely a qualitative assessment of whether or not a compound or extract possesses antimicrobial activity, mainly because of differences in physical properties, such as solubility, volatility and

diffusion characteristics in agar. The agar diffusion techniques assume that active compounds are soluble in the agar and therefore diffuse through the medium. This assumption is however not valid for non-polar compounds or highly charged molecules which may undergo ion exchange processes with the agar matrix (Cole, 1994).

4.1.2.2.2. Dilution methods (Minimum inhibitory concentration determination)

In the dilution method, the extract or compound, incorporated at a known concentration into a suitable agar based growth medium, is serially diluted using two fold-dilutions and is inoculated with the test organism (Cole, 1994). The lowest concentration at which the microorganisms do not grow is taken as the MIC. The method can be carried out in liquid as well as in solid media and growth of the microorganism can be measured in a number of ways. Turbidity can be estimated visually or obtained more accurately by measuring the optical density at 405 nm. However, test samples that are not fully soluble may interfere with turbidity readings, emphasizing the need for a sterility control, i.e., extract dissolved in blank medium without microorganisms. The liquid-dilution method also allows determining whether a compound or extract has a cidal or static action at a particular concentration. The minimal bactericidal or fungicidal concentration is determined by plating-out samples of completely inhibited dilution cultures and assessing growth (static) or no-growth (cidal) after incubation (Cos et al., 2006).

4.1.2.3. General considerations on antimicrobial assays

A review tackling the issue of the discovery of antimicrobial agents from medicinal plants revealed that still too many articles claim promising antimicrobial activities, despite major flaws in used methodologies (Rios and Recio, 2005). The most frequent are the omission of appropriate in-test controls, selection of target organism and growth medium, and the inclusion of unrealistically high assay dosages. Assays conducted with quantities higher than 1 mg/ml for extracts or 0.1 mg/ml for isolated compounds should be avoided, whereas the presence of activity is very interesting in the case of concentrations below 100 μg/ml for extracts and 10 μg/ml for isolated compounds. The choice of test microorganisms depends on the specific purpose of the investigation. In a primary screening, drug-sensitive reference strains are preferably used and should

represent common pathogenic species of different classes. Also, the panel should consist of Gram-positive and Gram-negative bacteria. American Type Culture Collection (ATCC) strains are well characterized and very popular for that purpose, but clinical field isolates may also be used if fully characterized. Also, it is important to use inoculates taken from actively growing colonies cultures in the logarithmic growth phase, since this is the growth stage at which the colony will be most homogeneous (Cole, 1994). Both positive and negative controls should be incorporated into the bioassay design. The negative control ensures that it is not the assay procedure itself which is responsible for any observed activity and that the tested microorganism is able to grow under the experimental conditions, whilst the positive control provides a marker against which the potency of an active extract can be measured. In addition, a sterility test control should be performed with the growth medium alone, to ensure that contamination by other microorganisms is prevented.

4.1.3. Objectives

P. lusitanica and *D. intermedia* have been used in traditional medicine and are known for their beneficial effects in fighting infections. Furthermore, because no scientific evaluation of any biological activity has been reported and the major compounds of their extracts have been identified, *P. lusitanica* and *D. intermedia* were selected to be submitted to biological assays. The aims of this chapter are to:

i) evaluate the AOC of extracts prepared from *in vitro* cultures of *D. intermedia* and *P. lusitanica*;

ii) investigate the antimicrobial activity of the extracts against a panel of human pathogenic microorganisms (including yeasts and bacteria) using the agar disc diffusion method followed by the determination of MICs;

iii) discuss the relation between the observed activities and the structure of the previously identified compounds.

4.2. EXPERIMENTAL

4.2.1. Plant material and sample preparation

The *P. lusitanica* methanol extract and the *D. intermedia n*-hexane, water and methanol extracts were prepared from *in vitro* cultured plant material according to the protocol described in section 3.2.1, except the lyophilized extracts were not cleaned using a SPE column as was done prior to the chemical analysis, but were included in the bioactivity assays as crude extracts. For the antioxidant assays the extracts were dissolved in phosphate buffer (75 mM, pH 7.0) and for the antimicrobial assays they were dissolved in their respective extraction solvents at the required concentration.

4.2.2. Antioxidant activity

4.2.2.1. Oxygen radical absorbance capacity (ORAC) assay

The peroxyl radical scavenging capacity of the prepared extracts was evaluated according to the protocol described by Gillespie et al. (2007). Fluorescein (Panreac, Spain, Barcelona) was used as the fluorescent probe and AAPH (Acros, Geel, Belgium) as a peroxyl radical generator. A black microplate (NUNC, Rochester, New York, USA) was loaded with 150 µL fluorescein (0.08 µM) and 25 µL of sample dilution (1-0.01 mg/mL), trolox standard (6.25-50 µM) or phosphate buffer (blank), and after a 10 min incubation period at 37ºC the reaction was initiated by adding 25 µL of AAPH (150 mM) to each well. The decrease in fluorescence of fluorescein was determined by collecting readings for excitation at 485 nm and emission at 530 nm every minute for 90 min with an Infinite 200 (Tekan, Grödig, Austria) microplate reader. The average area under the curve (AUC) of the blanks was subtracted from the AUC of each sample and standard to obtain the net AUC. The ORAC value for each extract was calculated using the respective net AUC and the regression equation obtained by plotting trolox standard concentrations against the net AUC (Figure 4.2.1). Results were expressed as trolox equivalents (TE) per gram of extract. Determinations were carried out three times and in triplicate, on each occasion and at each separate concentration of the standard and samples.

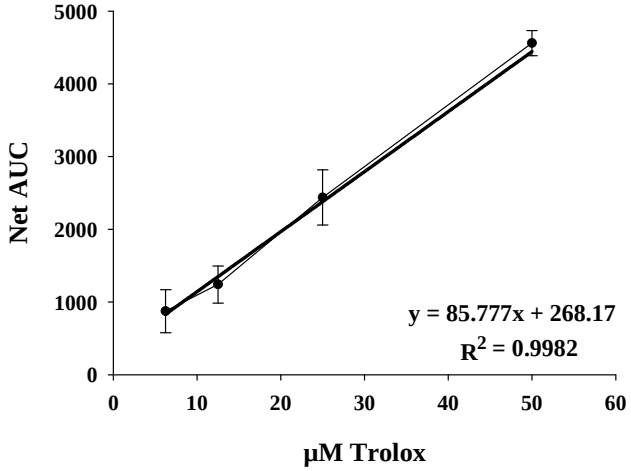


Figure 4.2.1 - Trolox standard curve for the ORAC assay.

4.2.2.2. Trolox equivalent antioxidant capacity (TEAC) assay

The radical scavenging capacity of the prepared extracts against the radical cation $ABTS^{+\bullet}$ was determined by the TEAC assay (Sigma-Aldrich, Steinheim, Germany) according to the procedure proposed by Re et al. (1999). The $ABTS^{+\bullet}$ stock solution was prepared by reacting an ABTS tablet (Sigma, USA) with a 2.45 mM potassium persulfate (Sigma-Aldrich, Steinheim, Germany) solution as the oxidant agent to give a final concentration of 7 mM, and was left to stand in the dark at room temperature for 12-16 h before use. The working solution of $ABTS^{+\bullet}$ was obtained by diluting the stock solution in phosphate buffer to give an absorption of 0.70 ± 0.02 at 734 nm. Afterwards, 10 µL of extract dilutions (1-0.1 mg/mL), trolox standards (0.1-0.4 mM) (Calbiochem, Darmstadt, Germany) or phosphate buffer (blank) were transferred to a clear 96-well microplate (NUNC, Rochester, New York, USA) and the reaction began with the addition of 190 µL of the $ABTS^{+\bullet}$ working solution. The absorbance reading was taken 1 min after initial mixing at 734 nm. The percentage of activity inhibition calculated as the decrease of the sample absorbance at 734 nm in relation to the control (Formula 4.2.1) was plotted as a function of Trolox concentration to obtain the standard curve (Figure 4.2.2).

$$\frac{Abs_{Blank} - Abs_{Sample}}{Abs_{Blank}} \times 100 \qquad \textbf{Formula 4.2.1}$$

The sample dilution that produced between 20-80% inhibition was used for TEAC calculation and results were expressed as TE per gram of extract.

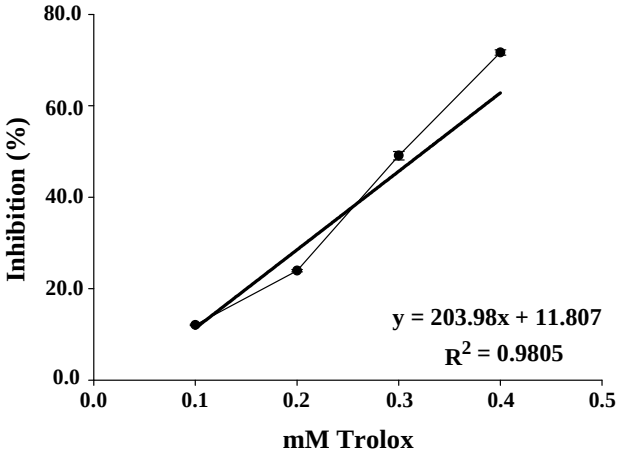


Figure 4.2.2 - Trolox standard curve for the TEAC assay.

4.2.2.3. Folin-Ciocalteu (F-C) assay

The F-C colorimetric method was used as described by Ainsworth et al. (2007) with slight modifications. In brief, 200 µL of 10% (v/v) F-C reagent (Fluka, Buchs, Switzerland) was added to 100 µL sample dilution (10-0.01 mg/mL) prepared in phosphate buffer (75 mM, pH 7.0), standard or phosphate buffer (blank) in a microtube. A 700 mM sodium carbonate solution (800 µL) (VWR, Leuven, Belgium) was added to each microtube and incubated for 2 h at room temperature. Thereafter, 200 µL of the content of each microtube was transferred to a clear 96-well microplate (NUNC, Rochester, New York, USA) and the absorbance was read at 765 nm. The standard curve was obtained plotting the gallic acid (Fluka, Buchs, Switzerland) concentrations ranging from 40 µM to 0.5 mM against the blank-corrected absorbance at 765 nm (Figure 4.2.3) and the results were expressed as gallic acid equivalents (GAE) per g of extract.

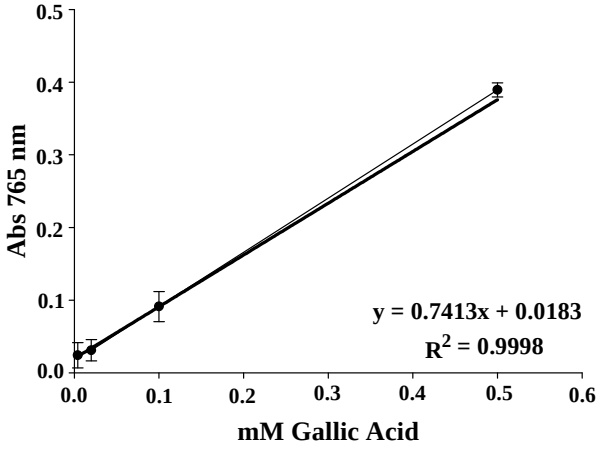


Figure 4.2.3 - Gallic acid standard curve.

4.2.3. Antimicrobial activity

4.2.3.1. Microorganisms

The prepared extracts were tested against a panel of bacteria and yeasts. The bacterial strains were acquired from the ATCC and included Gram-positive (*Staphylococcus aureus* ATCC 25923, *S. epidermidis* ATCC 12228 and *Enterococcus faecalis* ATCC 29212) as well as Gram-negative strains (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922*, Cronobacter sakazakii* ATTCC 29544 and *C. sakazakii* ATTCC BA-894). The tested yeasts included clinically isolated strains (*Candida albicans* YP0175, *C. famata* YP0011, *C. catenulata* YP0160, *C. guilliermondi* YP0170, *Trichosporon mucoides* YP0096 and *Cryptococcus neoformans* YP0186) and two reference strains of *C. albicans* (*C. albicans* ATCC 10231 and *C. albicans* ATCC 90028). Bacterial species were grown overnight at 37ºC in Plate Count Agar medium (PCA, Scharlau, Spain). Strains of *C. albicans* were grown overnight at 37ºC in Yeast Medium (YM, Scharlau) and the other yeasts were cultured in the same medium at 25ºC for 48 h.

4.2.3.2. Agar disc diffusion assay

The antimicrobial activity was evaluated according to the National Committee for Clinical Laboratory Standards (NCCLS) (1997) using the agar disc diffusion method. Briefly, a 24 or 48 h-old culture of selected bacteria/yeast was mixed with sterile physiological saline solution (0.85%) and the turbidity was adjusted to the McFarland scale 0.5 [~10^6 colony forming units (CFU) per mL]. Petri plates containing 20 mL of Mueller Hinton agar (MHA, Scharlau) were used for all tested bacteria and yeasts were cultured in MHA supplemented with 2% glucose (w/v) and 0.5 μg/L methylene blue dye (pH 7.2–7.4). The inoculum was spread on the surface of the solidified media and allowed to dry for 10 min. Filter paper discs (6 mm in diameter) were placed on the previously inoculated plates and impregnated with 20 μL of extract at 50 mg/mL (1 mg extract/disc). Chloramphenicol (30 μg/disc, Oxoid, UK) was used as positive control for bacteria (except for *P. aeruginosa* ATCC 27853 where gentamicin at 10 μg/disc was used) and amphotericin B (20 μg/disc, Sigma) for yeasts. Paper discs impregnated with

20 μL of saline solution, methanol, water or *n*-hexane were used as negative controls. Plates of bacteria and *C. albicans* strains were incubated at 37ºC for 24 h and the other yeasts at 25ºC for 48 h. The inhibition zone diameters (IZDs) were measured in mm. All the tests were performed in triplicate.

4.2.3.3. Minimum inhibitory concentration (MIC) determination

A broth dilution susceptibility assay was used, as recommended by NCCLS, for the determination of the MIC values (1999). The tests were performed in Mueller Hinton Broth medium (MHB, Scharlau) supplemented with 2% (w/v) glucose for the yeasts. Plant extracts, chloramphenicol and amphotericin B were serially-diluted with culture medium from a previously prepared stock solution. Each tube, containing 1 mL of extract/drug dilution, was inoculated with 200 μL of standard bacterial/yeast suspension; the inoculum concentration for standard MIC was 2×10^5 to 8×10^5 CFU/mL. Test tubes with methanol, water, *n*-hexane, DMSO and ethanol were also used as negative controls. For bacteria and *C. albicans* strains, tubes were incubated (under normal atmospheric conditions) at 37ºC for 24 h and for yeasts at 25ºC for 48 h. Results were expressed as the lowest concentration of extract which completely inhibited visible growth (turbidity on liquid media). The MIC was not determined for microorganisms that were inactive in the agar diffusion assay. All the tests were performed in triplicate.

4.2.4. Statistical analysis

The results of the antioxidant assays were subjected to one-way analysis of variance (ANOVA) to assess treatment differences using the SPSS statistical package for Windows (release 15.0; SPSS Inc., Chicago, IL, USA). Significant differences between means were determined using Duncan's New Multiple Range Test ($P = 0.001$).

4.3. RESULTS AND DISCUSSION

4.3.1. Antioxidant capacity

The methanol extract prepared from *P. lusitanica* and the *n*-hexane, water and methanol extracts prepared from *D. intermedia* were submitted to the ORAC, TEAC and F-C assays to determine their AOC (Figure 4.3.1). The results show that the *P. lusitanica* methanol extract has the highest AOC ($P < 0.001$), according to the ORAC and F-C assays, scoring values of 341.4 ± 18.3 $\mu mol_{TE}/g_{extract}$ and 677.7 ± 54.7 $\mu mol_{GAE}/g_{extract}$, respectively.

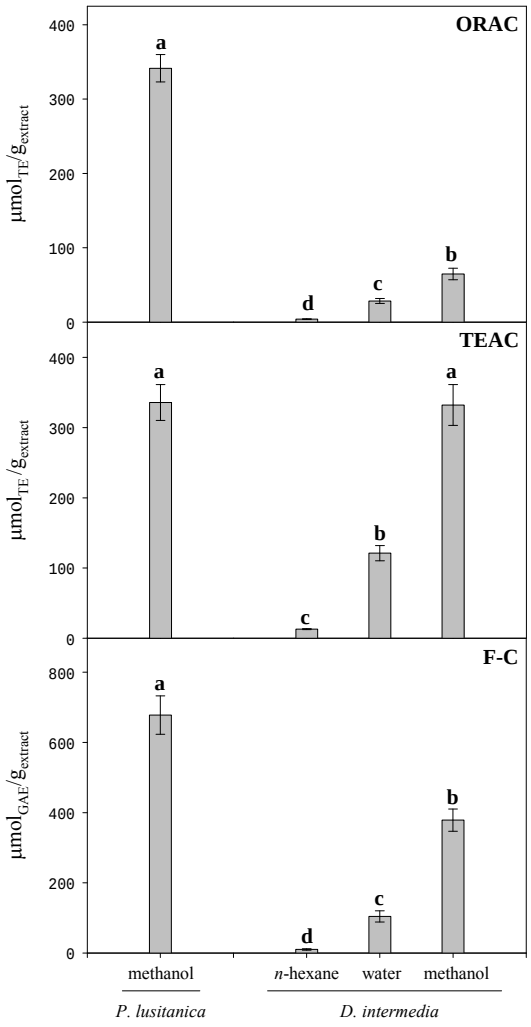


Figure 4.3.1 - AOC of *P. lusitanica* and *D. intermedia* extracts determined by the ORAC, TEAC and F-C assays. In each graph, columns with different letters are significantly different at $P < 0.001$ according to Duncan's multiple range test.

The same result was not observed in the TEAC assay as it indicated similar activity for the methanol extracts of *P. lusitanica* (335.8 ± 25.6 $\mu mol_{TE}/g_{extract}$) and *D. intermedia* (332.2 ± 29.1 $\mu mol_{TE}/g_{extract}$). This result might indicate a decreased affinity of the components of the *P. lusitanica* extract towards the $ABTS^{+\bullet}$ radical in comparison to the radical sources tested in the other assays. The largest difference in AOC between the *P. lusitanica* and *D. intermedia* extracts was obtained in the ORAC assay which suggests that the components of the *P. lusitanica* extract are better H donors and are therefore more effective at neutralizing radicals through the HAT mechanism. The major components of the *P. lusitanica* extract are acteoside and mussaenosidic acid (Section 3.3.1.4); however, it is assumable that the major contribution to the extract's AOC comes from acteoside and its derivatives considering that several studies have pointed out that this phenylethanoid glycoside is a very strong antioxidant and that the compounds belonging to the family of the iridoids generally have weak activity (Shahat et al., 2005; Harput et al., 2006; Es-Safi et al., 2007). In fact, Aligiannis et al. (2003) demonstrated that the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging capabilities of acteoside are comparable to that of butylated hydroxytoluene and α-tocopherol and suggested the potential of using acteoside as a natural protective agent against oxidative rancidity.

Structure-activity relationship studies have shown that phenolic hydroxyls play an important role in the antioxidant activity of acteoside and related phenylethanoid glycosides (Fu et al., 2008). The two catechol (*O*-dihydroxy) units belonging to the phenylethanoid and the caffeoyl moieties are very vulnerable to loss of a proton or an electron and capable of forming stable species due to resonance delocalization and are therefore at the base of the radical scavenging properties of acteoside (Figure 4.3.2).

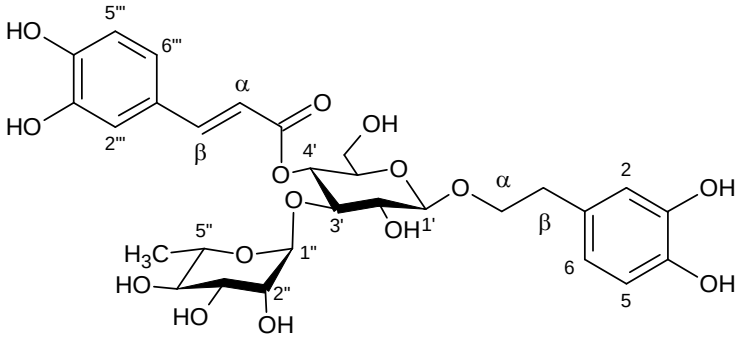
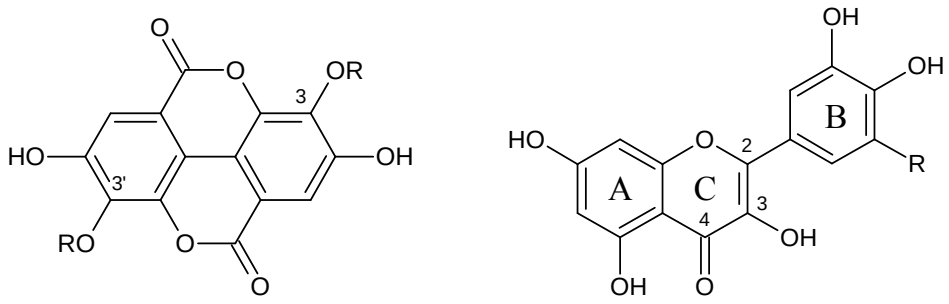


Figure 4.3.2 - Structure of acteoside.

In addition, the double bond at the olifinic chain of the caffeoyl moiety confers extra stability to the resulting free radicals (Es-Safi et al., 2007). Phenylethanoid glycosides bearing only two separate phenol moieties showed very low AOC in comparison to acteoside (Wong et al., 2001). The same author suggested that the antioxidant activities observed for acteoside are most likely attributable to their proton-donating capacities, supporting the relatively high AOC observed in the ORAC assay.

A recent review deals with the topic of naturally occurring phenylethanoid glycosides and their potential as leads for therapeutic activities (Fu et al., 2008). Besides the free radical scavenging capabilities of acteoside, the review article highlights a number of interesting biological activities. Acteoside has been reported to have significant neuroprotective activities against glutamate-induced toxicity in primary cultures of rat cortical cells (Koo et al., 2006); cytotoxic activity against HepG2 cells (Ahmed et al., 2009); and has been shown to inhibit HIV type-1 reverse transcriptase (Fu et al., 2006) and HIV-1 integrase *in vitro* (Kim et al., 2001). These findings suggest that acteoside might be an interesting lead structure to be further investigated and submitted to new biological assays.

The results presented in Figure 4.3.1 also show that the extracts prepared from *D. intermedia* have very different AOCs which is expectable considering their distinct compositions. The results show that in all assays the methanol extract has the greatest AOC (ORAC: 64.7 ± 7.8 $\mu mol_{TE}/mg_{extract}$; TEAC: 332.2 ± 29.1 $\mu mol_{TE}/mg_{extract}$; F-C: 378.6 ± 31.5 $\mu mol_{GAE}/mg_{extract}$). Phytochemical analyses revealed that the methanol extract was mainly composed by two groups of natural products, namely ellagic acid derivatives and flavonols (Section 3.3.2.3). Both classes of compounds have phenolic groups in their structures and therefore are expected to possess strong radical scavenging activity (Figure 4.3.3). When these compounds react with a free radical, it is the delocalization of the unpaired electron over the aromatic nucleus (resonance effect), that prevents the continuation of the free radical chain reaction (Tsao and Deng, 2004). The chemical structures of these compounds are suggestive of chain-breaking antioxidants since the hydrogen atom on the phenolic OH groups is prone to donation with subsequent formation of phenoxyl radicals (Laranjinha et al., 1994).



R=H: ellagic acid
R=CH_3: 3,3'-di-*O*-methylellagic acid

R=H: quercetin
R=OH: myricetin

Figure 4.3.3 - Aglycone structure of the major ellagic acid derivatives and flavonols identified in the *D. intermedia* methanol extract.

Flavonoids are known to be very efficient radical scavengers and this activity can be related with some determinant structural aspects (Middleton et al., 2000). Studies have shown that the catechol or pyrogallol (trihydroxy) group in the B ring in conjunction with the C2-C3 double bond of the C ring (Figure 4.3.3) confer great scavenging ability because of the stability of the produced phenoxyl radicals. In addition, the keto double bond at position 4 of the C ring, especially in association with the C2-C3 double bond, increases scavenger activity by delocalizing electrons from the B ring. Also, the 3-OH group on the C ring, in addition to the C2-C3 double bond and 4-oxo group, forms an extremely active scavenger, and compounds bearing this structural combination appear to have the best combination on top of the catechol group (Rice-Evans and Miller, 1998). These structural aspects explain why quercetin and myricetin are the flavonoids with the most efficient radical scavenging activity (Middleton et al., 2000). Several glycosilated derivatives of both flavonoids were identified in the methanol extract of *D. intermedia*, however, it is assumable that their activities will be somewhat compromised by the sugar moieties attached at the 3-OH group of the aglycone. In addition, the major compounds present in the methanol extract are ellagic acid and 3,3'-di-*O*-methylellagic acid and it is therefore assumable that they are responsible for the largest contribution to the extract's AOC profile. Despite the fact that ellagic acid bears two catechol groups in its structure, it has been reported to be a poorer radical scavenger in comparison to quercetin and myricetin (Kim and Lee, 2004). This might be due to internal hydrogen bonding between the oxygen atom of the carbonyl group present in the lactonic ring and the proximate phenolic OH group which reduces the ability of ellagic acid to donate a phenolic hydrogen atom to an attacking radical (Laranjinha et

al., 1994). In fact, it has been suggested that the presence of electron withdrawing substituents in phenolic molecules decreases reactivity with peroxyl radicals (Burton et al., 1985), which might explain the relative low reactivity of the methanol extract in the ORAC assay. In addition, it is expected that the AOC of 3,3'-di-*O*-methylellagic acid is even lower than ellagic acid, because of the two methylated phenolic hydroxyls which compromise its ability to react with free radicals. Overall, the *D. intermedia* methanol extract might have lower AOC in comparison to the *P. lusitanica* extract due to its more complex composition implicating a lower concentration of each individual antioxidant compound. In addition, the compounds with the highest reported antioxidant potential (myricetin and quercetin derivatives) belong to the minor components of the extract.

Figure 4.3.1 shows that the *D. intermedia n*-hexane extract had the lowest AOC (ORAC: 4.1 ± 0.5 $\mu mol_{TE}/mg_{extract}$; TEAC: 12.9 ± 0.7 $\mu mol_{TE}/mg_{extract}$; FC: 10.2 ± 1.9 $\mu mol_{GAE}/mg_{extract}$) ($P < 0.001$). Phytochemical analysis pointed out that the extract was composed by mainly one compound, the naphthoquinone plumbagin. The structure of plumbagin is based on a naphthalene ring substituted at positions 1 and 4 with two ketone groups, forming the quinone moiety (Figure 4.3.4).

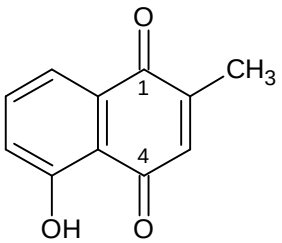


Figure 4.3.4 - Structure of plumbagin.

The two electron withdrawing ketone groups are responsible for the compounds' electrophilic nature which makes plumbagin unlikely to donate protons, but on the contrary, prone to act as a prooxidant. In fact, Murakami et al. (2010) showed that plumbagin was able to stimulate lipid peroxidation of microsomes from rat liver. Noteworthy, the water extract which was characterized by having a low content in secondary metabolites which could not be identified, showed superior AOC in comparison to the *n*-hexane extract (ORAC: 28.4 ± 3.4 $\mu mol_{TE}/mg_{extract}$; TEAC: 121.1 ± 10.8 $\mu mol_{TE}/mg_{extract}$; FC: 104.5 ± 16.1 $\mu mol_{GAE}/mg_{extract}$).

It is interesting to see that the obtained AOC values for the different *D. intermedia* extracts show a consistent order of activity between the tested methods (Figure 4.3.1), even though different reaction substrates and mechanisms are involved, suggesting that the antioxidant components follow a similar reaction mechanism with the free radicals and the F-C reagent. Although no related species to *P. lusitanica* and *D. intermedia* have been investigated for their AOC, rendering useless any comparison of results, the present results show that these species are sources of compounds with considerable antioxidant activity.

4.3.2. Antimicrobial activity

The antimicrobial activity of the methanol extract of *P. lusitanica* and the *n*-hexane, water and methanol extracts of *D. intermedia* was determined by the agar diffusion assay and by determination of MIC values against a panel of bacterial and yeast strains (Table 4.3.1). Except for the *P. lusitanica* extract, all the extracts showed significant inhibition of microbial growth based on the obtained inhibition zone diameter (IZD) values. For this reason the MICs were only determined for the *D. intermedia* extracts.

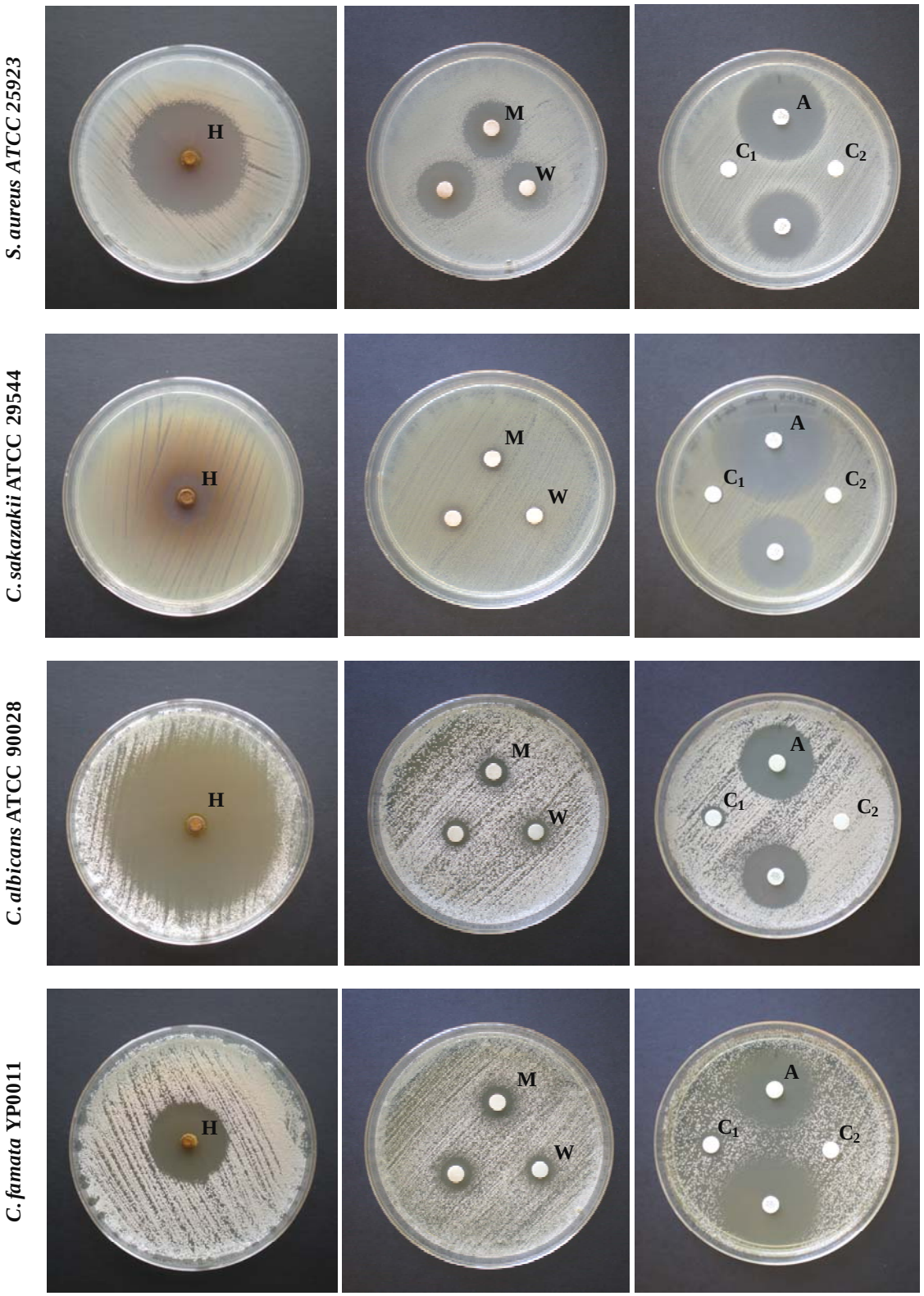
4.3.2.1. *D. intermedia*

Of the extracts prepared from *D. intermedia*, the *n*-hexane extract showed greater IZD values (14.3 ± 1.2 to 59.0 ± 0.6 mm) than water (8.7 ± 0.3 to 43.3 ± 1.3 mm) and methanol (9.0 ± 0.6 to 43.0 ± 0.6 mm) extracts (Table 3.4.1, Figure 4.3.5). The results obtained by the MIC determination assay further accentuate the fact that the *n*-hexane has by far the greatest antimicrobial activity. The *n*-hexane extract yielded MIC values under 100 μg/mL against nine of the tested strains, showing remarkable activity for a crude extract. In the case of *S. aureus* ATCC 25923 and *C. albicans* YP0175, the obtained MIC values for the *n*-hexane extract were over 100 fold inferior to those of the water and methanol extracts: 26.0 μg/mL, 13.0 μg/mL for the *n*-hexane extract, 5208.3 μg/mL, 5208.3 μg/mL for the water extract, and 5208.3 μg/mL, 2608.3 μg/mL for the methanol extract, respectively. These results were expectable considering that the main component of the *n*-hexane extract, the naphthoquinone plumbagin, is a potent antimicrobial agent for which activity had been previously demonstrated (Didry et al., 1998; Lim et al., 2007; Gonçalves et al., 2009).

Table 3.4.1. Antimicrobial activity of the *P. lusitanica* methanol extract and *n*-hexane, water and methanol extracts of *D. intermedia* determined by the agar disc diffusion and minimum inhibitory concentration (MIC) method.

	P. lusitanica	*D. intermedia*							
	Methanol	*n*- Hexane		Water		Methanol		Standard [a]	
Microorganism	IZD[b] (mm)	IZD[b] (mm)	MIC (µg/mL)	IZD[b] (mm)	MIC (µg/mL)	IZD[b] (mm)	MIC (µg/mL)	IZD[b] (mm)	MIC (µg/mL)
Bacteria									
E. faecalis ATCC 29212	-	21.0 ± 0.6	52.1	-	ND	-	ND	16.0 ± 0.6	33.3
S. aureus ATCC 25923	12.3 ± 0.9	43.3 ± 0.7	26.0	21.7 ± 1.8	5208.3	24.0 ± 1.5	5208.3	20.3 ± 0.3	8.3
S. epidermidis ATCC 12228	12.7 ± 0.7	50.7 ± 1.5	13.0	43.3 ± 1.3	650.0	43.0 ± 0.6	325.0	23.7 ± 0.3	8.3
C. sakazakii ATCC 29544	-	19.7 ± 1.2	208.3	9.0 ± 0.0	> 10000	9.7 ± 0.3	> 10000	23.0 ± 1.0	8.3
C. sakazakii ATCC BA-894	-	19.0 ± 0.6	208.3	9.0 ± 0.0	> 10000	9.0 ± 0.6	5208.3	24.0 ± 0.6	16.7
E. coli ATCC 25922	-	14.3 ± 1.2	208.3	11.3 ± 1.9	> 10000	13.7 ± 2.2	> 10000	24.0 ± 0.6	4.2
P. aeruginosa ATCC 27853	-	-	ND	-	ND	8.7 ± 0.3	> 10000	23.0 ± 0.6	ND
Yeast									
C. albicans ATCC 10231	-	41.7 ± 1.5	52.1	19.0 ± 0.6	5208.3	23.3 ± 2.2	5208.3	32.3 ± 1.2	0.1
C. albicans ATCC 90028	-	44.3 ± 0.3	26.0	9.7 ± 0.3	1300.0	11.0 ± 0.0	1300.0	30.7 ± 1.5	0.03
C. albicans YP0175	-	45.7 ± 0.7	13.0	14.7 ± 1.2	5208.3	15.0 ± 1.2	2608.3	30.3 ± 0.9	0.1
C. catenulata YP0160	-	38.3 ± 0.9	52.1	11.3 ± 1.2	2608.3	12.7 ± 1.5	1300.0	30.0 ± 0.0	0.13
C. famata YP0011	-	30.7 ± 1.5	104.2	11.0 ± 0.6	> 10000	12.0 ± 0.6	5208.3	27.0 ± 0.6	0.1
C. guillermondi YP0170	-	39.7 ± 0.3	52.1	8.7 ± 0.3	2608.3	10.3 ± 0.7	5208.3	28.7 ± 0.7	0.1
C. neoformans YP0186	-	59.0 ± 0.6	104.2	24.0 ± 0.6	1300.0	28.7 ± 0.7	650.0	32.0 ± 0.6	0.3
T. mucoides YP0096	-	34.0 ± 1.2	52.1	12.0 ± 1.5	5208.3	12.7 ± 1.2	2608.3	31.0 ± 1.0	0.1

[a] For agar diffusion method chloramphenicol (30 µg/disc) was used as control for bacteria, except for *P. aeruginosa* ATCC 27853 where gentamicin (10 µg/disc) was used, and amphotericin B for yeasts (20 µg/disc); [b] Extracts were tested at 1 mg/disc; IZD Inhibition zone diameter, including disk diameter of 6 mm; (-) inactive; ND not determined; values represent mean ± standard error of 3 repetitions.



Caption: **H** - *n*-hexane extract; **M** - methanol extract; **W** - water extract; **A** - reference antibiotic (chloramphenicol for bacteria, amphotericin B for yeasts); **C_1** - methanol; **C_2** - saline solution.

Figure 4.3.5 - Growth inhibition zones of selected microorganisms induced by *D. intermedia* extracts and reference antibiotics and solvents.

The chemical basis for the toxicity and pharmacology of quinones has been examined extensively and two primary mechanisms have been proposed to account for their actions (Inbaraj and Chignell, 2004; Rodriguez et al., 2004; Castro et al., 2008). Following the first mechanism, quinones can undergo one-electron reduction to semiquinone-free radicals by electron-transferring enzymes such as NADPH-cytochrome P450 reductase and mitochondrial NADH-ubiquinone oxidoreductase. These free radicals are oxidized back to quinones by transferring electrons to O_2, generating the superoxide anion and other ROS (Figure 4.3.6 A). This reduction and oxidation cycle of quinones is defined as redox cycling. Because of the catalytic nature of this redox process, one equivalent of quinone can generate multiple equivalents of superoxide and in this way overwhelm the protective, antioxidant systems in the cell.

Alternatively, quinones can act as potent electrophiles capable of reacting directly with protein thiol groups via 1,4-addition chemistry, leading to covalent modification of biological molecules at their nucleophilic sites and resulting in loss of function (Figure 4.3.6 B). Although the ability of quinones to act as either electrophiles or redox cycling agents is well established, the degree to which these properties contribute to their overall toxicity is unclear and will be highly dependent on their chemical properties and the conditions of cellular exposure. Determination of the contribution of these two mechanisms to the overall toxicity of many quinones is difficult since both mechanisms can operate under most experimental conditions (Rodriguez et al., 2004). Because the redox cycling mechanism can only occur in the presence of oxygen, the activity of plumbagin was tested against *Saccharomyces cerevisiae*, a facultative anaerobic organism, in the presence and absence of oxygen in an attempt to discriminate the mechanism responsible for the activity of plumbagin (Castro et al., 2004). The authors showed that plumbagin exerted mainly toxicity by binding with proteins and to a lesser extent by generating oxidative stress. It would be interesting to test the bioactivity of the *n*-hexane extract in the absence of oxygen in order to determine the toxicity mechanism exerted against the tested yeast strains.

The only microorganism tolerant to the *n*-hexane extract of *D. intermedia* was *P. aeruginosa* ATCC 27853, which comes as no surprise since it has a very high level of intrinsic resistance to virtually all known antimicrobials and antibiotics due to a

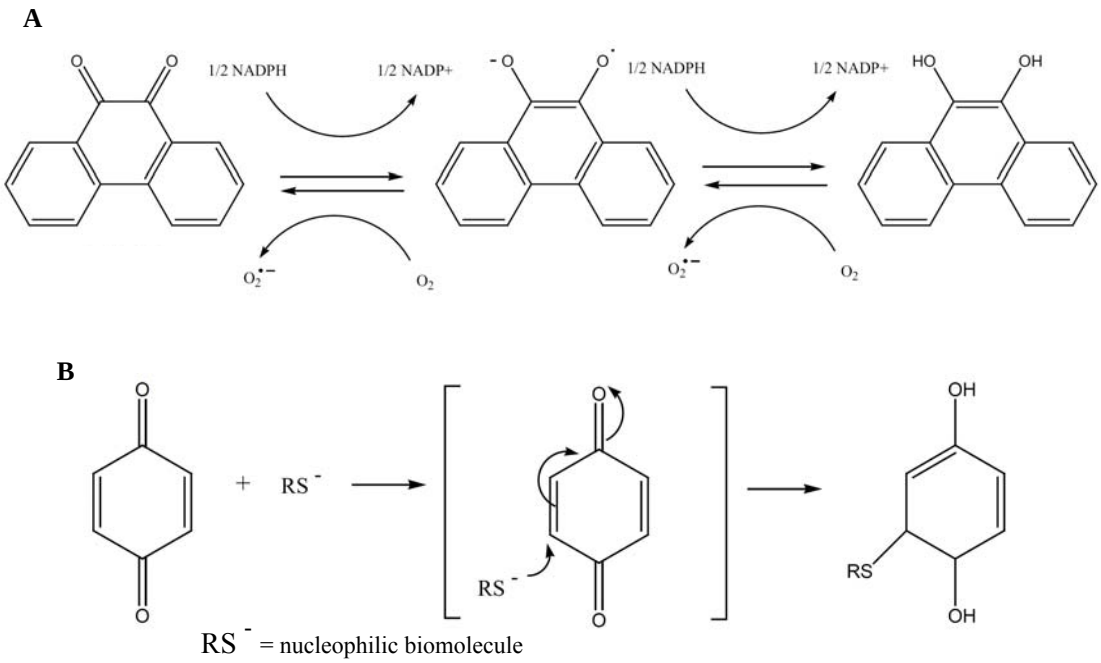


Figure 4.3.6 - Toxic reaction pathways of quinones: generation of reactive oxygen species by redox cycling mechanism (**A**); nucleoophilic 1,4-addition reaction (**B**) (Rodriguez et al., 2004).

combination of a very restrictive outer membrane barrier and efficient efflux mechanisms (Nikaido, 1999). This result could be confirmed by a work perfomed by Tegos et al. (2002), which could not detect the MIC at the limit of solubility of plumbagin (> 500 μg/mL) against *P. aeruginosa* as well. The standard chloramphenicol is not effective against this bacterial strain (Gonçalves et al., 2009) and gentamicin was used for the agar diffusion method instead.

In general, Gram-negative bacteria are less susceptible to antimicrobial products than Gram-positive because their cell walls present a more significant barrier to entry. As opposed to Gram-positive bacteria which have a more permeable outer peptidoglycan layer, Gram-negative bacteria have a efficient phospholipidic outer barrier, with narrow porin channels which limit the penetration of hydrophobic molecules and a low fluidity lipopolysaccharide leaflet which slows down the inward diffusion of lipophilic products (Simões et al., 2008). The presence of efflux systems consisting of multidrug resistance pumps (MDRs) coupled with low permeability of the outer membrane which restricts diffusion of antimicrobials into the cells is responsible for the very high intrinsic resistance of Gram-negative bacteria. The results presented in Table 4.3.1 are consistent with this knowledge, since the tested Gram-negative bacteria (*C. sakazakii* ATCC

29544, *C. sakazakii* ATCC BA-894, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853) were overall less affected by the *D. intermedia* extracts.

The IZD values presented in Table 3.4.1 also show that the water and methanol extracts were able to inhibit the growth of all tested microorganisms, except for *E. faecalis* ATCC 29212 and *P. aeruginosa* ATCC 27853 in the case of the water extract. Although showing less activity than the *n*-hexane extract, the water and methanol extracts have considerable activity, which could explain the use of *D. intermedia* for the treatment of various respiratory diseases (Melzig et al., 2001; Paper et al., 2005). Overall, the most susceptible bacterial strain to the *D. intermedia* extracts is *S. epidermidis* ATCC 12228 (MIC: 13.0 µg/mL, 650.0 µg/mL, 325.0 µg/mL for *n*-hexane, water and methanol, respectively) and *P. aeruginosa* ATCC 27853 the most tolerant (MIC: not determined for *n*-hexane and water; > 10000 µg/mL for methanol).

An interesting result was that, in contrast to the *n*-hexane extract, the methanol extract showed a slight activity against *P. aeruginosa*. This indicates that the growth inhibition is not only exerted by a different compound, but also that it is active by a different mechanism. Noteworthy, Krolicka et al. (2008) also reported activity of a methanol extract of *Dionaea muscipula*, which has a similar secondary metabolite profile as *D. intermedia* (Section 3.3.3; Pakulski and Budzianowski, 1996a; 1996b), against *P. aeruginosa*, while the chloroform extract containing plumbagin was ineffective. Some flavonoids, including myricetin, have been reported to have antimicrobial activity against Gram-negative and Gram-positive bacteria by inhibiting the synthesis of nucleic acids (Cushnie and Lamb, 2005). Therefore, the same author tested the bactericidal activity of myricetin and quercetin against *P. aeruginosa* which proved to be ineffective, suggesting that the ellagic acid derivatives might be responsible for the observed activity. Ellagic acid and its derivatives have been shown to have antimicrobial activity (Atta et al., 2001), which could explain the activity of the methanol extract against the panel of selected bacteria and yeasts. It is believed that the antimicrobial activity of ellagic acid and other phenolic phytochemicals is related with the fact that these compounds are weak acids capable of dissociating at the cell membrane, creating a proton gradient which alters the function of many proton pumps on the cell membrane and changes its resting potential (Mirzoeva et al., 1997; Choi and Gu, 2001). However, the reported activities are moderate and no activity was shown for

ellagic acid against *P. aeruginosa* (Reddy et al., 2007). Another possibility for the activity of the methanol extract against *P. aeruginosa* and other tested strains is a synergistic action between flavonoid glycosides or ellagic acid derivatives and plumbagin, which might have been extracted in residual amounts. For example, it has been shown that quercetin-3-rutinoside does not exhibit antibacterial activity but enhances significantly the antimicrobial properties of its aglycone quercetin (Arima et al., 2002). The phenomenum can be the result of cooperation of metabolites strongly influencing permeability of bacterial cell walls and of metabolites with effective antimicrobial activity. A number of plant metabolites have been identified which, although not being antimicrobial themselves, are capable of inhibiting MDRs, thereby potentiating the action of antimicrobials that would otherwise be extruded from the bacterial cell (Gibbons, 2005; Krolicka et al., 2008). In fact, ellagic acid has been shown to enhance the susceptibility of the Gram-negative bacterium *Acinetobacter baumannii* against a variety of antibiotics (Chusri et al., 2009). Taking into account the high resistance of *P. aeruginosa* against most antibiotics, it would be interesting to investigate this theory by fractionating the extract and testing these fractions independently and in combination against *P. aeruginosa* or by adding plumbagin to the crude extract to determine if its action against the pathogen is potentiated in a concentration dependent manner.

In the case of the yeasts, discrepant activities were observed for the *D. intermedia* extracts as well. Based on the obtained MIC values, the most tolerant strain to the *n*-hexane extract was *C. neoformans* YP0186 (104.2 μg/mL), while it was the most susceptible strain to the water and methanol extracts (1300.0 μg/mL and 650.0 μg/mL, respectively). This result suggests that more than one group of compounds with different action mechanisms are responsible for the activity. Overall, the most susceptible yeast to the *D. intermedia* extracts is *C. albicans* ATCC 90028 (MIC: 26.0 μg/mL, 1300.0 μg/mL, 1300.0 μg/mL for *n*-hexane, water and methanol extracts, respectively) and the most tolerant is *C. famata* YP0011 (MIC: 104.2 μg/mL, > 10000 μg/mL, 5208.3 μg/mL for *n*-hexane, water and methanol extracts, respectively).

4.3.2.2. *P. lusitanica*

In contrast to the *D. intermedia* extracts, the methanol extract of *P. lusitanica* showed little activity against the panel of microorganisms (Figure 4.3.7). Only the Gram-positive bacteria *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 12228 showed susceptibility with inhibition zones of 13.3 ± 0.9 mm and 12.7 ± 0.7 mm (Table 4.3.1), respectively. The tested Gram-negative bacteria and yeasts were tolerant to the *P. lusitanica* methanol extract and therefore the MIC's were not determined for this extract. However, moderate antimicrobial activity has been reported for the major extract components, namely acteoside (Didry et al., 1999) and iridoid glucosides structurally related to mussaenosidic acid (Ishiguro et al., 1983). Interestingly, removal of the sugar moiety of the iridoid glucoside aucubin by treatment with β-glucosidase resulted in a potent antimicrobial capable of inhibiting the growth of several Gram-negative and Gram-positive bacteria (Davini et al., 1986). This seems to be related with the plant's strategy to store anti-infective agents which become only active after endogenous enzymatic hydrolysis induced by herbivore attack (Section 3.3.1.4; Konno et al., 1999). For this reason, it could prove worthwhile to test the corresponding aglycones of the iridoids identified in *P. lusitanica* to determine their effective antimicrobial potential.

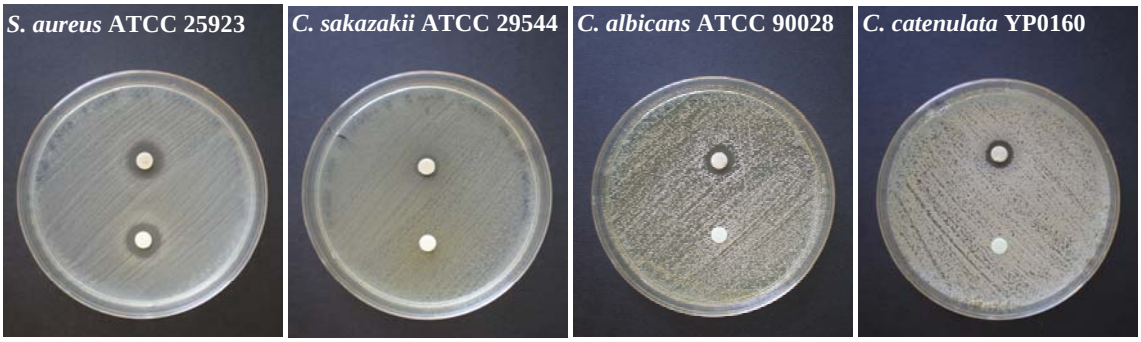


Figure 4.3.7 - Growth inhibition zones induced by *P. lusitanica* methanol extract (top) against selected bacterial and yeast strains.

4.3.3. Evaluation of antimicrobial assays

When comparing the results obtained by the agar diffusion method and the MIC determination method one can see that they are not always coherent. For example,

C. neoformans was one of the most tolerant yeast strains to the *n*-hexane extract according to the obtained MIC value (104.2 μg/mL), while showing the largest IZD (59.0 ± 0.6 mm). This can be explained by the fact that the strains which present the largest IZDs are not always the most sensitive, as antimicrobial effectiveness of a compound is compromised by factors such as extract solubility, the diffusion range in the agar and the evaporation of the extract's solvent (Hernández et al., 2005). For this reason, the MIC method should prevail over agar diffusion methods in evaluating the antimicrobial activity of an active principle. Also, the fact that the agar diffusion method does not highlight the higher activity of the *n*-hexane extract of *D. intermedia*, to the same extent as the dilution method, can be explained by the low solubility of *n*-hexane as solvent carrier in an aqueous based agar media.

4.3.4. Potential of *D. intermedia* metabolites as antimicrobial agents

Plant antimicrobials are not used as systemic antibiotics at present. Although there are a significant number of phytochemical classes with antibacterial potential, none has reached clinical application (Tegos et al., 2002; Gibbons, 2005; Simões et al., 2008). In most cases, the concentrations required for phytochemicals to exert activity are too high to be clinically relevant, i.e., the reported MICs for plant antimicrobials are often in the range of 100 to 1000 μg/ml, orders of magnitude higher than those of common broad-spectrum antibiotics from bacteria or fungi (MICs 0.01 to 10 μg/ml). Those rare plant antimicrobials that are effective and that have broad-spectrum activities, like pyrithione of *Polyalthea nemoralis* (Han et al., 1981), are fairly toxic antiseptics. The case of plumbagin is similar, despite its relatively broad range of activity and high effectiveness, plumbagin is an unlikely candidate as an antimicrobial agent, due to its toxicity and low therapeutic selectivity (Kayser et al., 2003). The remaining major secondary metabolites produced by *D. intermedia*, namely ellagic acid derivatives and flavonoid glucosides are reported in literature as antimicrobial compounds and showed activity against the panel of selected microorganisms, however their activities are possibly too weak to be considered as potential candidates for drug development.

It is generally accepted that phytochemicals are less potent anti-infectives than agents of microbial origin (Yamada, 1991; Cushnie and Lamb, 2005). Nevertheless, research programs combining phytochemical screening and biological assays continue to be of

importance as new classes of antimicrobial drugs are urgently required (Levy, 1998; Cowan, 1999). Future optimisation of these compounds through structural alteration may allow the development of a pharmacologically acceptable antimicrobial agent or group of agents (Cushnie and Lamb, 2005). Despite the fact that the activity identified by an *in vitro* test does not necessarily confirm that a plant extract is an effective medicine, nor a suitable candidate for drug development, it does provide basic understanding of its efficacy and in some cases toxicity (Alviano and Alviano, 2009). In addition, phytochemicals should continue to be considered as a source of potential antimicrobials following the ecological rationale that antimicrobial natural products are produced to protect the plant from pathogenic microbes in its environment (Gibbons, 2005).

A new insight concerning the potential of plant derived antimicrobials has been given by Tegos et al. (2002). The authors showed a striking increase in antimicrobial activity of several plant metabolites by disabling the multidrug resistance pumps (MDRs) in Gram-negative bacteria and suggested that plant antimicrobials can be developed into effective, broad-spectrum antibiotics in combination with inhibitors of MDRs. For instance, the activity of rhein, a metabolite produced by *Rheum officinalis*, was potentiated 100- to 2,000-fold, depending on the bacterial species, by disabling the MDRs. Comparable results were obtained for plumbagin. This strategy was inspired on *Berberis* plants which besides the strong antimicrobial berberine, were shown to produce the MDR disruptors 5'-methoxyhydnocarpin D and pheophorbide A, which facilitate the penetration of berberine into attacking microorganisms. *Berberis* plants do not have known bacterial pathogens, suggesting that they have developed means of delivering their antimicrobials into bacterial cells, following a similar mechanism as the putative synergistic action between the *D. intermedia* compounds previously described in section 4.3.2.1. The study performed by Tegos et al. (2002) shows that plant antimicrobials are potentially as effective as conventional antibiotics produced by bacteria and fungi if they are delivered into the pathogen cell. The potentiation of these metabolites by MDR inhibitors opens the possibility for the development of combination therapy.

4.4. CONCLUSIONS

The AOC of extracts prepared from *P. lusitanica* and *D. intermedia* was evaluated by the ORAC, TEAC and F-C assays. The results indicated that the methanol extract of *P. lusitanica* has the highest AOC, which is possibly due to one of its major components, acteoside. Acteoside is a secondary metabolite which has shown activity against several biological targets and is a potential candidate for further studies. The *D. intermedia* methanol extract also showed considerable AOC which can be explained by the combined activity of ellagic acid derivatives and flavonoid glucosides. The same extracts were also subjected to antimicrobial assays, in which their capacity to inhibit the growth of several bacteria and yeasts were evaluated by the agar disc diffusion and MIC methods. In this case the most effective extract was the *n*-hexane extract of *D. intermedia* inhibiting the growth of all tested microorganisms, except *P. aeruginosa*. The observed activity can be explained by the fact that the major component of the extract is plumbagin, a naphthoquinone reported to have remarkable antimicrobial potency.

The bioactivity of plumbagin is most likely explained by its electrophilic nature and its ability to bind to proteins and other biomolecules, but also by its capacity to induce the production of ROS in cellular organisms by the redox cycling mechanism, which explains the low AOC values obtained in the antioxidant assays. Despite the promising results of this preliminary assay, plumbagin is an unlikely candidate due to its toxicity and low therapeutic selectivity. Interestingly, the methanol extract was able to inhibit slightly the growth of the multidrug resistant *P. aeruginosa*, possibly by a membrane destabilizing effect induced by the ellagic acid derivatives. Considering the high resistance of this microorganism to most antibiotics it would be interesting to determine the underlying mechanism. The *P. lusitanica* extract showed little activity against the panel of microorganisms and was only able to inhibit the growth of two bacteria, namely *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 12228.

This chapter dealt with the preliminary bioactivity screening of extracts prepared from *P. lusitanica* and *D. intermedia*. Overall it was shown that both species are sources of antioxidant compounds and that the metabolites produced by *D. intermedia* have

considerable antimicrobial activity. Despite the fact that the assays did not result in any potential candidate for drug development, nor underline any potential specific application, is it important to continue to submit phytochemicals to screening programs taking into perspective the vast diversity of chemical structures derived from plants and the amount of drugs derived from them which are currently in use.

4.5. REFERENCES

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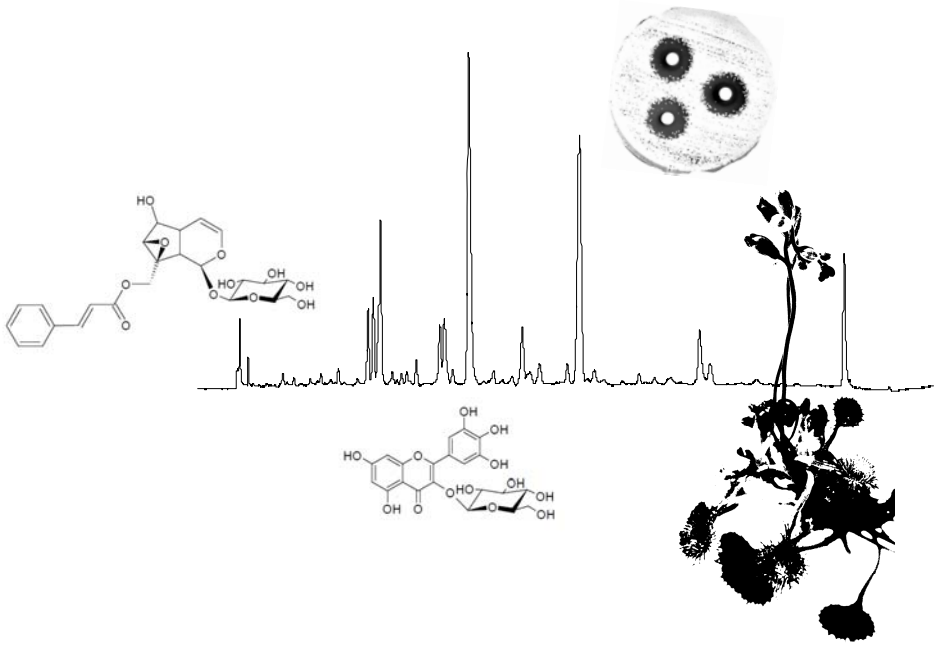
CHAPTER 5

METHOD DEVELOPMENT FOR THE BIOPROSPECTION OF PLUMBAGIN FROM MICROPROPAGATED *D. INTERMEDIA*



5.1. INTRODUCTION

5.1.1. Biocompound extraction from plants

Many higher plants are major sources of useful secondary metabolites which are used in pharmaceutical, agrochemical, flavour and aroma industries (Debnath et al., 2006; Karuppusamy et al., 2009). Attempts to synthesise plant-derived active principles have largely been successful, but in most cases this has proved to be uneconomic in comparison to isolation from plant material. Camptothecin (*Camptotheca acuminata*), podophyllotoxin (*Podophyllum hexandrum* and *P. peltatum*), vinblastine and vincristine (*Catharanthus roseus*) are examples of anticancer compounds that are extracted from their natural sources and not synthesized chemically on a commercial scale due to their complex structure with several chiral centres (Wink et al., 2005). Often, the respective plants are grown in plantations as conservation problems may arise when they are harvested from nature. However, inconsistent production and low yields, as usually secondary metabolites account for less than 1% of the plants' dry weight, make this approach unfeasible in the long term (Georgiev et al., 2009).

In response to these issues, *in vitro* culture technology has become an attractive and cost-effective alternative for the production of high value plant-derived metabolites. Plant cell and tissue cultures can be established routinely under sterile conditions from explants, such as plant leaves, stems, roots and meristems for both multiplication and extraction of secondary metabolites purposes, as they produce and accumulate many of the same valuable chemical compounds as the parent plant in nature. Secondary metabolite production in plant cell and tissue culture has the advantage of being reliable, predictable, generated on a continuous year-round basis without seasonal constraints, and in some cases yields may exceed the ones found in nature (Debnath et al., 2006; Karuppusamy et al., 2009).

In a limited number of instances production of high-value phytochemicals in non-organized *callus* and suspension cultures using bioreactors was successfully implemented. Paclitaxel is an alkaloid that was first isolated from the bark of *Taxus brevifolia*, during a screening program of the United States National Cancer Institute, and is currently produced by plant cell fermentation by Phyton Biotech (Germany). Sold

under the trademark Taxol®, paclitaxel is used in cancer chemotherapy to treat lung, ovarian and breast cancer and is a good example of commercially viable production of secondary metabolites using biotechnological approaches (Wink et al., 2005).

However, undifferentiated callus and suspension cultures very often fail to accumulate the compounds of interest. This situation occurs when the metabolite is only produced in specialized plant tissues or glands in the parent plant and production requires more differentiated microplant or organ cultures. A prime example is the production of ginseng saponins (*Panax ginseng*), for which root culture is required as these saponins are produced specifically in the roots (Karuppusamy, 2009). It is assumed that the genes that encode proteins of secondary metabolite biosynthesis, transport or storage are regulated in a cell- or tissue-specific way as is the situation for most of the genes regulating development and metabolic functions. It is likely that the secondary metabolite related genes are turned off in undifferentiated tissues, which would explain the failure of *callus* and suspension cultures to produce these in significant quantities (Wink, 1987; de Luca and St. Pierre, 2000). On the other hand, organized tissue cultures such as shoot and root cultures, as well as hairy roots, hardly ever fail to synthesize secondary metabolites. Karuppusamy (2009) presents an extensive review on the secondary metabolites produced by *in vitro* tissue, organ and cell cultures. From a literature survey, there has only been one example of commercial production of a higher plant natural product by plant tissue culture and that is the case of *Lithospermum erythrorhizon* cultures, which are used to produce the antiseptic dye shikonin (Philipson, 1994).

5.1.2. The naphthoquinone plumbagin

5.1.2.1 Importance of plumbagin

Plumbagin is the most efficient secondary metabolite isolated so far from carnivorous plants (Eilenberg et al., 2005). This naphthoquinone has received an enormous amount of attention in pharmacological research due to its antimalarial (Likhitwitayawuid *et al.*, 1998), antimicrobial (Didry et al., 1994; Didry et al., 1998, Gonçalves et al., 2009a), antifungal (Ribeiro de Paiva et al., 2003), anticancer (Parimala and Sachdanandam, 1993; Sugie et al., 1998), antimutagenic (Durga et al., 1992), cardiotonic (Itoigawa et

al., 1991), hypolipidemic and antiatherosclerotic effects (Sharma et al., 1991). Despite the broad range of biological activities of plumbagin, its high cytotoxicity and relative low therapeutic selectivity are the major disadvantages that limit its medical application (Kayser et al., 2003). To circumvent this problem several synthetical plumbagin derivatives have been examined to identify products exerting plumbagin-like activity and lower toxicity (Ogihara et al., 1997; Hazra et al., 2002; Tandon et al., 2004; Tandon et al., 2006). Nevertheless, it has been shown recently that at subtoxic concentrations, plumbagin is proving to be an effective agent against several pharmacological targets and has regained the interest of researchers in the field of drug discovery in the past years (Checker et al., 2009; Son et al. 2009; Shieh et al., 2010) (Figure 5.1.1).

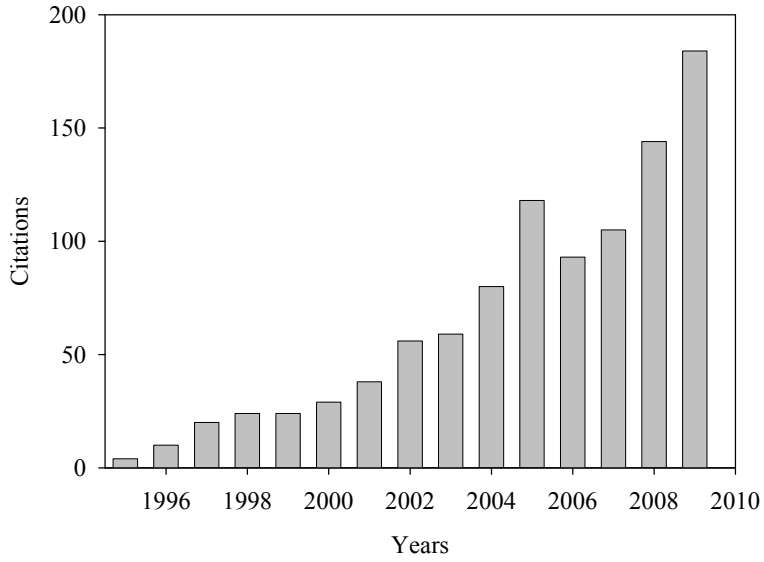


Figure 5.1.1 - Citation number in the period of 1995-2009 of published items with the following search criteria: topic="plumbagin", areas="pharmacology and pharmacy" or "medicinal chemistry". Citation report generated by Web of Science (ISI Web of Knowledge).

Plumbagin and several of its analogues also exert a strong antifeedant activity against *Spodoptera litura* due to a combination of both high volatility and high redox potential (Tokunaga et al., 2004). Furthermore, the inhibition of insect ecdysis and chitin synthetase by plumbagin suggests that plumbagin and its closely related derivatives may serve as environmentally friendly insect control agents and be of value to the agrochemical industry (Kubo et al., 1983).

5.1.2.2. Occurrence and biological significance of plumbagin

Naphthoquinones are one of the groups of secondary metabolites widespread in nature. Plumbagin is produced by a disperse and heterogeneous group of plant families and can be found in members of the Droseraceae (Marckzack et al., 2005), Plumbaginaceae (Ribeiro de Paiva et al., 2003), Nepenthaceae (Rischer et al., 2002), Ebenaceae (Dzoyem et al., 2007) families and also in *Drosophyllum lusitanicum* (Grevenstuk et al., 2008) and *Triphyophyllum peltatum* (Bringmann et al., 2000), which belong to the monotypic families Drosophyllaceae and Dioncophyllaceae, respectively. The production of naphthoquinones is common in carnivorous plants but not exclusive to this group of plants. The ability of naphthoquinone synthesis is not limited to higher plants. Naphthoquinones have also been found in fungal organisms in genera such as *Fusarium*, *Aspergillus*, *Cladosporium*, *Microsporium*, *Mollisia*, *Penicillium*, *Trichophyton* and *Verticillium* and *Actinomycetes* of the genus *Streptomyces* (Medentsev and Akimenko, 1998). Plumbagin, however, has not been found in these organisms to date.

The biosynthetic pathway of plumbagin (Figure 5.1.2) has been studied in plants and it was established that it is synthesized by the acetate-malonate pathway using L-alanine as precursor (Durand and Zenk, 1974). The fact that many plants accumulate this naphthoquinone in significant amounts suggests that it plays an important role in the plants' interaction with its environment (Tokunaga et al., 2004).

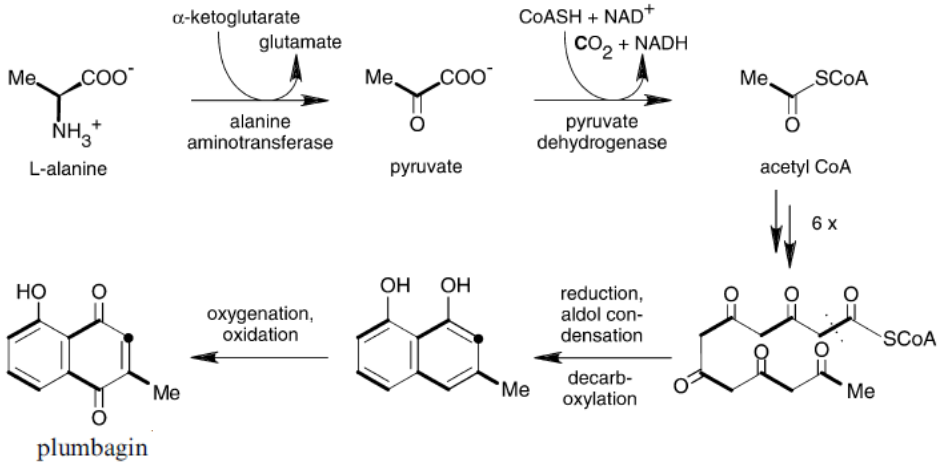


Figure 5.1.2 - Proposed biosynthetic pathway of plumbagin (Rischer et al., 2002).

Besides the effects of plumbagin on vertebrates, there are many reports on its allelochemical effects on organisms of interest in the ecological context. Plumbagin acts as an antifeedant agent on herbivorous insects: it is capable of repelling even adapted *Lepidoptera* (Villavicencio and Perez-Escandon, 1994) and insects feeding on plants containing a critical dose of plumbagin die either immediately (Gonçalves et al., 2008) or during the next ecdysis due to the inhibiton of ecdysteroid (Joshi and Sehnal, 1989) and chitin synthetase production (Kubo et al., 1983). Plumbagin is responsible for fungicidal activity against plant pathogenic species, acts as a potent phytoalexin against parasitic plants (Bringmann et al., 1999) and inhibits the germination of seeds from other species (Spencer et al., 1986; Gonçalves et al. 2009b). These findings support the importance of the production of plumbagin and related naphthoquinones, improving the plants' fitness and conferring an adaptive advantage over other plants (Rischer et al., 2002).

5.1.2.3. Chemical and physical characterization of plumbagin

The chemical structure of naphthoquinones is based on a bicyclic structure, more specifically on a naphthalene skeleton, substituted at positions C1 and C4. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone; Figure 3.3.2.3) is substituted at position C2 and C5 by a methyl and hydroxyl group, respectively. At Standard Temperature and Pressure (STP) conditions it presents a physical state of a yellow crystalline powder and with a melting point of 78-79 ºC it is considered to be a moderately polar compound (Merck 1997). Plumbagin is slightly soluble in hot water; soluble in alcohol, acetone, chloroform, benzene, and acetic acid (Merck, 1997)

5.1.2.4. The exploitation of plumbagin

At present, the most exploited source of plumbagin is the roots of *Plumbago* spp. However, these plants grow quite slowly and the roots suitable for extraction take years to grow (Komaraiah et al., 2003). Moreover, conventional propagation of the plant is rather difficult and insufficient to meet the growing demand owing to the poor germination of seeds and death of young seedlings under natural conditions (Verma et al., 2002). Attempts to produce plumbagin synthetically proved to be commercially

ineffective (Ichihara et al., 1980; Wurm and Gurka, 1986), and therefore alternatives for the production of plumbagin based on *in vitro* techniques should be sought for.

Nahálka et al. (1996) showed that cell suspension cultures of *D. lusitanicum* are capable of producing large quantities of plumbagin in a short amount of time. However, vitality of the suspension was relatively low because of cell plasmolysis and compromised the process viability (Nahálka et al., 1998). Plumbagin is produced mainly in the roots of *Plumbago* spp., therefore, researchers used another approach using hairy root cultures in the expectation that it might lead to high levels of plumbagin production (Verma et al., 2002; Gangopadhyay et al., 2008). Hairy roots are obtained by transforming root cultures with *Agrobacterium rhizogenes*, resulting in a phenotype that is characterized by fast hormone-independent growth, lack of geotropism and genetic stability (Veena and Taylor, 2007). Despite achieving promising biomass production rates, the plumbagin content was too low for commercial exploitation.

Komaraiah (2003) used an improved method to produce plumbagin using *Plumbago indica* (same species as *P. rosea*) cell cultures. The cells were immobilized in calcium alginate while elicitated using chitosan and plumbagin was recovered simultaneously using *in situ* adsorption. Elicitors are signal compounds of plant defence responses, which can therefore enhance the production of secondary metabolites. The most frequently used elicitors are fungal carbohydrates, yeast extract and chitosan (Karuppusamy, 2009). The immobilized cell system enhanced plumbagin production, possibly due to increased degree of differentiation or cell to cell contact (Komaraiah et al., 2001). *In situ* direct extraction also increased plumbagin recoveries by reducing the toxic effects of plumbagin on the cells and feed back inhibition of secondary metabolite synthesis. However, *Plumbago* plants might not be the most adequate source of plant material for the bioprospection of plumbagin because its content in field specimens is lower when compared to other plumbagin bearing plants, such as *D. lusitanicum* for instance (Grevenstuk et al., 2008). *Drosera* spp. were never seriously considered for plumbagin prospection due to their low biosynthesis of this naphthoquinone (Crouch et al., 1990), nevertheless, this issue is controversial since higher recoveries have been obtained from *Drosera* plants in comparison to *Plumbago* plants (Marczak et al., 2005; Krolicka et al., 2008; Putalun et al., 2010).

5.1.3. Methods for plant secondary metabolite extraction

5.1.3.1. Solvent extraction

5.1.3.2. Maceration

Maceration is the simplest form of solid-liquid extraction from plants and consists of leaching the compounds contained in the plant matrix by immersion in the extracting solvent. Due to reduced mass-transfer rates, high solvent volumes and long extraction times are required. Stirring can be used to enhance mass-transfer. The choice of extracting solvent is preponderant as different solvents will yield different extracts and extract compositions. Several solvents have been used to extract plumbagin, including methanol and chloroform, however *n*-hexane seems to be the most adequate solvent as it provides high recoveries and degree of purity (Grevenstuk et al., 2008; Babula et al., 2009).

5.1.3.3. Soxhlet extraction

Soxhlet extraction is a general and well established technique used for the isolation and enrichment of analytes of medium and low volatility and thermal stability (Romanik et al., 2007). In a conventional Soxhlet system, plant material is placed in a thimble-holder and filled with solvent. When the liquid reaches the overflow level, a siphon aspirates the solution of the thimble-holder and unloads it into the distillation flask, carrying extracted solutes into the bulk liquid. The solvent is separated from the solute in the solvent flask by distillation and the condensed solvent passes back into the plant solid bed, until complete extraction is achieved (Wang and Weller, 2006). Soxhlet extraction is one of the oldest techniques for isolating metabolites from plant material but continues to be the main reference for evaluating the performance of other solid-liquid extraction methods due to its simplicity and exhaustive extraction. It surpasses in performance other conventional extraction techniques except for the extraction of thermolabile compounds, as the extraction usually occurs at the solvents' boiling point for long periods leading to the thermal decomposition of the target compounds. Other drawbacks such as long extraction times and high solvent volumes can be pointed out (Wang and Weller, 2006).

5.1.3.4. Ultrasound Assisted Extraction (UAE)

Ultrasounds are waves with frequencies ranging from 16 kHz to 1 GHz acting as mechanical vibrations in a solid, liquid and gas (Luque-Garcia and Luque de Castro, 2003). The mechanical effects of ultrasound induce a greater penetration of solvent into cellular materials and can also disrupt biological cell walls, facilitating the release of contents. These effects are attributed to the phenomenon called acoustic cavitation. If the ultrasound intensity is sufficient, the expansion cycle can create cavities or micro-bubbles in the liquid. Once formed, these bubbles will absorb the energy from the sound waves, grow during the expansion cycles and recompress during the compression cycle. The increase in pressure and temperature caused by the compression leads to the collapse of the bubbles, which generates shock waves that pass through the solvent, enhancing the mass transfer within the system (Toma et al., 2001; Yang and Zhang, 2008). Therefore, efficient cell disruption and effective mass transfer are considered to be the major factors leading to the enhancement of extraction with ultrasonic power (Mason et al., 1996).

Two general designs of ultrasound-assisted extractors are ultrasonic baths or closed extractors fitted with an ultrasonic horn transducer. The average time of ultrasonic extraction typically ranges from a few to 60 min, and the recoveries are comparable to those obtained after several hours of Soxhlet extraction (Szentmihályi et al., 2002; Chemat et al., 2004). The extraction is carried out at room temperature, which makes it suitable for the extraction of thermally labile analytes. The need for separation of the extract from the sample following the extraction is a disadvantage of this technique (Romanik et al., 2007).

5.1.3.5. Supercritical fluid extraction (SFE)

Solvent extraction techniques are cost effective but present considerable shortcomings as to what concerns safety and environmental issues. These techniques require large volumes of expensive, high purity organic solvents which not only increase operating costs but also cause additional environmental problems during post-processing of the extracts for solvent elimination (Wang and Weller, 2006). For instance, *n*-hexane is listed as No. 1 on the list of 189 hazardous air pollutants by the US Environmental

Protection Agency (Mamidipally and Liu, 2004). Thus, there is an increasing demand for new extraction techniques with shortened extraction time, better selectivity, reduced organic solvent consumption, and increased pollution prevention. Supercritical fluid extraction (SFE) was introduced as an environmentally responsible and efficient extraction technique for solid materials and extensively studied for separation of active compounds from plants (Jarvis and Morgan, 1997; Pourmortazavi and Hajimirsadeghi, 2007). Supercritical state is achieved when the temperature and the pressure of a substance are raised over its critical value (Figure 5.1.4). Supercritical fluids have several advantages over liquid solvents: they penetrate plant material samples as well as gases due to their high diffusion coefficients and low viscosity and surface tension; and at the same time their dissolving power is similar to liquids (Romanik et al., 2007). Furthermore, the dissolving power of a supercritical fluid solvent depends on its density, which is highly adjustable by changing the pressure and temperature, meaning that the extraction can be selective to some extent by controlling the density of the medium. After extraction the material is easily recovered by simply depressurizing, allowing the supercritical fluid to return to gas phase and evaporate leaving no or little solvent residues (Abbas et al., 2008).

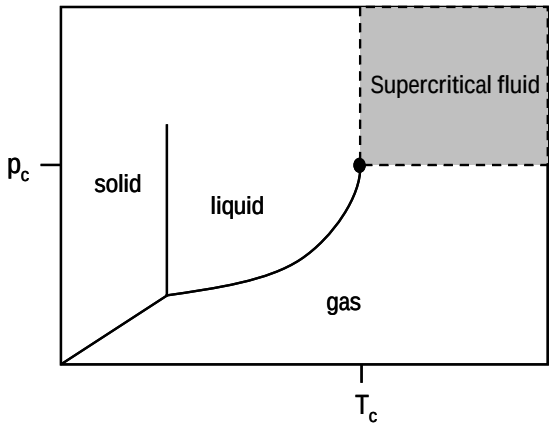


Figure 5.1.4 - Phase diagram for a single substance: Pc, critical pressure; Tc, critical temperature (Brunner, 2005). Supercritical state is achieved when the temperature and the pressure of a substance are raised over its critical value.

The most common extracting agent is carbon dioxide, because of its low cost, low toxicity, and favourable critical parameters (Tc= 304 K, Pc= 7.3 MPa; Wang and Weller, 2006). CO_2 as a nonpolar substance is capable of dissolving nonpolar or moderately polar compounds and is especially well suited for the isolation of substances

of low and medium polarity and high volatility (Romanik et al., 2007). A mixture of CO_2 with modifiers (polar organic solvents) is used for the extraction of polar substances. The modifiers increase the solubility of analytes, preventing them from adsorbing on the active sites of sample matrix.

5.1.3.5. SFE Operation

A SFE process can be carried out in different modes of operation. The majority of cases concerns extraction from solids, which is usually carried out in batch and single stage mode (Brunner, 2005). A simplified generic SFE system is shown in Figure 5.1.4. A typical batch extraction proceeds as follows, raw material is charged in the extraction tank which is equipped with temperature controllers and pressure valves at both inlet and outlet to keep desired extraction conditions. The extraction tank is pressurized with the fluid by a pump, which is also needed for the circulation of the fluid in the system. The fluid and the dissolved components are transferred to the separator where the solvation power of the fluid is decreased by increasing the temperature, or more likely, decreasing the pressure of the system. The product is then collected via a valve located in the lower part of the separators and the fluid is further regenerated and cycled (Wang and Weller, 2006).

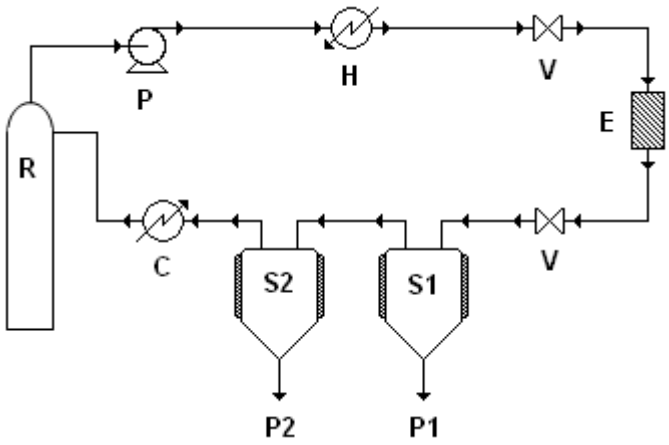


Figure 5.1.4 - Schematic diagram of a SFE system: CO_2 reservoir (R); CO_2 feed pump (P); heat exchanger (H); pressure valve (V); extraction vessel (E); separator 1 (S1); separator 2 (S2); product 1 (P1); product 2 or waste (P2); condenser (C); (adapted from Wang and Weller, 2006).

5.1.3.5. Operation parameters in SFE

There are many variables to be considered in SFE and method development can be a demanding task. One initial area that must be assessed is the solubility of the analyte to be extracted in the supercritical extracting fluid. This can be investigated by spiking an inert medium, usually celite or sand, with the analyte of interest (Pourmortazavi and Hajimirsadeghi, 2007). However, solute solubility in the fluid alone does not necessarily guarantee successful extraction and other parameters need to be optimized.

5.1.3.5.1. Plant matrix

The effect of matrix on SFE is of critical importance since the rate of extraction also depends on the rate of diffusion of the supercritical fluid through the sample matrix and the influence of the analyte-matrix adsorption effects (Modey et al., 1996). Different factors such as the particle size, shape, surface area, porosity, moisture and the nature of the matrix will affect the extraction results. As a general rule, decreasing the particle size of solid matrices leads to a higher surface area, making extraction more efficient. Yet, excessive grinding may hinder the extraction due to readsorption of the analytes onto matrix surfaces (this could be avoided by increasing the flow rate) and pressure drop inside the extraction chamber (Pourmortazavi and Hajimirsadeghi, 2007).

Even though the solubility of water in CO_2 is limited (0.3%), the content in water of the plant sample can cause restrictor plugging upon the fluid depressurization, which may freeze out as the fluid evaporates (Modey et al., 1996). Therefore, the sample matrix is usually dried before extraction, preferably by freeze-drying, as oven drying may result in solute volatilization (Pourmortazavi and Hajimirsadeghi, 2007). However, in some cases water can be beneficial to extraction, increasing the polarity of the fluid and enabling higher recoveries of relatively polar species. Water can also aid in the extraction process by opening pores and swelling the matrix, thereby allowing the supercritical fluid better access to analytes and aid in the flow trough the matrix (Pourmortazavi and Hajimirsadeghi, 2007).

5.1.3.5.2. Effect of pressure and temperature

Solubility of a substance in a supercritical fluid is the sum of two factors: the volatility of the substance, which is a function of temperature; and the solvating effect of the supercritical fluid, which is a function of fluid density. Therefore, solubility is controlled experimentally by selecting the extraction pressure and temperature (Modey et al., 1996). Once the experimental conditions at which the solute begins to partition in to the supercritical fluid are reached, the fluid pressure is the main parameter that influences the extraction efficiency. An elevation of operating pressure at a given temperature results in an increase of fluid density, which means an enhanced solubility of the solutes. It is often desirable however, to extract the sample just above the point where the analyte becomes soluble, minimizing the extraction of interfering compounds (Wang and Weller, 2006). The temperature should be above the critical temperature and chosen taking into consideration the thermal stability of the solutes. Higher temperatures often improve extraction recoveries owing to increased solute diffusion coefficients in the fluid with increasing temperature. However, the effect of a temperature elevation is difficult to predict because of its dependence on the nature of the sample. For a non-volatile solute, an increase in temperature would result in lower extraction recovery owing to a decrease in solubility because the density of CO_2 decreases when the temperature is increased at constant pressure (Pourmortazavi and Hajimirsadeghi, 2007).

5.1.3.5.3. Extraction time and flow rate

The extraction time should be sufficient to maximize SFE extraction, however, extraction is never complete in a finite time, being in general initially rapid but then tailing off with time: a 99% removal of a particular analyte may require an extraction period of up to ten times that needed to remove the first 50% (Modey et al., 1996). The speed of the supercritical fluid flowing through the cell has a strong influence on the extraction efficiencies. Lower flow rates usually enhance extraction efficiency because the fluid is able to penetrate the matrix deeper at slower velocity. The fluid speed can be expressed by the linear velocity, which is strongly dependent on the flow rate and the cell geometry (Pourmortazavi and Hajimirsadeghi, 2007).

5.1.3.6. SFE of plumbagin

The low polarity and high volatility of plumbagin makes SFE an interesting technique for extracting the naphthoquinone, especially when one takes into account that as a rule of thumb, the solubilizing properties of supercritical carbon dioxide are compared to *n*-hexane (Modey et al., 1996). Plumbagin has been previously extracted by SFE from the roots of *Plumbago scandens* (Rodrigues et al., 2006). The authors determined the solubility of several naphthoquinones and determined that for plumbagin a maximum solubility of 13.1 g/L was obtained at 40 ºC and 13.5 MPa (the highest pressure tested). The results also show that the greatest amount of plumbagin is extracted during the initial 20 min and that after 120 min the extraction has nearly reached completion. *P. scandens* roots were extracted at 40 ºC and 20 MPa and a plumbagin mass fraction of 0.193% was obtained from fresh roots.

5.1.4. Evaluation of extraction efficiency

In order to evaluate the effectiveness of an extraction method it is important to accurately determine the concentration or purity, and the recovery of the final product. A survey prepared by Rao and Nagaraju (2003) indicated that in the field of pharmaceutical quality control, HPLC has been the main technique used for analysis of impurities in drugs. In most cases, reversed-phase mode with UV absorbance detection is used, as this method provides the best available reliability, analysis time, repeatability and sensitivity. Although isocratic elution is preferred over gradient elution by most analysts due to simpler method transfer between instruments and laboratories, shorter column reequilibration time and lower baseline noise (Schellinger and Carr, 2006), good results have been obtained using a gradient elution to quantify plumbagin from *Plumbago zeylanica* (Wang et al., 2005). The authors used a mobile phase composed of water and methanol and peaks were detected at 254 nm.

5.1.5. Solid Phase Extraction (SPE) procedure

SPE is a sample preparation technique for chromatographic analysis and is used for sample extraction, concentration and cleanup, increasing reproducibility of analysis and column life-span. SPE has replaced liquid/liquid extraction as it prevents problems such

as incomplete phase separations, less-than-quantitative recoveries, use of expensive, breakable specialty glassware, and disposal of large quantities of organic solvents. Furthermore, SPE has the ability to isolate and enrich both volatile and non-volatile analytes, eliminate emulsion formation (common in liquid/liquid extraction) and foaming (common in gas/liquid extraction). SPE involves adsorption of sample components on the surface of a solid sorbent (aminopropyl or octadecyl stationary phases, bonded to silica gel), followed by elution with a selected solvent. SPE is carried out in glass or polypropylene columns or on extraction disks. A wide selection of sorbents enables substantial selectivity of the enrichment process and allows not only the isolation of analytes but also the removal of interferences (Romanik et al., 2007). In the context of this work, SPE was used as a sample purification step for the isolation of plumbagin, taking into account the results obtained in Chapter 3.

SPE is a process that generally involves four steps (Figure 5.1.5). After choosing the most adequate sorbent type, the SPE tube is conditioned with a water miscible organic solvent to wet the surface of the sorbent and penetrate bonded alkyl phases, allowing water to wet the silica surface efficiently. The sample is transferred to the tube and is washed with solvent to remove unwanted, weakly bonded retained materials. Finally, the tube is rinsed with a solvent that elutes compounds of interest and leaves the maximum of impurities behind. The eluate is collected and the tube cleaned for further use.

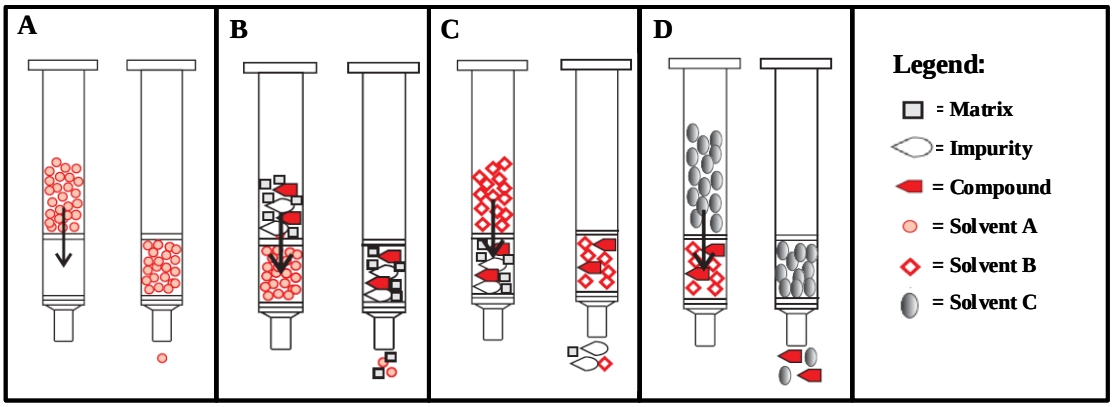


Figure 5.1.5 - Schematic diagram of the process for the separation of impurities from the compound of interest using SPE: sorbent conditioning (A); sample transfer (B); packing washing (C); Elution of compounds of interest (D) (Adapted from Supelco Bulletin 910 - Guide to Solid Phase Extraction, Sigma-Aldrich).

5.1.5. Objectives

Plumbagin is a natural product with commercial value which is obtained from cultivated plants. In Chapter 3 it was observed that high purity plumbagin could be recovered in high amounts from *D. intermedia* using a simple procedure. This chapter deals with the development of a method for the bioprospection of plumbagin from micropropagated *D. intermedia* plants to determine whether the process can be viable and therefore the specific objectives of this chapter are to:

i) monitor the growth of *D. intermedia* cultures over time to determine the biomass production rate;

ii) compare plumbagin production levels with other producing species;

iii) compare plumbagin extraction efficiency of different methods;

iv) evaluate the viability of SPE for product recovery and purification.

5.2. EXPERIMENTAL

5.2.1. Biomass production

D. intermedia plant material was produced according to the micropropagation protocol developed in section 2.3.3. The biomass increment of *D. intermedia* cultures was monitored in order to determine the optimum harvesting period. The culture growth index was registered at 2 week intervals (in quadruplicate) during 16 weeks of culture according to Formula 5.2.1:

$$\text{Growth index} = \frac{\text{Final fresh weight - Initial fresh weight}}{\text{Initial fresh weight}} \qquad \textbf{Formula 5.2.1}$$

Separate cultures with the same culture time as the ones used for plumbagin extraction were dried until constant weight (in quadruplicate) to determine its water content for dry weight yield determination.

5.2.2. Plant material extraction

All extraction procedures were performed with 5 g of fresh micropropagated *D. intermedia* plantlets (in triplicate) harvested at the optimum growth period. Prior to extraction, the plant material was ground in a mortar with liquid nitrogen to reduce particle size. Solvent extractions were performed with *n*-hexane using the same volume of solvent (150 mL) to compare extraction efficiency. CO_2 without modifiers was the solvent of choice for SFE.

5.2.2.1. Solvent extraction

The plant material was extracted twice for 24 h by maceration at room temperature under mechanical stirring. Soxhlet extraction (Figure 5.2.1 A) was performed until exhaustion to prevent eventual thermal degradation of plumbagin. Thus, plant material was extracted until verifying loss of yellow coloration of the solvent in the extractor (indicating dissolved plumbagin), which coincided with 1 h extractions (extraction cycles lasting between 3-4 min). For UAE, the plant-solvent mixture was placed in an

Erlenmeyer flask and immersed into a Bandelin Sonorex Super RK103H (Bandelin Electronic, Berlin, Germany) ultrasound bath operating at 35 kHz (Figure 5.2.1 B,C). A period of 1 h sonication at room temperature was chosen to guarantee maximum product recovery. The water of the bath was renewed after each extraction to prevent overheating.

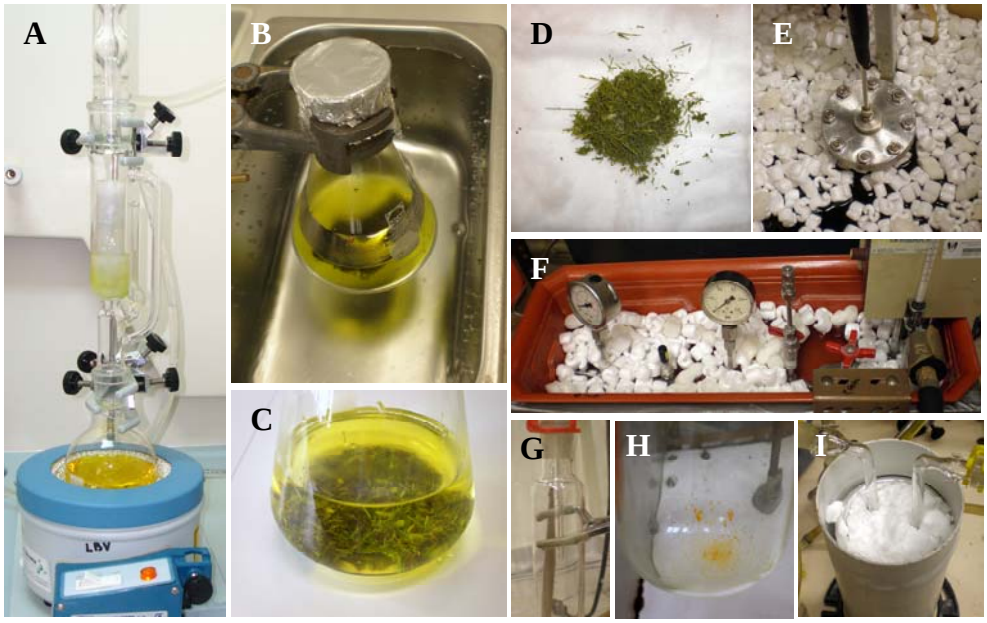


Figure 5.2.1 - Extraction methods used for recovering plumbagin from micropropagated *D. intermedia*: Soxhlet extraction (A); plant matrix and solvent mixture in ultrasound bath (B); solvent after 1 h extraction by UAE (C); ground material prior to extraction by SFE (D); closed extraction vessel (E); decompression zone (F); first collection vessel (G); recovered plumbagin in first collection vessel (H); second collector placed in Dewar flask (I).

5.2.2.2. Supercritical fluid extraction (SFE)

5.2.2.2.1. General experimental setup

Extraction procedure was carried out in a semi-batch flow extraction apparatus built at Instituto Superior Técnico, Technical University of Lisbon (Esquível and Bernardo-Gil, 1993). The schematic diagram is shown in Figure 5.2.2. Compressed carbon dioxide (6 MPa) is refrigerated in a cold bath at approximately 0 ºC and is fed to a Haskell reciprocating liquid pump operated by compressed air. Liquefied carbon dioxide flows through a coil in a water bath where it is brought to the desired extraction temperature, prior to being fed to a 100 mL stainless steel tubular extractor with the following

dimensions: 9.4 cm in height and 2.13 cm internal diameter. The extractor is immersed in the same thermostatic water bath to maintain extraction temperature. The outlet stream of the extraction vessel is depressurized in two stages to atmospheric pressure across two micrometering valves (Figure 5.2.1 F). The piping between the valves is placed in a heated water bath (40 ºC) and the extract is collected in the first receiver as it is thrown out of solution when the carbon dioxide expands (Figure 5.2.1 G,H). A second collector is immersed in a Dewar flask containing a freezing mixture consisting of acetone supersaturated with solid carbon dioxide where water and the volatile fraction of the extract are collected (Figure 5.2.1 I). The solute-free carbon dioxide leaving the collectors is vented through an Ariete Compact gas flow meter.

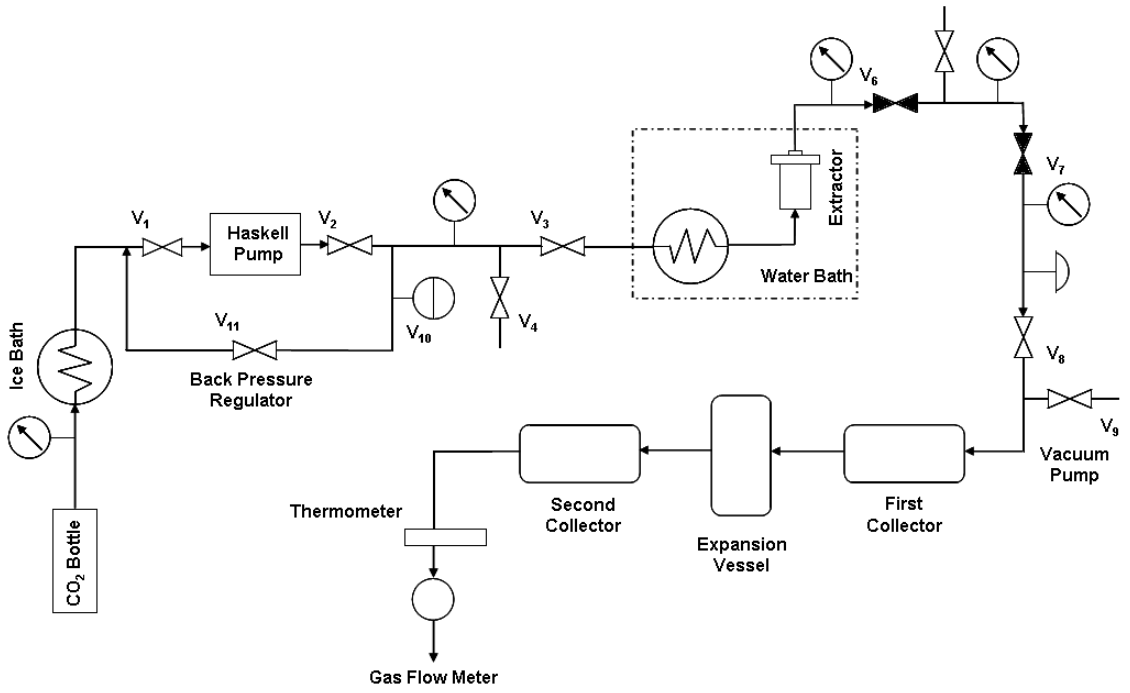


Figure 5.2.2 - Schematic diagram of the SFE apparatus (adapted from Esquível and Bernardo-Gil, 1993).

5.2.2.2.2. SFE operation

The operation parameters were based on a protocol that was optimized for extraction of plumbagin from *Plumbago scandens* (Rodrigues et al., 2006). Before each extraction the plant material was placed in the extractor between two pads of washed glass wool to prevent downward dissipation of the plant matrix or entrainment of solid particles in the stream of compressed carbon dioxide (Figure 5.2.1 D,E). Then, CO_2 was pumped until the extraction pressure of 20 MPa was reached. The extraction temperature was set to

40 ºC and after ensuring that there was no leak in the equipment, the expansion valves were opened and a steady stream of the solvent was allowed to pass upward through the bed of plant material until reaching an extraction time of 2 h. The valve settings were adjusted to give a flow rate of approximately 2 L/min. The extract deposited in the tubing around the expansion valves was recovered by washing the system with *n*-hexane at the end of each experiment. The *n*-hexane was sucked out with a vacuum pump and collected in a glass receiver. The CO_2 was supplied by ARLIQUIDO-Portugal in bottles as a 99.5% pure fluid.

5.2.3. Sample treatment

All extracts, except the one obtained by Soxhlet extracion, were filtered (Whatman nº 1, Springfield Mill, England) centrifuged (Heraeus Megafuge 1.0R, Osterode, Germany) and the supernatants were evaporated under vacuum on a rotary evaporator at 40 ºC. The extraction yields were registered and the dry samples were dissolved in a 50% acetonitrile solution in water at 2.5 mg/mL for further analysis.

5.2.4. SPE procedure

A SPE column (SUPELCLEAN™ LC-18 Packing; 60 mL; 10 g) was used to clean an aliquot of each sample. The distinct yellow coloration of plumbagin makes it possible to elute plumbagin selectively from a SPE column using an appropriate gradient. Before each SPE procedure the column was activated with 100 mL methanol, washed with 100 mL acetonitrile, and equilibrated with 100 mL of 30% acetonitrile in water solution. Then, the sample was loaded onto the column and the most polar impurities were eluted with 100 mL of 30% acetonitrile in water solution (Figure 5.2.3 A, B). A volume of 100 mL of 50% acetonitrile in water solution was used to elute plumbagin and collected (Figure 5.2.3 C,D). The remaining extract was eluted from the column with 100% acetonitrile and discarded (Figure 5.2.3 D). The column was washed with 100 mL of acetonitrile, 100 mL of chloroform and 100 mL of methanol for further use. The collected sample was evaporated under vacuum to remove the organic solvent and the water removed by lyophilisation. The recovery yields were registered by weighing the dry sample. Parameters such as extraction yield, sample purity and product recovery were calculated to evaluate process efficiency.

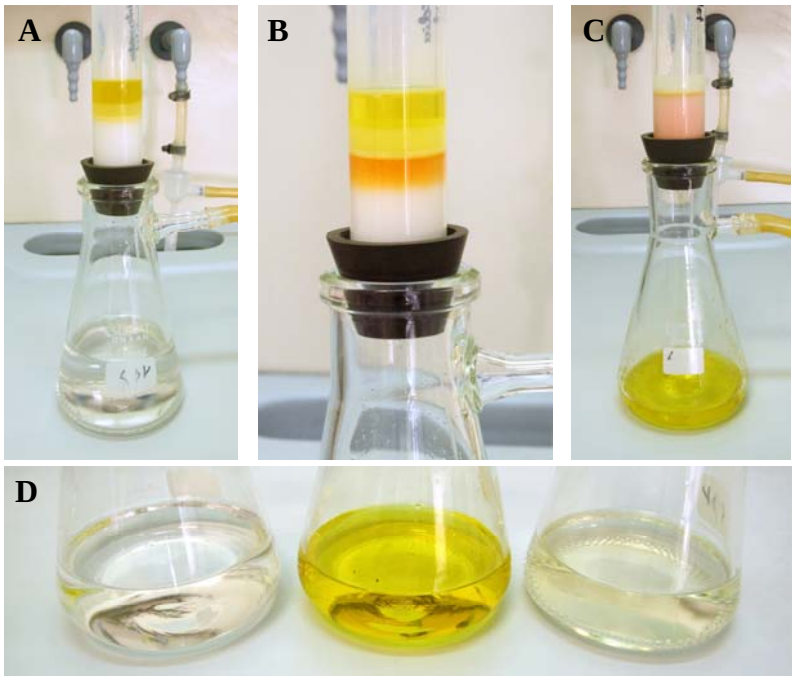


Figure 5.2.3 - Sample loaded onto the SPE column (A); SPE column eluted with 30% acetonitrile in water solution (B); SPE column eluted with 50% acetonitrile in water solution (C); recovered fractions: 30% (left), 50% (center) and 100% (right) acetonitrile in water fractions (D).

5.2.4. Plumbagin quantification

To determine the content in plumbagin the obtained samples were analyzed using a HPLC-DAD system. The analyses were carried out on an Agilent 1100 series liquid chromatography system (Agilent Technologies, Waldbronn, Germany), composed by the following modules: vacuum degasser (G1322A), quaternary pump (G1311A), autosampler (G1313A), thermostated column compartment (G1316A) and diode array detector (G1315B). Data acquisition and instrumental control were performed using LC3D ChemStation (Agilent Technologies) software. Analyses were performed on a Mediterranean Sea 18 column (150 mm × 4.0 mm, 5 μm particle size; Teknokroma, Barcelona, Spain) adapted with a Ultraguard Sea 18 (10 × 3.2 mm; Teknokroma, Barcelona, Spain) precolumn. The mobile phase consisted of acetonitrile (A) and water (B). The applied gradient was: 0-30 min, 10-90% A; 30-35 min, 90-100%; 35-40 min hold at 100%; 40-45 min 100-10%; and hold at 10% for 15 min. The flow rate was 0.5 mL/min and the injection volume 10 μL. The analyses were performed at 25 ºC and the detector was set at 254 nm. The crude extracts and SPE products were injected at 1.0 mg/mL and 0.5 mg/mL, respectively. The plumbagin concentration of the samples was determined using the standard external method. Calibration solutions of plumbagin

(Sigma, Steinheim, Germany) were prepared in 50% acetonitrile solution in water at 0.2; 0.4; 0.6; 0.8 mg/mL and injected in triplicate. A standard curve was obtained plotting the concentration of the calibration solutions against their peak areas (Figure 5.2.4).

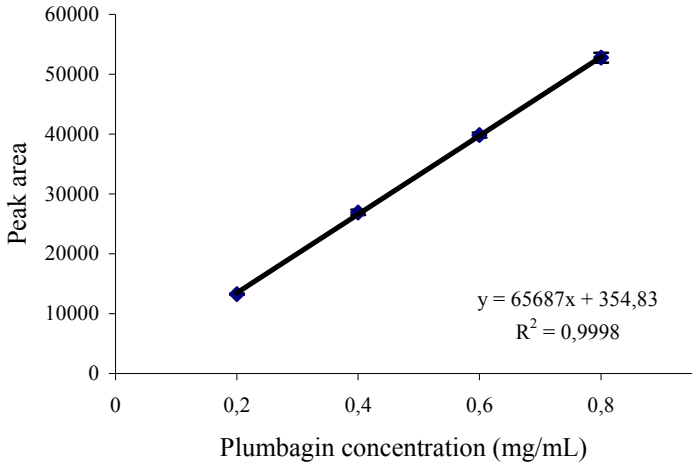


Figure 5.2.4 - Plumbagin standard curve.

The content of plumbagin or purity of the samples was calculated using Formula 5.2.2:

$$\text{Purity}_{\text{sample}}\ (\%) = \frac{\text{m(Plumbagin}_{\text{sample}})}{\text{m(Sample)}} = \frac{\text{Injection volume} \times [\text{Plumbagin}_{\text{sample}}]}{\text{Injection volume} \times [\text{Sample}]}$$ **Formula 5.2.2**

[Plumbagin$_{sample}$] – Sample plumbagin concentration determined by the standard curve; [Sample] – Concentration of the injected sample.

5.2.5. Statistical analysis

The data was subjected to analysis of variance (ANOVA) to assess if there were significant differences between the extraction procedures. Significant differences between means were determined using Duncan's New Multiple Range Test ($P = 0.05$). All calculations were performed with SPSS statistical package for Windows (release 11.0, SPSS Inc., Chicago, IL, USA).

5.3. RESULTS AND DISCUSSION

5.3.1. Evaluation of biomass production

The growth rate of *D. intermedia* cultures was monitored in order to determine the optimum harvesting time for product recovery (Figure 5.3.1). The results show that the biomass increases slowly during the first 6 weeks of culture, period after which the cultures grow more vigorously, reaching a 9.70 ± 0.94 fold increase in biomass after 10 weeks of culture.

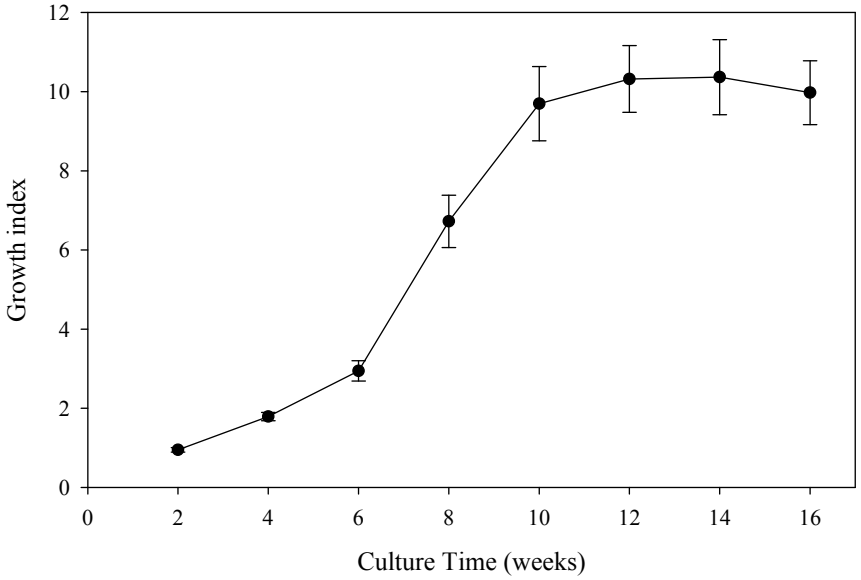


Figure 5.3.1 - Growth index of *D. intermedia* cultures during a 16 weeks culture period.

The efficiency of *in vitro* culture methods is usually evaluated in terms of the capacity of an explant to generate new shoots, instead of biomass production. It is therefore difficult to assess the efficiency of *D. intermedia* biomass production in the tested growth conditions as these results are available only for few *Drosera* species. However, *D. intermedia* does seem to grow more vigorously than *Drosera capensis*. After 4 weeks of growth in ½MS medium, biomass gain of *D. capensis* control cultures amounted to 1.193 ± 0.035 g FW/g initial FW (Krolicka et al., 2008), while for *D. intermedia* a gain of 2.79 ± 0.10 g FW/g initial FW (result not shown; 1.74 ± 0.10 fold biomass increment) was scored. The same author also reported a two fold increase in biomass production after addition of jasmonic acid, meaning that the biomass production of *D. intermedia* could be further enhanced.

The biosynthesis of plumbagin was not monitored over time, which could lead to misassumptions concerning the optimum harvesting time, as the production of plumbagin can vary depending on the growth phase. However, Verma et al. (2002) showed that the highest plumbagin concentration found in the roots of *P. zeylanica* hairy cultures coincided with the period with the highest biomass production. The authors reported a 21 fold increase in biomass yield after 6 weeks of culture. The higher biomass production yields reported for *P. zeylanica* cultures are expectable since hairy roots have higher growth rates and no growth regulators were used when producing *D. intermedia* cultures.

5.3.2. Evaluation of extraction methods

In vitro produced *D. intermedia* plant material was extracted with *n*-hexane using maceration under mechanical stirring, Soxhlet extraction, UAE and by SFE using supercritical carbon dioxide as solvent. The results expressing extraction yield, purity and plumbagin content obtained for each extraction method were calculated in terms of fresh weight (FW) and are presented in Table 5.3.1. The content in plumbagin of the obtained extracts is also presented in terms of dry weight (DW) in order to allow comparisons with other publications.

Table 5.3.1 - Extraction parameters of plumbagin from *D. intermedia* using different extraction methods.

	Extraction yield ($mg_{extract}$/g FW)	**Purity (%)**	**Plumbagin content (mg/g FW)**	**Plumbagin content (mg/g DW)***
Maceration	5.23 ± 0.61 a	42.60 ± 2.36 b	2.21 ± 0.17 c	17.51 ± 1.40 c
Soxhlet	5.63 ± 0.14 a	47.43 ± 1.24 b	2.67 ± 0.04 b	21.18 ± 0.44 b
UAE	5.14 ± 0.54 a	61.88 ± 5.59 a	3.12 ± 0.05 a	24.78 ± 0.55 a
SFE	5.93 ± 0.44 a	43.14 ± 3.00 b	2.54 ± 0.14 bc	20.15 ± 1.13 bc

Values represent mean ± standard error of 3 repetitions. For each parameter values with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test. * FW : DW ratio of *D. intermedia* cultures = 7.9 ± 0.13 : 1.

The results show that the extraction methods are equivalent as to what concerns the extraction yield but that there exist differences in the purity of the extracts obtained by the different methods. The extract obtained by UAE is the most concentrated in plumbagin (61.88 ± 5.59%) and therefore affords the highest content in plumbagin (3.12 ± 0.05 mg/g FW) ($P < 0.05$), despite its relative low extraction yield (5.14 ± 0.54

$mg_{extract}$/g FW). The amount of plumbagin extracted by UAE is superior to that obtained by Soxhlet extraction (2.67 ± 0.04 mg/g FW) ($P < 0.05$), which is surprising as the latter is performed until the plant matrix is exhausted, meaning that the amount of plumbagin contained in the plant matrix should be entirely extracted. UAE has been frequently used to reduce extraction times while maintaining similar extraction yields to Soxhlet extraction (Hemwimol et al., 2006; Williams et al., 2006; Jadhav et al., 2009), but few cases have reported superior yields when using UAE. However, it is interesting that the amount of plumbagin extracted from *Drosera binata* was also higher using UAE instead of Soxhlet extraction (Marczak et al., 2005).

This result might be explained by the fact that the solvent is able to penetrate deeper in to the plant cells due to the effect of the ultrasounds and is therefore able to extract plumbagin that is inaccessible when using Soxhlet extraction. Scanning electron microscopy micrographs of plant material after extraction with UAE show that samples irradiated with ultrasound reveal structural changes in comparison to samples extracted by maceration (Yang et al., 2008). The appearance of pits on the plant cell surface confirms that the ultrasonic exposure causes the cells to rupture more readily. It is also possible that the amount of plumbagin recovered with Soxhlet extraction was inferior in comparison to that obtained by UAE because the extraction time (1 h) was insufficient and therefore limiting. However, it has been demonstrated that prolonged extraction times lead to a decrease in the extraction of plumbagin, possibly due to thermal decomposition (Ribeiro de Paiva et al., 2004). For this reason the plant material was extracted until the solvent in the extractor lost its yellow coloration and was therefore no longer extracting plumbagin. The total extraction time was short in comparison to typical Soxhlet extractions due to the high volatility of *n*-hexane, allowing a higher number of extraction cycles in a shorter time, when compared to other frequently used solvents.

Nevertheless, in this work the greatest advantage of UAE over the other tested methods for obtaining plumbagin from *D. intermedia* plants is the increased purity of the obtained crude extract. It is unclear however, why fewer contaminating metabolites are co-extracted with plumbagin when using UAE. Furthermore, it is possible that the extraction process can be enhanced by adjusting other operation parameters. Increasing ultrasonic power can result in increased extraction yields, as has been reported

(Smelcerovic et al., 2006), or reduce extraction time if plumbagin is being recovered completely. The operating temperature also influences extraction efficiency and can therefore be optimized as well in order to maximize product recovery. An extraction period of 1 h was chosen for UAE to compare its efficiency with Soxhlet extraction and because extraction reaches exhaustion for most materials after this time (Fulzele and Satdive, 2005; Hemwimol et al., 2006; Smelcerovic et al., 2006).

The extract obtained by SFE afforded a considerable amount of plumbagin (2.54 ± 0.14 mg/g FW), with comparable yields to Soxhlet ectraction ($P < 0.05$). The highest extraction yield was obtained with SFE (5.93 ± 0.44 $mg_{extract}$/g FW), despite the difference not being statistically different from the other extraction methods, and yet the amount of plumbagin recovered is inferior to the ones recovered with UAE and SE (3.12 ± 0.05 mg/g FW and 2.67 ± 0.04 mg/g FW, respectively). This means that other undesired compounds are being co-extracted with the supercritical solvent.

It seems unlikely that plumbagin recoveries can be greatly increased by altering the operating conditions, as the extraction temperature and pressure were held at the optimal values for plumbagin extraction (Rodrigues et al., 2006). An elevation of the extraction pressure may enhance extraction by increasing the fluid density and therefore its solvating power, but the risk of co-extracting other undesired compounds is also increased. The SFE extraction was performed for 2 h because it was reported by Rodrigues et al. (2006) that the extraction had reached exhaustion after this time. Because the extracted plant material (*P. scandens* roots) is a woodier and harder matrix for the solvent to penetrate it was assumed that the same extraction time would be sufficient in this study. On the other hand, the extracted amounts of root sample were smaller and therefore it is difficult to exclude that the extraction time was not limiting. However, extraction may be optimized using organic modifiers and adjusting other parameters such as flow rate and particle size. At low flow rates the effect of axial dispersion is important and extraction efficiency decreases, on the other hand, if flow rates are too high the extraction efficiency is hampered as well due to shorter residence times (Pourmortazavi and Hajimirsadeghi, 2007). This means that there is an optimum flow rate at which the extraction should be performed. Decreasing particle size could also enhance extraction as it will increase surface area, allowing a better contact with the solvent and enhancing mass transfer.

Another aspect is that in the SFE setup used in this work, organic solvent is required to flush the extract out of the piping and to dissolve the extract that is deposited in the first receiver flask. This can lead to product losses besides representing a considerable consumption of organic solvent. It would be of interest to control operating conditions in such a way that the extract would keep in solution until reaching the receiver flask. This can be achieved by placing the flow restrictor in the collection solvent, and depressurizing the supercritical CO_2-extract mixture directly in contact with the solvent. In this setup it is important that the restrictor is heated to prevent obstructions caused by solvent that freezes out during decompression. This would reduce even more the use of organic solvents and pose an important step for an eventual scaling-up process.

The results presented in Table 5.3.1 also show that maceration is the least efficient method as it extracts the smallest amount of plumbagin (2.21 ± 0.17 mg/g FW) ($P < 0.05$). The low efficiency and long extraction times makes maceration an inadequate method for the extraction of plumbagin. These results are expectable as maceration usually affords lower yields in comparison to other extraction methods. It is interesting to see that the amount of plumbagin recovered using maceration (2.21 ± 0.17 mg/g FW) is not statistically different from the one using SFE (2.54 mg/g FW ± 0.14) ($P < 0.05$), despite the considerable absolute difference. This can be explained by the fact that plant materials are not completely homogeneous, resulting in variations of active compound contents in different extracts from the same plant material (Smelcerovic et al., 2006). However, these issues are greatly reduced when using micropropagated plant material, as can be confirmed by comparing the experimental deviations obtained in a similar study conducted with field specimens of *D. lusitanicum* (Grevenstuk et al., 2008).

Another extraction technique that bears some similarities with UAE and has been implemented successfully in many cases is microwave assisted extraction (MAE). The method is based on absorption of microwave energy (electromagnetic radiations with a frequency from 0.3 to 300 GHz) by molecules of polar chemical compounds. Microwaves offer a rapid delivery of energy to the solvent and solid plant matrix mixture with subsequent heating of the mixture. Because water within the plant matrix absorbs microwave energy, cell disruption is promoted by internal superheating, which facilitates desorption of chemicals from the matrix (Wang and Weller, 2006). However, the high operating temperatures (150-190 ºC) make this method unfeasible for the

extraction of plumbagin as it would lead to high product losses due to thermal decomposition.

Although all extractions were performed with fresh plant material, the content in plumbagin is presented on fresh weight and dry weight basis in order to allow comparison with previous publications. Despite the fact that most extraction methods use dry plant material, for the purpose of this work the extractions were performed with fresh plant material because the extraction of plumbagin is greatly hampered when using dry material (Verma et al., 2002; Marczak et al., 2005; Grevenstuk et al., 2008). Marczak (2005) hypothesized that plumbagin may be more strongly bound to the dry plant matrix instead of being decomposed during the drying process. The author showed that it was possible to extract plumbagin from dried material with methanol, even after it had been extracted with chloroform, possibly due to easier disruption of intermolecular interactions with the more polar solvent. When performing extractions with fresh samples, higher yields were obtained with chloroform than with the other solvents.

5.3.3. *D. intermedia* as a source of plumbagin

The naphthoquinone plumbagin is currently exploited from plants of the *Plumbago* genus. However, when comparing the recoveries presented in Table 5.3.1 with the contents in plumbagin of *Plumbago* spp. reported in literature, one can conclude that *D. intermedia* could be an alternative source of plumbagin. The content in plumbagin obtained by maceration with ethyl acetate of dried *P. zeylanica* roots varies between 0.629 and 4.975 mg/g according to Wang et al. (2005). For the same species, Hsieh (2005) reported a recovery of 13.40 ± 1.30 mg/g DW after extracting the plant material with boiling ethanol. Another author reported recoveries of 1.50, 1.91 and 1.40 mg/g DW obtained from roots of *Plumbago auriculata*, *P. indica* and *P. zeylanica*, respectively, obtained by Soxhlet extraction with acetone (Mallavadhani et al., 2002). Despite the fact that in these reports the plant material was dried prior to extraction and the extraction procedures are diversified, the recoveries are in some cases considerably lower than those obtained from *D. intermedia*. The lowest plumbagin recovery was 17.51 ± 1.40 mg/g DW, obtained by maceration.

Roots of *P. scandens* were extracted by SFE with plumbagin contents ranging from 0.056 to 1.93 mg/g FW depending on the time period between collection and extraction (Rodrigues et al., 2006). Using the same extraction method and experimental conditions 2.54 ± 0.14 mg/g FW were obtained from *D. intermedia*. It is worth underlining that the results mentioned above were obtained from field specimens of *Plumbago* spp. and might therefore be overestimated. The differences of the recoveries reported in literature for plants of the *Plumbago* genus, even for those of the same species, are in part due to the different extraction procedures but also due to geographical and seasonal factors which affect the content in secondary metabolites, underlining one of the advantages of using *in vitro* cultures for bioprosprection, as it is a more reliable and qualitatively consistent source of plant material.

Several biotechnological approaches have been used to improve the production yield of plumbagin of *Plumbago* spp., while simultaneously preventing harvesting of the whole plant. Hairy root cultures have been established from *P. zeylanica* (Verma et al., 2002) and *P. indica* (Gangopadhyay et al., 2008) with the intent of increasing the growth rate and plumbagin production. The hairy root cultures resulted in increased plumbagin yields in comparison to untransformed roots (8.40 mg/g DW from *P. zeylanica*; 6.18 mg/g DW from *P. indica*), however, the obtained plumbagin contents are inferior to those obtained from *D. intermedia* cultures (Table 5.3.1). Another approach used to improve the production of plumbagin was to immobilize cell cultures of *P. indica* in calcium alginate while being elicitated with chitosan and collecting plumbagin by *in situ* adsorption, thereby reducing the feedback inhibition of secondary metabolite production (Komaraiah et al., 2003). This way, recoveries of 92.13 mg/g of dry cell weight (DCW) were obtained, which is nearly four times more than the highest recovery obtained from *D. intermedia* (24.78 ± 0.55 mg/g DW). In the cited study, the step of plumbagin elicitation with chitosan alone was responsible for a production increment of over six times, meaning that the production of plumbagin by *D. intermedia* could be enhanced considerably as well using adequate elicitors. In a different study the production of plumbagin by *Drosera burmanii* suffered a 3.5-fold increase over control due to elicitation by yeast extract application (Putalun et al., 2010).

D. intermedia produces significant amounts of plumbagin, even when compared to plants of the same genus. *D. intermedia* seems to produce higher levels of the

naphthoquinone then the eight *in vitro* cultured *Drosera* species evaluated by Marczak (2005). In this study, the highest plumbagin recovery was obtained from *D. binata* (12.4 mg/g DW) using UAE with chloroform as extracting solvent. Using the same extraction technique, higher recoveries were obtained from *D. intermedia* (24.78 ± 0.55 mg/g DW). It has to be stated that chloroform can give higher recoveries, although this increment usually comes at cost of the extract purity, because a greater amount of undesired compounds are co-extracted (Grevenstuk et al., 2008).

The plumbagin production levels of *D. intermedia* seem to be similar to those reported for *D. lusitanicum* (Grevenstuk et al., 2008). Field specimens were extracted with *n*-hexane using Soxhlet extraction and UAE affording recoveries of 2.42 ± 0.39 mg/g FW and 1.52 ± 0.39 mg/g FW, respectively. The recovery of plumbagin using Soxhlet extraction is comparable to the one obtained from *D. intermedia* (2.67 ± 0.04 mg/g FW). The plumbagin content of the extract obtained by UAE is lower compared to the one obtained from *D. intermedia* (3.12 ± 0.05 mg/g FW), but this can be explained by the fact that an experimental setup with a horn transducer was used to deliver ultrasounds to the *D. lusitanicum* matrix, instead of an ultrasound bath. In this setup, the ultrasounds are only delivered efficiently to the plant matrix close the horn transducer, leading to lower recoveries. In a different work, cell suspension cultures were established from *D. lusitanicum*, yielding high amounts of plumbagin (35 mg/g Fresh Cell Weight) (Nahálka et al., 1996). The cell suspension culture was able to produce over 10 times more than the mother plant, but the cultures underwent a strong plasmolysis short after being transferred to the liquid medium, making this approach unviable. *D. lusitanicum* is a species endemic to the Iberian Peninsula and northern Morocco and is in risk of eminent extinction; therefore harvesting this plant from the wild for plumbagin extraction is unfeasible and irresponsible from an ecological perspective. An *in vitro* culture protocol has been developed for this species (Gonçalves et al., 2005), but *D. intermedia* seems to grow easier and more vigorously in these conditions.

5.3.4. Evaluation of the SPE purification procedure

An aliquot of each sample obtained from the different extraction methods was concentrated using a SPE column to investigate the potential of using SPE to purify the

crude extracts. The main product was plumbagin in all cases which was confirmed based on comparison of retention time and UV spectral data with an authentic standard (Figure 5.3.1).

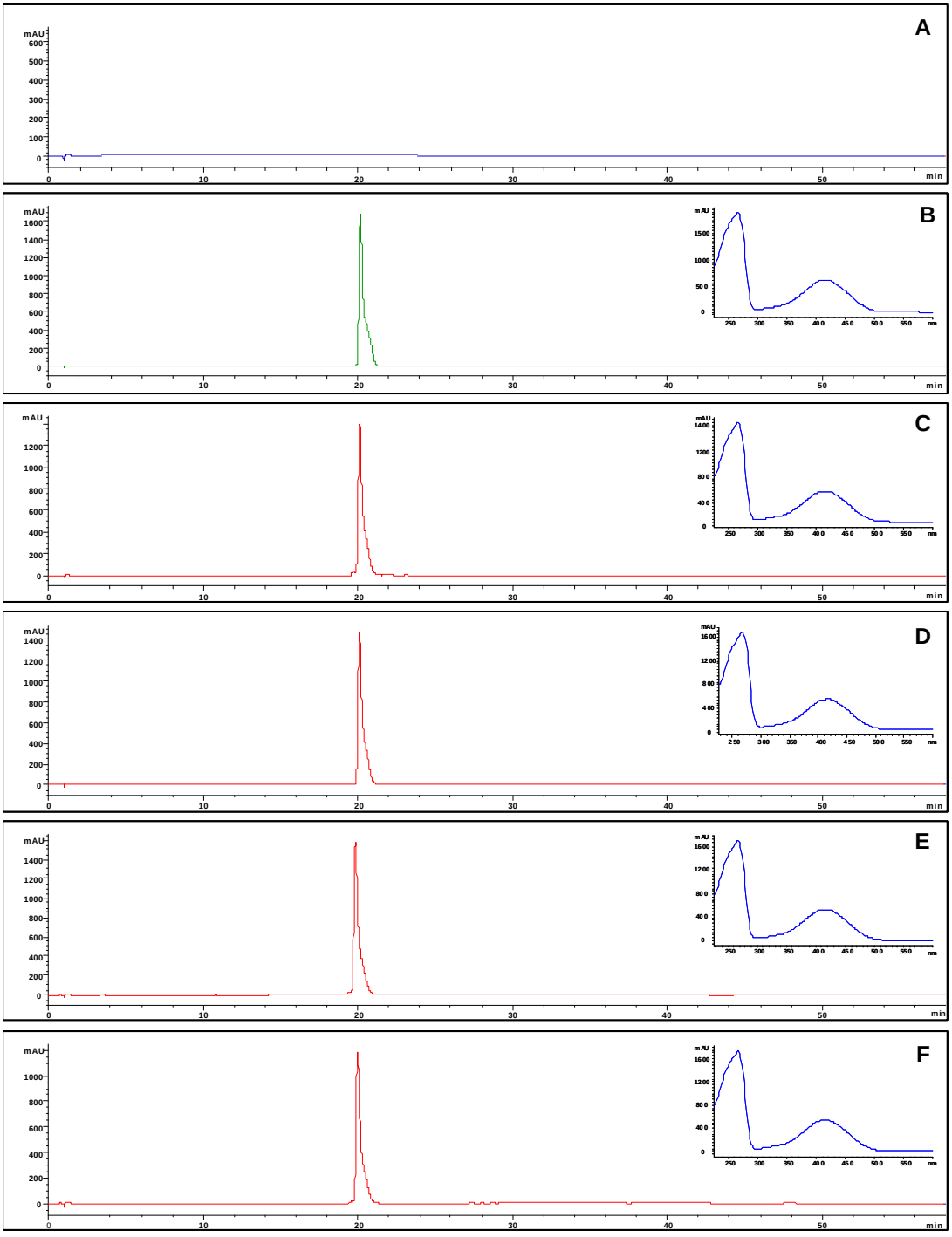


Figure 5.3.1 - Chromatograms and respective UV-DAD spectrum at peak apex position of: blank run (acetonitrile) (A); plumbagin standard 0.6 mg/mL (B); maceration (C); Soxhlet (D); UAE (E) and SFE (F) samples at 0.5 mg/mL after SPE purification.

The results expressing SPE yield (ratio between the obtained product and the mass of extract subjected to purification), product purity and SPE recovery (percentage of plumbagin recovered from the total amount of plumbagin that was subjected to purification) are presented in Table 5.3.2.

Table 5.3.2 - Purification of plumbagin from *D. intermedia* extracts using SPE.

	SPE yield (mg product/mg extract)	Purity (%)	SPE recovery (%)
Maceration	0.37 ± 0.03 b	94.30 ± 1.80 b	82.58 ± 2.31 a
Soxhlet	0.37 ± 0.01 b	99.91 ± 0.09 a	78.49 ± 2.13 a
UAE	0.54 ± 0.06 a	99.51 ± 0.49 a	86.31 ± 2.40 a
SFE	0.32 ± 0.04 b	83.90 ± 3.23 c	71.58 ± 3.44 b

Values represent mean ± standard error of 3 repetitions. For each parameter values with different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

The results show that the highest extraction yield was obtained from the extract prepared by UAE (0.54 ± 0.06 mg product/mg extract) ($P < 0.05$). This result is expectable because the extract was the most concentrated, and therefore the content in plumbagin represents a larger fraction of the crude extract in comparison to the other extracts. The results also show that the purity of the obtained SPE products is very high and in some cases purities over 99.5% were achieved. The products obtained from the Soxhlet and UAE extracts were the most pure ($P < 0.05$), with purities of 99.91 ± 0.09% and 99.51 ± 0.49%, respectively. The SPE products with the highest purity were the ones obtained from the purest crude extracts. This shows how important it is to use the purest possible extract in an initial stage, even sometimes at cost of the absolute recovery, because it enhances the efficiency of the subsequent purification steps.

The SPE recovery parameter represents an indirect measure of product loss during the SPE procedure which is unavoidable in sample purification procedures. For the SPE products of extracts prepared by solvent extraction, product recoveries between 78.49 and 86.31% were achieved. These recoveries are quite promising taking into account that the procedure has not been thoroughly optimized. Different stationary phases and gradients can be applied to increase the amount of plumbagin recovered while minimizing the co-elution of impurities at the same time.

The SPE procedure was less effective for the extract prepared by SFE; for which the lowest purity (83.90 ± 3.23%) and SPE recovery (71.58 ± 3.44%) was obtained ($P < 0.05$). This means that the impurities extracted by SFE are different in nature than those obtained by solvent extraction and are more difficult to remove using SPE. It seems that these impurities have a similar polarity to that of plumbagin and are therefore co-eluted with the chosen gradient. On the other hand, the recoveries are relatively low as well, suggesting that some product is bound to the contaminants and is lost in the SPE waste. This procedure needs to be optimized in order to be implemented in the purification of the extracts prepared by SFE. Perhaps an initial approach would be to characterize the co-extracted contaminants that are obtained together with the purified product to efficiently adapt the gradient, and to analyze the SPE waste to understand where the remaining plumbagin that is not recovered is lost.

Figure 5.3.2 shows the overall process of plumbagin extraction from *D. intermedia* and the subsequent SPE purification step. The results are expressed in relation to the initial fresh weight of the plant material to show the amount of absolute plumbagin that can be produced according to this procedure. It can be seen that the combined UAE and SPE procedure can be used to produce 2.74 mg of plumbagin (purity = 99.5%) per gram of fresh *D. intermedia* plant material.

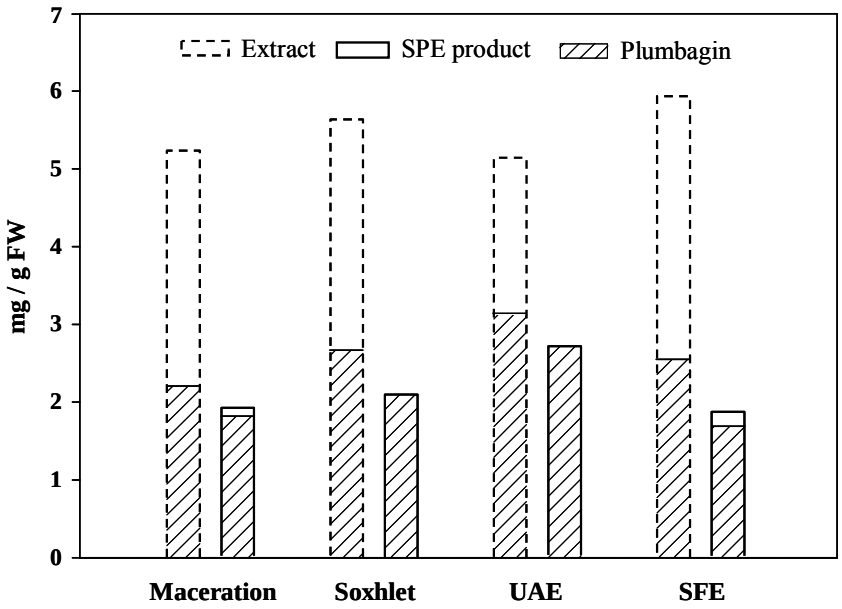


Figure 5.3.2 - Extraction, SPE yields and respective plumbagin contents per gram of fresh *D. intermedia* plant material.

The graphic shown in Figure 5.3.2 illustrates well that nearly all the impurities (white area) are removed from the crude extract in one single step. This is very desirable because any additional step will result in product loss and reduction of the overall extraction yield. A different approach for obtaining plumbagin from *P. indica* is reported by Kapadia (2005). The method consists in obtaining a crude extract by cold maceration of powdered root material with acetone and precipitating plumbagin by adding water to the acetone extract, making use of the hydrophobic nature of plumbagin. The residue was filtered, taken in chloroform and the concentrated chloroform extract was subjected to column chromatography. Using this procedure, 1.65 g of plumbagin were obtained starting from 100 g of plant material (16.5 mg/g DW). The total yield is lower than the highest yield obtained in this study (2.74 mg/g FW ~ 21.75 mg/g DW), but it is not possible to compare the efficiency of the purification procedures because the author does not present yields of the intermediary steps. Nevertheless, based on the presented results it can be said that SPE is an efficient procedure to isolate plumbagin from a crude extract with high recoveries and final purities.

5.4. CONCLUSIONS

Overall the best method for the extraction of plumbagin from *D. intermedia* is UAE, as it provides the greatest recovery of plumbagin in a short period of time. The increased extract purity in comparison to the other tested methods is also of interest because it smoothes the progress of posterior purification procedures. This way, plumbagin can be extracted on a commercial scale by simply applying ultrasound to the pre-leached mixture for a short period of time. Optimizing an extraction procedure with SFE can be a daunting task, but for an initial approach the obtained results are encouraging, affording considerable plumbagin recoveries with the advantage of avoiding the use of hazardous organic solvents for extraction. Therefore, the optimization of plumbagin extraction using supercritical fluids is worthwhile investigating due to the environmental advantages that SFE offers over conventional methods.

The results also show that *D. intermedia* plants produce increased amounts of plumbagin in comparison to the *Plumbago* spp. on a fresh weight basis. Additionally, *D. intermedia* cultures grow fast and could therefore represent an alternative for the bioprospection of plumbagin. Taking into consideration that no growth regulators or elicitors were used it is possible that the production of plumbagin can be further enhanced. Furthermore, extracting plant metabolites from *in vitro* cultures is desirable when high yields can be obtained. Plant materials are not completely homogeneous and are affected by seasonal and geographical factors, and this may be one reason for the variations in active compound contents in different extracts from the same plant material (Smelcerovic et al., 2006). Micropropagated plant material is more reliable and homogeneous on the other hand, and this might have contributed to lower experimental error deviations when compared to experiments conducted with field specimens (Grevenstuk et al., 2008).

The SPE procedure is very effective in concentrating the sample and removing impurities; in some cases purities of over 99% could be obtained while maintaining considerable recoveries (over 75% for products of extracts prepared by solvent extraction). However, it did not show the same efficiency for the extracts prepared by SFE and has to be optimized in order to be considered a viable alternative.

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GENERAL CONCLUSIONS

The plant kingdom represents an immeasurable source of chemical diversity which is still largely unexplored and is of utmost importance to drug discovery. Collectively, plants produce a remarkably diverse array of over 100,000 low-molecular-mass natural products, also known as secondary metabolites, which are important to medicine not only for their direct pharmacological effects but also in their role as template molecules for the production of new drug substances. However, plant biodiversity is declining at an unprecedented rate and conservation measures are urgently needed. *In vitro* techniques have found increasing use in the conservation of threatened plants in recent years and this trend is likely to continue as more species face risk of extinction.

Carnivorous plant species are becoming increasingly scarce in the wild due to depletion of natural habitats. Yet, at the same time the interest in these plants has been a constant, greatly due to the interest of biologists in trying to unfold the secrets of their characteristic carnivorous habits and because they have been shown to produce valuable secondary metabolites. As natural populations of carnivorous plant species occurring in Portugal are fragile and cannot provide sufficient material for research activities, the development of micropropagation protocols for the studied species was crucial. In Chapter 2 of this thesis the establishment of *in vitro* cultures of *P. vulgaris*, *P. lusitanica*, *D. rotundifolia* and *D. intermedia* is discussed. In general it was found that these species thrive in media with reduced macronutrient concentrations, which is in good agreement with their natural habit. Also, they are relatively indifferent to supplementation with plant growth regulators (PGRs), and in some instances these had a negative effect on growth. Except for *D. rotundifolia*, all species formed shoots and roots simultaneously in the proliferation phase, which might be an indication of high levels of endogenous growth hormones. Two rapid and efficient protocols were developed for *P. lusitanica* and *D. intermedia* which provided sufficient plant material to achieve the goals set for the subsequent phases of this study. The micropropagation protocols for *P. vulgaris* and *D. rotundifolia* have to be further optimized. In the case of *P. vulgaris*, the viability of the shoots was low, while for *D. rotundifolia* the produced shoots were small and unable to produce roots. Nevertheless, the cultures initiated *in vitro* represent an active germplasm collection which is of value in the case of extinction of local populations in the wild and for possible reintroduction programs.

Chapter 3 dealt with the chemical characterization of extracts prepared from micropropagated *P. lusitanica* and *D. intermedia* plant material. For this purpose, state of the art HPLC-MS and HPLS-SPE-NMR equipment was used, capable of providing on-line structural information sufficient for structure elucidation directly from essentially crude extracts without having to recur to large scale preparative procedures for compound isolation. The interface of an automated SPE unit between the chromatographic separation and the NMR equipment allowed for the selective extraction and concentration of chromatographic peaks to SPE cartridges for subsequent spectroscopic analysis. Following this approach it was possible to determine unambiguously the structure of most of the major secondary metabolites of *P. lusitanica* and *D. intermedia* using minimal amounts of material. The secondary metabolites identified in the *P. lusitanica* methanol extract were grouped into iridoid glucosides and phenylethanoid glycosides. In turn, the major components of the *D. intermedia* methanol extract were ellagic acid derivatives and flavonoid glucosides, while the *n*-hexane extract was exclusively composed of plumbagin. However, further experiments are needed to confirm the structure of a compound with a naphthoquinone-like structure from *D. intermedia* and an iridoid glucoside from *P. lusitanica*, as partial structural information suggests that their structures might not have been hitherto reported. *P. lusitanica* was investigated for its secondary metabolite profile for the first time and *D. intermedia* was only investigated for its content in naphthoquinone-related compounds, and therefore this work is a contribution to the better understanding of the biochemistry of these plants. Also, from a chemotaxonomical perspective, the secondary metabolites identified in *P. lusitanica* and *D. intermedia* were in agreement with previous chemical studies performed in species of the Lentibulariaceae and Droseraceae families, respectively.

There are several approaches to natural product-based drug discovery; however the most appropriate seems to combine chemical screening procedures and biological assays in detriment of bioassay guided isolation procedures, because compounds with other interesting activities might be missed out. Also the dereplication of compounds present in the extracts in early stages of research projects is important because it ensures that novelty is brought into the isolation process and that only the most promising lead-structures are selected for further investigation. Because it is unfeasible to perform a large number of biological assays at once, as a starting point, it was decided to perform

a preliminary screening of the biological activities of the *P. lusitanica* and *D. intermedia* extracts by evaluating their antioxidant and antimicrobial activity. Determination of a samples' antioxidant capacity (AOC) is a valuable assay because it can give an indication of activity against several other biological targets. Antibacterial activity was evaluated due to the urgent need to find new sources of anti-infective agents. Because the extracts were previously characterized it was possible, to some extent, to deduce the structure activity relationships behind some activities. The results pointed out that the methanol extract of *P. lusitanica* has the highest AOC, which is possibly due to one of its major components, acteoside. Because acteoside has previously shown activity against several biological targets it is a potential candidate for further study. Determinant structural aspects of acteoside, such as two catechol units, seem to confer high free radical scavenging potential to the molecule. In addition, the compound is considered to be nontoxic, because it has been detected as a component of several edible plants. The methanol extract of *D. intermedia* also showed considerable antioxidant activity which can be explained by the combined activity of ellagic acid derivatives and flavonoid glucosides.

The *D. intermedia* extracts were the most effective in the antimicrobial activity assays, particularly the *n*-hexane extract, which showed activity against a diverse panel of microorganisms and scored very low MICs for a crude extract. The observed activity could be explained by the fact that the extract was in great part composed of plumbagin, for which antimicrobial potency had been previously reported. It is believed that the activity of plumbagin is mainly related with covalent binding to biomolecules and to a lesser extent due to generation of ROS. However, it is unclear whether plumbagin has a great enough margin of safety for pharmacological use. The *D. intermedia* methanol extract also showed considerable antimicrobial activity, and interestingly, was able to inhibit the growth of the only strain resistant to the *n*-hexane extract, namely the multidrug resistant *P. aeruginosa*. Considering the high resistance of this microorganism to most antibiotics it would be interesting to determine the underlying mechanism. The *P. lusitanica* extract showed little activity against the panel of microorganisms. Although the performed biological assays did not point out any potential application for the prepared extracts it is important to pursue further screening programs considering the vast chemical diversity of phytochemicals and the enormous contribution plants have provided to the current state of medicine.

During the chemical analysis of the *n*-hexane extract prepared from *D. intermedia* it became clear that, using a relatively straightforward procedure, large amounts of nearly pure plumbagin could be obtained from micropropagated *D. intermedia* plants. Due to the commercial value of the naphthoquinone and the failed attempts of production by chemical synthesis, the potential of plumbagin bioprospection from *in vitro* cultures of *D. intermedia* was evaluated. The results showed that, besides the relatively high biomass production rates obtained, *D. intermedia* plants seem to produce higher amounts of plumbagin in comparison to the current source of exploitation (*Plumbago* spp.). Also, it is expected that the production of plumbagin can be further enhanced considering that no growth regulators or elicitors were used in this study. Besides the advantages of obtaining plumbagin from micropropagated plants in terms of quality control, harvesting plumbagin from naturally growing or cultivated plants would be obviated. Comparison of several extraction methods showed that the most efficient way to extract plumbagin consists on applying ultrasound to a pre-leached mixture of micropropagated plant material and *n*-hexane for a short period of time. Alternatively, following a more environmental approach, plumbagin can be extracted using supercritical carbon dioxide, avoiding the use of hazardous organic solvents for extraction. Despite yielding lower recoveries, SFE provided encouraging results for an initial attempt and method development might be worthwhile investigating due to the advantages that SFE offers over conventional methods. The potential of using SPE to purify the crude extracts was evaluated and results showed that the procedure is very effective in concentrating plumbagin and removing impurities in one single step. The SPE procedure applied to the UAE extract can be used to produce plumbagin in large amounts with a purity of nearly 100% and very low product losses.

In conclusion, this work provided a more in-depth study of the carnivorous plant species *P. lusitanica*, *P. vulgaris*, *D. rotundifolia* and *D. intermedia*, and hopefully contributed to a better understanding of their *in vitro* culture, biochemistry as well as to the development of methods for discovering secondary metabolites with potential as lead structures for drug development in a sustainable approach. Given some of the remarkable aspects of these plants mentioned in this work it is crucial that further efforts for the conservation of these species are undertaken.