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(FOR EVALUATION OF THE ACT DOCTORAL THESIS)

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DOCTORANDO *(candidate PHD)*: **CORREIA MATÍAS, DIOGO HENRIQUE**
D.N.I./PASAPORTE (*Id.Passport*): ******3452**
PROGRAMA DE DOCTORADO *(Academic Committee of the Programme)*: **D325-CIENCIAS DE LA SALUD**
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En el día de hoy 02/12/16, reunido el tribunal de evaluación, constituido por los miembros que suscriben el presente Acta, el aspirante defendió su Tesis Doctoral **con Mención Internacional** (*In today assessment met the court, consisting of the members who signed this Act, the candidate defended his doctoral thesis with mention as International Doctorate*), elaborada bajo la dirección de (*prepared under the direction of*) PATRICIA DIAS MENDOÇA RIJO // ANA CATARINA BECO PINTO REIS // ANA M. DÍAZ LANZA.

Sobre el siguiente tema *(Title of the doctoral thesis)*: *ISOLATION, MODELING AND PHYTOSOME FORMS OF ANTIMICROBIAL AND ANTIPROLIFERATIVE COMPOUNDS FROM PLECTRANTHUS SPP.ECTRANTHUS SPP*

Finalizada la defensa y discusión de la tesis, el tribunal acordó otorgar la CALIFICACIÓN GLOBAL[1] de (**no apto, aprobado, notable** y **sobresaliente**) *(After the defense and defense of the thesis, the court agreed to grant the GLOBAL RATING (fail, pass, good and excellent)*: SOBRESALIENTE / EXCELLENT

Alcalá de Henares, a 2 de diciembre de 2016

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Con fecha 21 de diciembre de 2016 la Comisión Delegada de la Comisión de Estudios Oficiales de Posgrado, a la vista de los votos emitidos de manera anónima por el tribunal que ha juzgado la tesis, resuelve:

☒ Conceder la Mención de "Cum Laude"
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La Secretaria de la Comisión Delegada

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Juan Ramón Velasco Pérez

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Secretario del Tribunal: NATALIO GARCÍA HONDUVILLA.
Directores de Tesis: PATRICIA DIAS MENDOÇA RIJO // ANA CATARINA BECO PINTO REIS // ANA M. DÍAZ LANZA

Doctoral Program in Health Sciences

ISOLATION, MODELING AND PHYTOSOME FORMS OF ANTIMICROBIAL AND ANTIPROLIFERATIVE COMPOUNDS FROM *PLECTRANTHUS SPP*.

Doctoral thesis presented by:

Diogo Henrique Correia Matias

Alcalá de Henares, 2016

DEPARTAMENTO DE
CIENCIAS BIOMEDICAS

ESCOLA DE CIÊNCIAS E
TECNOLOGIAS DA SAÚDE

En virtud del acuerdo de colaboración con la Universidade Lusófona de Humanidades e Tecnologias

Programa de Doctorado en Ciencias de la Salud

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AISLAMIENTO, MODELIZACIÓN Y FITOSSOMAS DE COMPUESTOS ANTIMICROBIANOS Y ANTIPROLIFERATIVOS DE *PLECTRANTHUS* SPP.

ISOLAMENTO, MODELAÇÃO E FORMAS FITOSSOMAIS DE COMPOSTOS ANTIMICROBIANOS E ANTIPROLIFERATIVOS DE *PLECTRANTHUS SPP*.

Tesis doctoral presentada por:

Diogo Henrique Correia Matias

Directores:

Dra. Patrícia Rijo

Dra. Catarina Reis

Dra. Ana Mª Díaz-Lanza

Alcalá de Henares, 2016

Universidad de Alcalá

DPTO. CIENCIAS BIOMÉDICAS
Unidad Docente de Farmacología
Facultad de Medicina
Universidad de Alcalá
Ctra. Barcelona, Km. 33,600
28871 Alcalá de Henares (Madrid)
Telf.: 91 885 45 49 - Fax: 91 885 45 91
e-mail: dpto.biomedicas@uah.es

D. FRANCISCO ZARAGOZÁ GARCÍA, CATEDRÁTICO DE FARMACOLOGÍA Y DIRECTOR DEL DEPARTAMENTO DE CIENCIAS BIOMÉDICAS DE LA UNIVERSIDAD DE ALCALÁ,

CERTIFICA:

Que el trabajo titulado: "Isolation, modeling and phytosome forms of antimicrobial and antiproliferative compounds from *Plectranthus spp.*" ha sido realizado por D. Diogo Henrique Correia Matias, bajo la dirección de las Dras. Ana María Díaz-Lanza, Profesora Titular de Farmacología del Dpto. de Ciencias Biomédicas, de la Universidad de Alcalá, Patricia Mendonça Rijo Professora Auxiliar Y Professora de Química Farmacêutica y Catarina Pinto Reis Professora Auxiliar Y Professora de Tecnologia Farmacêutica na Escola de Ciências e Tecnologias da Saúde da Universidade Lusófona de Humanidades e Tecnologias de Lisboa, respectivamente y cumple todos los requisititos para su defensa pública como Tesis Doctoral.

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Universidad de Alcalá

DEPARTAMENTO DE CIENCIAS BIOMÉDICAS
Campus Universitario
Ctra. de Barcelona Km. 33,600
E-28871 Alcalá de Henares (Madrtid)
Teléfono: 91 885 46 57

PATRÍCIA MENDONÇA RIJO PROFESSORA AUXILIAR Y PROFESSORA DE QUÍMICA FARMACÊUTICA Y CATARINA PINTO REIS PROFESSORA AUXILIAR Y PROFESSORA DE TECNOLOGIA FARMACÊUTICA NA ESCOLA DE CIÊNCIAS E TECNOLOGIAS DA SAÚDE DA UNIVERSIDADE LUSÓFONA DE HUMANIDADES E TECNOLOGIAS DE LISBOA, RESPECTIVAMENTE Y ANA MARÍA DÍAZ-LANZA, PROFESORA TITULAR DE UNIVERSIDAD DEL ÁREA DE FARMACOLOGÍA DEL DEPARTAMENTO DE CIENCIAS BIOMÉDICAS DE LA FACULTAD DE FARMACIA DE LA UNIVERSIDAD DE ALCALÁ

CERTIFICAN:

Que la memoria para optar al Grado de Doctor, elaborada por Diogo Henrique Correia Matias, cuyo título es:

"Isolation, modeling and phytosome forms of antimicrobial and antiproliferative compounds from Plectranthus spp."

ha sido realizada bajo su dirección y que reúne todos los requisitos necesarios para su juicio y calificación.

Y para que así conste, firman el presente certificado en Alcalá de Henares a nueve de mayo de dos mil dieciséis.

Universidade Lusófona de Humanidades e Tecnologias
Escola de Ciências e Tecnologias de Saúde

Patrícia Rijo

Universidade Lusófona de Humanidades e Tecnologias
Escola de Ciências e Tecnologias de Saúde

Catarina Pinto Reis

Ana Mª Díaz-Lanza

UNIVERSIDAD DE ALCALÁ DEPARTAMENTO DE CIENCIAS BIOMÉDICAS

Dedicado aos meus pais e avós que fizeram de mim o que sou hoje.

“Science, my boy, is made up of mistakes,
but they are mistakes which it is useful to make,
because they lead little by little to the truth.”

Jules Verne, Journey to the Centre of the Earth

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Abstract

Natural products have been used as a valuable source of new lead drugs. The *Plectranthus* L'Her (Lamiaceae) genus possess a wide diversity of ethnomedicinal uses which indicate the presence of bioactive molecules. Also, innovative methods for natural drug delivery, as phytosome, have showed to be a promising strategy for the improvement of delivery and stability.

In this work, three plants from the *Plectranthus* genus were studied: *P. madagascariensis*, *P. neochilus* and *P. porcatus*. Several extracts were prepared by the combination of extraction methods (infusion, decoction, microwave, ultrasound, maceration and supercritical fluid extraction) with different polarity solvents (water, acetone, methanol and $scCO_2$). Those extracts were profiled by HPLC-DAD and the main components were identified, including polyphenols (caffeic acid, chlorogenic acid and rosmarinic acid), diterpenes (7α-acetoxy,6β-hydroxyroyleanone and coleon U) and flavones (rutina y naringenina).

The prepared extracts were screened for their antimicrobial (Gram positive and negative bacteria and yeasts), antioxidant (DPPH radical scavenging activity) and cytotoxic activities (MDA-MB-231 cell line). Extracts from *P. madagascariensis* (acetone maceration and acetone ultrasound) and *P. neochilus* (acetone ultrasound) showed antibacterial effects against Gram positive bacteria strains, namely, *Bacillus subtilis, Staphylococcus aureus* and *S. epidermidis* and a Gram negative bacteria strain, *Klebsiella pneumonia* (MIC values 1.95-250 µg/mL). The ultrasound extract of *P. madagascariensis* prepared with acetone showed potent antibacterial effect against *Staphylococcus* spp., including a methicillin-resistant strain (MRSA), with MIC values ranging from 1.95 to 7.81 µg/mL. These results validate the traditional uses of such plants as anti-infectious agents. All methanolic extracts showed potent antioxidant effects at 100 ng/mL (60.8-89.0%). The maceration acetone extract from *P. madagascariensis* showed moderate cytotoxic effects in the MDA-MB-231 breast cancer cell line with IC_{50} of 64.52 µg/mL.

The organic solvent extracts from *P. madagascariensis* were the most bioactive and thus characterized (identification and quantification) using HPLC-DAD. Furthermore, the compounds were identified by authentic standard overlay: rosmarinic acid, 7α,6β-dihydroxyroyleanone, 7α-acetoxy-6β-hydroxyroyleanone and coleon U. A diterpenic compound was isolated from the ultrasound acetonic extract of *P. madagascariensis* and spectroscopically characterized (^{1}H- and ^{13}C-NMR) as 7α-formyloxy-6β-hydroxyroyleanone. This was the first time this compound was isolated from this plant. The cytotoxic effect of the identified compounds was evaluated in a battery of cancer cell lines (MDA-MB-231, MCF-7, HCT116, NCI-H460 and MCR-5). The diterpenic compounds showed moderate to potent cytotoxicity in the majority of tested cell lines. A high selectivity for cancer cell lines was observed for 7α,6β-dihydroxyroyleanone and 7α-acetoxy-6β-hydroxyroyleanone with selectivity index of 4.3 and 3.2, respectively.

The combination of the observed results and literature data afforded the establishment of new structure-activity relationships of roylenanone abietanic

compounds. The relevance of lipophilicity and of the presence of an electron donating group at 6 and/or 7 positions was observed.

The antibacterial acetonic ultrasound extract from *P. madagascariensis* identified in the initial screening was selected for incorporation into a phytosomal formulation and subsequently coated by chitosan. Phytosomes were amorphous, uniform in shape as shown by AFM and SEM, and with an average size of 1082 ± 363 nm and zeta potential of +20.59 ± 12.02 mV. The encapsulation of the antibacterial extract was determined by HPLC (57.7±0.06%) and the chemical interactions between the formulation components was cofirmed by DSC and DRIFTS. Such phytosomes showed a sustained release of the extract **4** and lower skin-like permeation fluxes. An improvement up to a 4-fold factor in the anti-Staphylococci activity (MIC values 0.98-31.25 µg/mL) was observed. The safety of such formulation was verified by *in vitro* human keratinocytes cytotoxicity assays and by *in vivo* acute and sub-chronic dermal irritation tests in mice.

This study showed the potential of the *Plectranthus* genus as source of lead antibacterial and antiproliferative agents and validate the ethnomedicinal uses of the studied plants. The isolated abietane diterpenes obtained from *P. madagascariensis* possess promising selective cytotoxic effects, namely, aginst the lung cancer lines tested. Also, the developed formulation of extract of *P. madagascariensis* corresponds to a potent topical antibacterial candidate with a broad spectrum of activity.

Keywords: *Plectranthus*; Diterpenes; Cytotoxicity; Antibacterial; Phytosome.

Resumen

Los productos naturales han sido una valiosa fuente de potenciales fármacos. Las plantas del género *Plectranthus* L'Her (Lamiaceae) poseen una amplia diversidad de usos etnomedicinales, que son una indicación de la presencia de potenciales fármacos en su composición. Los innovadores nanocarregadores de productos naturales, como el fitosoma, han demostrado ser una estrategia prometedora para la mejora de su veiculación y estabilidad.

En este trabajo, tres plantas del género *Plectranthus* fueron estudiadas: *P. madagascariensis*, *P. neochilus* y *P. porcatus*. Varios extractos fueron preparados por la combinación de métodos de extracción (infusión, decocción, maceración, microondas, ultrasonido o extracción con fluido supercrítico) utilizando disolventes de diferente polaridad (agua, acetona, metanol o dióxido de carbono supercrítico). Los extractos preparados fueron perfilados por HPLC-DAD y se identificaron los majores componentes principales, incluyendo polifenoles (ácido cafeico, ácido clorogénico y ácido rosmarínico), diterpenos (7α-acetoxi-6β-hidroxiroileanona y coleona U) y flavonas.

Los extractos preparados han sido evaluados por su actividad antimicrobiana (bacterias Gram positivas, Gram negativas, levaduras), antioxidante (captación de radicales por DPPH) y citotóxica (células MDA-MB-231). Los extractos de *P. madagascariensis* (maceracion y ultrasonido con acetona) y *P. neochilus* (ultrasonido com acetona) mostraron efectos antibacterianos contra *Bacillus subtilis*, *Staphylococcus aureus*, *S. epidermidis* (bacterias Gram positivas) y *Klebsiella pneumoniae* (bacterias Gram negativas). Lo extracto de ultrasonido de *P. madagascariensis* obtenido con acetona reveló efectos antibacterianos potentes en *Staphylococcus* spp., incluyendo una cepa resistente a la meticilina, con valores de concentración mínima inhibitoria en el rango de 1,95 a 7,81 μg/mL. Esto está de acuerdo con los usos tradicionales de tales plantas como agentes anti-infecciosos. Cada extracto metanólico mostró potentes efectos antioxidantes en una concentración de extracto de 100 ng/mL (60,8-89,0%). El extracto de maceración de *P. madagascariensis* mostró algunos efectos citotóxicos en la línea celular de cáncer de mama MDA-MB-231 con IC_{50} de 64,52 mg/mL.

Dado que los extractos más bioactivos se obtuvieron de *P. madagascariensis*, aquellos se caracterizaron con más detalle con la identificación y cuantificación de los principales compuestos en los extractos. Cuatro compuestos fueron identificados por comparación con estándares: ácido rosmarínico, 7α,6β-dihidroxiroyleanona, 7α-acetoxi-6β-hidroxiroileanona y coleona U. Se aisló un compuesto diterpénico del extracto ultrasonido de acetona de *P. madagascariensis* que se caracterizó por RMN ^{1}H y ^{13}C como 7α-formiloxi-6β-hidroxiroileanona. Esto supone el aislamiento por primera vez, de este compuesto en esta planta. Los efectos citotóxicos de los compuestos determinados en una batería de líneas celulares de cáncer y en conjugación con los datos disponibles de la literatura proporcionaron el establecimiento de algunas relaciones estructura-actividad de la estructura abietanica de la roilenanona. Se observó

cierta selectividad para líneas celulares de cáncer de 7α,6β-dihidroxiroileanona y 7α-acetoxi-6β-hidroxiroileanona con el índice de selectividad de 4,3 y 3,2 respectivamente.

El extracto más antibacteriano resultante del cribado inicial fue seleccionado para su incorporación en una formulación fitosomal y posteriormente recubierto por el quitosano. Los fitosomas obtenidos eran amorfos, uniformes en forma (SEM y AFM), con un tamaño medio de 1082 ± 363 nm y lo potencial zeta de 20,59 ± 12,02 mV. La eficiencia de encapsulación se determinó por HPLC (57,7 ± 0,06%). La interacción entre los componentes de la formulación se demostró mediante DSC y DRIFTS. Los fitosomas mostraron una liberación sostenida y el flujo de membrana de baja con una cierta mejora de la actividad anti-estafilococos (concentración mínima inhibitoria de 0,49 a 31,25 mg/ml). La seguridad de dicha formulación se demuestra *in vitro* por la baja citotoxicidad en queratinocitos humanos y *in vivo* con ensayos de irritación cutánea aguda y sub-crónica en ratones.

Este estudio demostró el potencial del género *Plectranthus* como fuente de nuevos agentes antibacterianos y de tratamiento del cáncer, justificándose algunos de los usos etnomedicinales de esas plantas. Los diterpenos del tipo abietano aislados a partir de *P. madagascariensis* poseen prometedores efectos citotóxicos selectivos contra algunas líneas de cáncer. Además, la formulación desarrollada de extracto de *P. madagascariensis* corresponde a un candidato antibacteriano tópico potente con un amplio espectro de actividad.

Palabras-chave: *Plectranthus*; diterpenos; citotoxicidad; antibacterianos; fitosoma.

Resumo

Os produtos naturais têm sido uma valiosa fonte de novos produtos farmacêuticos. As plantas de género *Plectranthus* L'Her (Lamiaceae) possuem uma ampla diversidade de usos etnomedicinais, que correspondem a uma indicação da presença de potenciais fármacos na sua composição. Métodos inovadores para a veiculação dos produtos naturais, como os fitossomas, demostraram ser uma estratégia promissora para a melhora da veiculação e estabilidade destes produtos.

Três plantas do género *Plectranthus* foram estudadas: *P. madagascariensis*, *P. neochilus* e *P. porcatus*. Extratos foram preparados pela conjugação de técnicas de extração como infusão, decocção, maceração, micro-ondas, ultrassons ou de extração por fluidos supercríticos, utilizando como solventes água, acetona, metanol ou dióxido de carbono supercrítico. Os extratos preparados foram perfilados por HPLC-DAD e identificados alguns dos principais componentes, por comparação com padrões, verificando-se na sua constituição polifenois, diterpenos e flavonoides.

Os mesmos extratos foram avaliados em termos das suas atividades antimicrobiana (bactérias de Gram positivo, Gram negativo e leveduras), antioxidante (captura de radicais) e citotóxica (células MDA-MB-231). Extratos de *P. madagascariensis* (maceração e ultrassons em acetona) e *P. neochilus* (ultrassons em acetona) apresentaram efeitos antibacterianos contra *Bacillus subtilis*, *Staphylococcus aureus*, *S. epidermidis* e *Klebsiella pneumoniae*. O extrato de ultrassons de *P. madagascariensis* obtido com acetona apresentou potentes efeitos antibacterianos em espécies de *Staphylococcus*, incluindo uma estirpe resistente à meticilina, verificando-se uma concentração mínima inibitória no intervalo de 1,95 a 7,81 µg/mL. Esta atividade encontra-se de acordo com os usos tradicionais destas plantas como agente anti-infecioso. Os extratos metanolicos apresentaram potentes efeitos antioxidantes a uma concentração de extrato de 100 ng/mL (60,8-89,0%). O extrato de maceração em acetona de *P. madagascariensis* apresentou alguns efeitos citotóxicos na linha celular de cancro de mama MDA-MB-231 com um IC_{50} de 64,52 mg/mL. Uma vez que os extratos mais bioativos foram obtidos a partir de *P. madagascariensis*, estes foram caracterizados em maior detalhe com identificação e quantificação dos principais compostos nesses extratos. Quatro compostos foram identificados por comparação com padrões: ácido rosmarinico, 7α,6β-dihidroxiroyleanona, 7α-acetoxi-6β-hidroxiroileanona e coleona U. Um composto diterpenico foi isolado a partir do extrato de ultrassons com acetona de *P. madagascariensis* e caracterizado por RMN de ^{1}H e ^{13}C como 7α-formiloxi-6β-hidroxiroileanona. Este composto foi isolado pela primeira vez a partir desta planta. Os efeitos citotóxicos dos compostos purificados foram determinados numa bateria de linhas celulares de cancro e em conjugação com dados da literatura, foram estabelecidas algumas relações de estrutura-atividade na estrutura abietanica do tipo roileanona. Foi observada alguma seletividade para linhas celulares de cancro por parte da 7α,6β-dihidroxiroileanona e 7α-acetoxi-6β-hidroxiroileanona com com índice de seletividade de 4,3 e 3,2 respetivamente.

O extrato com maior potência antibacteriana identificado na triagem inicial foi selecionado para sua incorporação numa formulação fitossomal com posterior encapsulação por quitosano. Os fitossomas obtidos apresentavam-se amorfos, uniformes na forma (SEM e AFM), com um tamanho médio de 1082 ± 363 nm e potencial zeta de 20,59 ± 12,02 mV. A eficiência de encapsulação determinada por HPLC (57,7 ± 0,06%). A existência de interações entre os componentes da formulação foi analisada por A ocorrência de encapsulação foi demonstrada por DSC e DRIFTS. Os fitossomas demonstraram uma libertação sustentada com redução da permeação trans-cutanea. Uma melhoria em até 4 vezes na atividade anti-estafilococo (concentração mínima inibitória de 0,49 a 31,25 mg/ml). A segurança desta formulação foi demonstrada por baixa citotoxicidade em queratinocitos humanos *in vitro* e por irritação negligenciável *in vivo* nos ensaios de irritação cutânea aguda e sub-crónica em ratinhos.

Este estudo demonstrou o potencial do género *Plectranthus* como fonte de novos agentes antibacterianos justificando-se alguns dos usos etnomedicinais destas plantas. Os diterpenos do tipo abietano isolados a partir de *P. madagascariensis* possuem efeitos citotóxicos com alguma seletividade face a algumas linhas celulares de cancro. A formulação desenvolvida a partir do extrato de *P. madagascariensis* corresponde a um promissor candidato a antibacteriano tópico com amplo espectro de atividade e elevada potência.

Palavras-chave: *Plectranthus*; diterpenos; citotoxicidade; antibacterianos; fitossoma.

Abbreviations and acronyms list

AFM – atomic force spectroscopy

AK – actinic keratosis

AuNPs – gold nanoparticles

BMP-2 – bone morphogenetic protein 2

cAMP – cyclic adenosine monophosphate

Cmax – maximum concentration in plasma

CMC – carboxymethyl cellulose

CO_2 – carbon dioxide

FT-IR – Fourier transform infrared spectroscopy

DAD - diode array detector

DAG – diacylglycerol

DLS – dynamic light scattering

DMSO – Dimethyl sulfoxide

DPPH – 2,2-diphenyl-1-picrylhydrazyl

DSC – differential scanning calorimetry

EGCg – epigallocatechin-3-O-gallate

EMA – European Medicines Agency

FDA – Food and Drug Administration Agency of United States of America

GRAS – generally recognized as safe

HER2 – human epidermal growth factor receptor 2

HPLC – High pressure liquid chromatography

HTS – high throughput screening

Jmax – maximum flux

LOD – limit of detection

LOQ – limit of quantification

MDR – multidrug resistant

MIC – minimum inhibitory concentration

MRSA – methicillin-resistant *Staphylococcus aureus*

MS – mass spectroscopy

MWAE – microwave assisted extraction

NDDS – novel drug delivery systems

NF-kB – nuclear factor kappa B

NMR – nuclear magnetic resonance

PCS – photon correlation spectroscopy

PdA – phosphatidic acid

PdC – phosphatidylcholine

PdE – phosphatidylethanolamine

PdI – phosphatidylinositol

PdS – phosphatidylserine

PI3K – phosphoinositide 3-kinase

PKA – protein kinase A

PKCδ – protein kinase C delta

PLA – polylactic acid

PMAE – pressurized microwave assisted extraction

PPC – phyto-phospholipidic complex

Rf – retention factor

RUNX2 – runt-related transcription factor 2

$scCO_2$ – supercritical carbon dioxide

SCF – supercritical fluid

SCFE – supercritical fluid extraction

SEDS – supercritical fluid solution enhanced dispersion

SEM – scanning electron microscopy

SFMAE – solvent-free microwave assisted extraction

SIRT1 - silent information regulator 1

STAT3 – signal transducer and activator of transcription 3

TB – *Mycobacterium tuberculosis*

TEM – transmission electron microscopy

THF – tetrahydrofuran

TLC – thin layer chromatography

UHPLC – ultra-high-pressure liquid chromatography

USAE – ultrasound assisted extraction

UV – ultraviolet

VEGF – vascular endothelial growth factor

WHO – World Health Organization

XRD – x-ray powder diffraction

ζP – Zeta potential

Publications and communications list

Articles (Web of Knowledge indexed)

- Rijo, P, Matias, D, Fernandes, AS, Simões, MF; Nicolai, M; Reis, CP. Antimicrobial Plant Extracts Encapsulated into Polymeric Beads for Potential Application on the Skin. Polymers (Basel). 2014, 6, 479–490. (IF2015 2.944, Polymer Science Q1) [Annex 9];
- Matias, D; Rijo, P; Reis, CP. "Phytosomes as biocompatible carriers of natural drugs" submitted for publication in Current Medicinal Chemistry (IF2015 3.455, Pharmacology and Pharmacy Q1).

Other articles

- Pereira, M; Matias, D; Pereira, F; Reis, CP; Simões, MF; Rijo, P. Antimicrobial screening of *Plectranthus madagascariensis* and *P. neochilus* extracts. Biomed Biopharm Res 2014; 12(1): 127–138 [Annex 10];
- Matias, D; Roque, L; Simões, MF; Diaz-Lanza, A; Rijo, P; Reis, CP. *Plectranthus madagascariensis* phytosomes: formulation optimization. Biomed Biopharm Res 2015; 12(2): 223–231 [Annex 11].

Books chapters

- Matias D. *et al*. Natural products as lead Protein Kinase C modulators for cancer therapy. Studies in Natural Products Chemistry (Elsevier) (accepted, in press).

Conference proceedings (in Web of Knowledge indexed journals)

- Matias, D; Pereira, F; Nicolai, M; Roberto, A; Saraiva, N; Fernandes, AS; Simões, MF; Lanza, A D; Reis, CP and Rijo, P. 2014. "Abietane Diterpenes from *Plectranthus Madagascariensis*: A Cytotoxicity Screening." Planta Med 80 (16): P1L152. (IF 1.990, Plant Sciences Q2);
- Matias, D; Pereira, F; Pereira, M; Simões, MF; Lanza, AD; Reis, CP and Rijo, P. 2014. "Antimicrobial screening of *Plectranthus madagascariensis* Benth. extracts." Planta Med 80 (16): P1L102. (IF 1.990, Plant Sciences Q2);
- Matias, D; Nicolai, M; Costa, J; Saraiva, N; Fernandes, AS; Simões, MF; Lanza, A D; Reis, CP and Rijo, P. 2015. "Cytotoxicity Screening of *Plectranthus* Spp. Extracts

and Individual Components in MDA-MB-231 Cells." Toxicology Letters 238 (2): S240 (IF 2015 3.522, Toxicology Q1).

Oral communications

- Matias, D, et al. "Bioactive abietane diterpenes from *Plectranthus* spp. extracts and its encapsulation into a novel phytosomal formulation" at 11º Encontro Nacional de Química Orgânica / 4º Encontro de Química Terapêutica; 1-3 Dez 2015, Porto (Portugal);
- Matias, D; Roque, L; Simões, MF; Diaz-Lanza, AM; Bernardes, C; Piedade, M; Viana, AS; Rijo, P and Reis, CP. "Phytosome as leading strategy for natural compounds delivery" at Nano2016.pt – III Simposio de Nanociencia e nanotecnologia biomédica; 15 Abr 2016, Lisbon (Portugal);
- Matias, D; Simões, MF; Diaz-Lanza, AM; Reis, CP and Rijo, P. "*Plectranthus* spp. antimicrobial and antiproliferative components and their inclusion into phytosomal formulation" at 6th CIPAM – International Congress of Aromatic and Medicinal Plants; 29 May to 1 Jun 2016, Coimbra (Portugal).

Poster communications

- Matias D, Nicolai M, Roque L, Reis CP, Simões MF, Rijo P. *Encapsulation of Plectranthus madagascariensis extract into polymeric beads*. Nano2013.pt - II Simpósio Nacional de Nanociência e Nanotecnologia Biomédica. P14. Lisbon, 11 Oct 2013;
- Rijo P, Matias D, Simões MF, Nicolai M, Roque L, Reis CP. New approach to improving efficacy of herbal extracts. 9th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology. 31 Mar – 3 Apr 2014;
- Diogo Matias, Filipe Pereira, Margarida Pereira, Maria Fátima Simões, Ana M. Diaz-Lanza, Catarina Pinto Reis, Patricia Rijo. *Antimicrobial screening of Plectranthus madagascariensis Benth. Extracts*. Planta Med 2014; GA2014; 80 - P1L102
- Diogo Matias, Filipe Pereira, Marisa Nicolai, Amilcar Roberto, Nuno Saraiva, Ana Sofia Fernandes, Maria Fátima Simões, Ana Diaz Lanza, Catarina Pinto Reis, Patricia Rijo. *Abietane diterpenes from Plectranthus madagascariensis: a cytotoxicity screening*. Planta Med 2014; GA2014; 80 - P1L152.

- M. Pereira, F. Pereira, D. Matias, M. F. Simões, C. P. Reis and P. Rijo. Antimicrobial screeening of *Plectranthus madagascariensis* and *Plectranthus neochilus* extracts. International conference in antibiotic research (ICAR). Madrid, 1-3 Oct 2014.
- Diogo Matias, L Roque, MF Simões, AM Diaz-Lanza, CP Reis, P Rijo. "Chitosan encapsulated phytosomes of *Plectranthus madagascariensis*: an innovative antibacterial system for topical application" at International Society for Biophysics and Imaging of the Skin World Congress (ISBS 2016); 31th May to 3rd June 2016, Lisbon (Portugal).

Grants and awards

- Honourable mention in Oral communication at 6th CIPAM – International Congress of Aromatic and Medicinal Plants; 29 May to 1 Jun 2016, Coimbra (Portugal);
- PADDIC 2013-2014 grant from ALIES-COFAC as part of the PhD program in Health Sciences from U Alcalá and U Lusófona.

Chapter I


physic-chemical characteristics
diterpenes antitumoral
purposes chemotherapeutic advances
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1. State of the art, hypothesis and objectives

1.1. Natural products as new drugs source

1.1.1. Historic perspective

The medicinal use of plants has been directly related to human evolution. Primitive hominids, as the Neanderthals, should have been aware of the therapeutic utility of various plants. Pollen vestiges of medicinal plants have been found in their graves, dated more than 60,000 years old[1]. The first archeologic evidences of plant extract production consisted in several extraction pots, similar to modern Soxlet extractors, dated about 3,500 BC, back to ancient Mesopotamian. Ancient Sumerian texts, from about 2,100 BC, described elaborated methods of bulk extract production using water and oils as solvents. The Egyptian *Ebers Papyrys*, from around 1,550 BC, contains the description of more than 700 sources of natural medicinal products and their indications[2]. Later, the roman physician Pedanius Dioscorides compiled *De Materia Medica* which included the description of about 600 plant based medicines[1]. At the end of Roman empire, Galen, a Greek physician and pharmacist left a registry of 540 medicines based in plants and introduced the notion that herbal components could be not only beneficial but also harmful depending on dose and extracted components[2]. Even though the ancient knowledge of the therapeutic properties of plants, only in XVIII and XIX centuries the first purified compounds were isolated from plants. Morphine was isolated by Sertürner in 1804 from the opium powder extracted from *Papaver somniferum* and it became the first purified natural product commercialized (by Merck, 1826)[2,3]. In subsequent years diverse natural products were isolated from plants and some of them are still currently used as the cases of atropine from *Atropa belladonna*, caffeine from *Coffea arabica*, colchicine from *Colchicum autumale* or salicin from *Salix alba*[2]. The increasing knowledge about drug chemical structures and the evolution of chemical synthesis led to a growing interest of the pharmaceutic industries in the drug total chemical synthesis, over the isolation from natural sources. The 1990s advent of combinatory chemistry and the implementation of high throughput screening (HTS) programs led to an abrupt reduction of the pharmaceutic industry in phytochemistry research programs[3,4]. Those new techniques were expected to create a surge of new active substances discovered, which did not occur. Also, the total number of approved new chemical entities had some of the lowest values in the past two decades[5], besides of an increasing spending in research and development. In this period, only one *de novo* new chemical entity was reported to the public domain resulting from the use of combinatory chemistry and HTS (the antitumor sorafenib)[5]. Those disappointing results from HTS have been related to the chemical characteristic of the compounds constituting the databases subject of HTS. Feher and Schmidt[6] have disclosed the main differences between drug molecules, natural products and combinatorial chemistry compounds. They noted that molecules obtained from combinatory synthesis had a lower number of chiral centres, a less

prevalent presence of complex ring systems and a substantial difference in the degree of saturation and number and distribution of the heteroatoms in comparison to natural products and drug molecules[6]. Those authors suggest that if the constitution of HTS databases would be based on nature-like molecules, the new chemical entity discovery process would have an improved hit-ratio[6]. Such approach have been recently adopted in some groups[5,7] and even some natural product databases have been disclosed to the public[8]. Newman and Cragg have comprehensively reviewed the natural products, semi-synthetic and natural inspired molecules that have been approved by the Food and Drug Administration (FDA)[4,5,9,10]. On their most recent review, they verified that from all 1,135 new drugs approved in the 1981 to 2010 period, about 50% have natural origin (fully natural, derivatives or mimics)[5]. Those reviews along with other compilations of the natural product impact on clinical used drugs, firm the statement that the natural products are the most reliable source of new drug molecules[3,11,12]. The value of natural products in drug discovery has been highlighted in the recent Nobel prize attribution to Dr. William C. Campbell and Professor Satoshi Ōmura[13,14] for their discovery of avermectins, fermentation products of *Streptomyces avermitilis*, as active drugs against a number of parasitic diseases including river blindness and lymphatic filariasis, and to Professor Youyou Tu for his discovery of artemisinin as the active component of the *Artemesia annua*, and that resulted in a key reduction of malaria morality[15,16].

Considering that up to 95% of the Earth's biodiversity was still not studied for any biologic activity, a virtually infinite natural chemical diversity is yet available for use in further drug discovery[17].

1.1.2. The pharmacologic relevance of natural products

The metabolism of plants resulted in the production of numerous compounds that can be classified according its functions as primary or secondary metabolites[18]. Primary metabolites are directly involved in normal growth, development and reproduction processes while secondary metabolites are not absolutely necessary for the survival of the plant but play an important role in interspecies defences, intra/interspecies interaction and environmental adaptation[18,19] . Some of the most relevant plant secondary metabolites are terpenoids, alkaloids and phenolic compounds[18]. The plant secondary metabolites have contributed to the development of new drugs in three ways: being new drug entities with good pharmacologic activity and adequate pharmacokinetics and toxicological characteristics in the unmodified state; as source of lead compounds which present some biologic activity but could be modified by hemi-synthesis to obtain more potent drug candidates; and by revealing new targets and action mechanisms which could further be used to the development of synthetic analogues[20].

Modern pharmaceutical research comprehends a multi-disciplinary approach involving chemists', biologists' and pharmacists' contribution to obtain leading drug candidates. To find such bioactive compounds in higher plants, a targeted approach, with a careful plant to study selection, based in ethnobotanical references or

chemotaxonomic studies would assure the highest hit rate[21]. The existence of bibliographic and/or local references to the traditional medicinal use of some plants are powerful indicators to the presence of bioactive compounds in those plants (i.e. the discovery of artemisinin from the traditionally used Chinese plant *Artemesia annua* for Malaria treatment[15]). The alternative approach could be focused on a specific class of molecules, with a known biologic activity and that could be taxonomically characteristic of some plant genus (e.g. some taxanes produced by *Taxus brevifolia* are also produced in other *Taxus* species[22]).

A large number of methods could be used for the extraction of natural products from vegetal matrixes and were discussed in detail in some reference books and articles[21,23–25]. In this work, solid-liquid extractions using organic and/or aqueous solvents were the most frequently employed methods but also non-conventional techniques employing supercritical fluids were also applied. The choice of a method of extraction is dependent on the plant material characteristics and on the quality and quantity of compounds to be extracted[23,24]. The extraction process could be exhaustive/total, extracting as much compounds as possible, or selective, extracting preferentially one type of desired metabolites. In total extractions is recommended the use of a polar solvent, namely, an alcoholic solvent which increase the permeability of the cellular wall facilitating the extraction of secondary metabolites. In selective extractions, the selection of solvent should follow the "like-dissolves-like" principle: polar solvents extract preferentially polar compounds (flavonoids, polyphenols, tannins and some alkaloids) while nonpolar solvents solubilize lipophilic compounds (fatty acids, waxes, sterols, along with some alkaloids, some terpenoids and some coumarins)[23,24].

A solid-liquid extraction is a dynamic process involving a series of simple steps (Figure **1**). Initially, the contact of the plant material with the solvent conduct to its penetration into the solid matrix. This step was generally very fast due to capillary forces acting on a porous solid. When the solvent reaches the cellular structures some of the plant

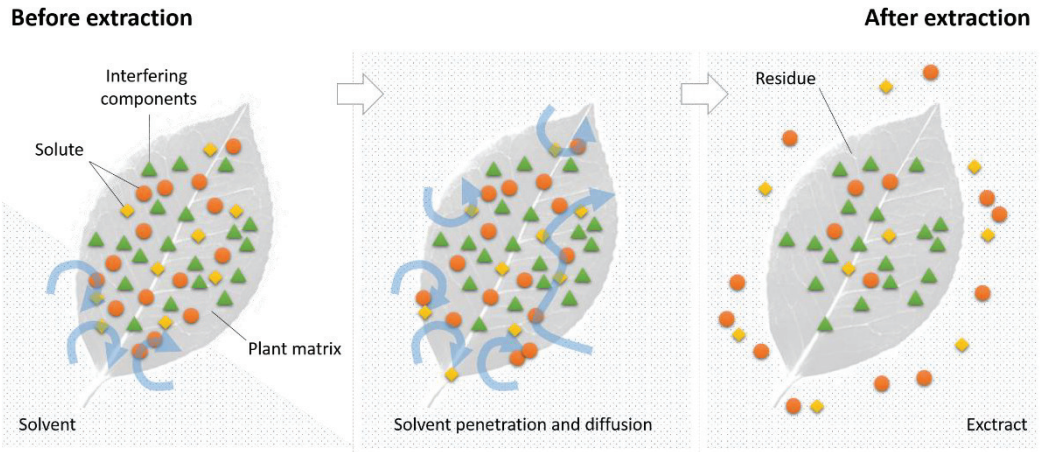

Figure 1. Representation of the extraction process and its basic terminology.

components were dissolved and migrate by diffusion to the solid-liquid interface at an approximately constant velocity. At this step, interactions with the solid matrix limit the mass transfer but the solute was gradually incorporated into the bulk solution by convection (natural or forced). The extraction rate is low at this period and the limiting step of the process.

1.2. *Plectranthus* genus (Lamiaceae): chemistry and bioactivities

Lamiaceae family comprises 236 genera and about 7000 species of flowering plants, most of them with aromatic characteristics[26]. Many are known for their ethnobotanical uses and essential oil production as the cases of sage (*Salvia* genus), basil (*Ocimum* genus) and mint (*Mentha* genus). Other important genus in this family is *Plectranthus* L'Her (tribe Ocimeae, subfamily Nepetoideae). This genus comprises more than 300 species and is mainly distributed through tropical and sub-tropical regions of Africa, Asia and Australia[27]. The morphologic identification of *Plectranthus* plants could be puzzling due to the lack of clear-cut morphological criteria to distinguish among species within the genus[28]. Also species classified originally in the closely related genus *Isodon*, *Solenostemon*, *Englerastrum* and the former genus *Coleus* are now part of the *Plectranthus* genus due to phylogeny studies with resource to genetic tolls[29]. This genus is actually considerer paraphyletic, as currently circumscribed, and three clades have been phylogenetically established: a sigmoid *'Coleus'* clade, including the species of the former Coleus genus; a sigmoid *'Plectranthus'* clade; and a straight *'Plectranthus'* clade[30]. *Plectranthus* is an economically important genus due to their horticultural, floricultural and ethnomedicinal uses[27,28]. Most *Plectranthus* species are robust, prolific and many possesses attractive leaves or flowers which justifies their gardening uses for over more than 100 years[31,32]. However more than 85% of the *Plectranthus* citations in literature are due to their uses in traditional medicine (as reviewed by Lukhoba et al. 2006[27]) and for the biologic activities of their extracts or isolated compounds that includes anti-infectious[33–35], antioxidant[36,37], anti-inflammatory[38] and cytotoxic[39–41] effects.

1.2.1. Ethnopharmacological uses

Plectranthus species have been used for their medicinal properties namely in digestive[42,43], skin[44], infective[45,46] and respiratory[47] conditions, among others. The species more frequently cited for their ethcnomedicinal uses were *P. barbatus* Andr. (formerly known as *Coleus forskohlii*) used in gastric problems, wounds, skin allergy, colds, cough, abdominal pain, rheumatic, bacterial, fungal and viral infections[27,45,46,48,49] and *P. amboinicus* (Lour.) Spreng (syn of *C. aromaticus*), used in dyspepsia, indigestion, diarrhoea, skin ulcerations, burn, skin allergies, chronic cough, asthma, bronchitis, fevers, headaches, muscular-skeletal pain, and infections[27,42,43]. The biologic activities and phytochemistry of *P. barbatus* have been extensively studied and reviewed[48,49], and many pharmacologic activities could be justified by the presence of the bioactive labdane diterpene forskolin (**1**). This compound is a specific activator of the adenylyl

cyclase which result in cardiotonic, bronchodilator, antihypertensive, anti-inflammatory and platelet aggregation inhibitory effects[50]. In *P. amboinicus*, several studies support the medicinal uses with the activity of their extracts but the link between its pharmacologic properties with the traditional uses have not yet been clearly established[51–53]. This example emphasises the importance of the phytochemical study of medicinal plants for the validation of their traditional uses by the identification of the bioactive secondary metabolites.

1.2.2. Phytochemical insights of the *Plectranthus* genus

The *Plectranthus* genus, as a member of the Nepetoideae subfamily, is chemotaxonomically characterized by the presence of the caffeic acid esters, rosmarinic acid (**2**) and the nepetoidins A (**3**) and B (**4**) (Figure **2**) with absence or scarce presence of iridoids and caffeoyl phenylethanoid glycosides[54]. This genus is rich in essential oils (more than 0.5% of the dry weight as volatile oil) with a major yield of monoterpenoids[54,55]. But the more frequently studied compounds from the genus *Plectranthus* are diterpenoids, mainly found in the coloured leaf-glands[56]. This chemical profile is similar to some genus of the Nepetoideae subfamily, as *Salvia* spp.[54]. The abundance of essential oils[57] along with other antibacterial[58], antifungal[54] and antioxidant[54,58] secondary metabolites was suggested to offer an improved environmental protection[54].

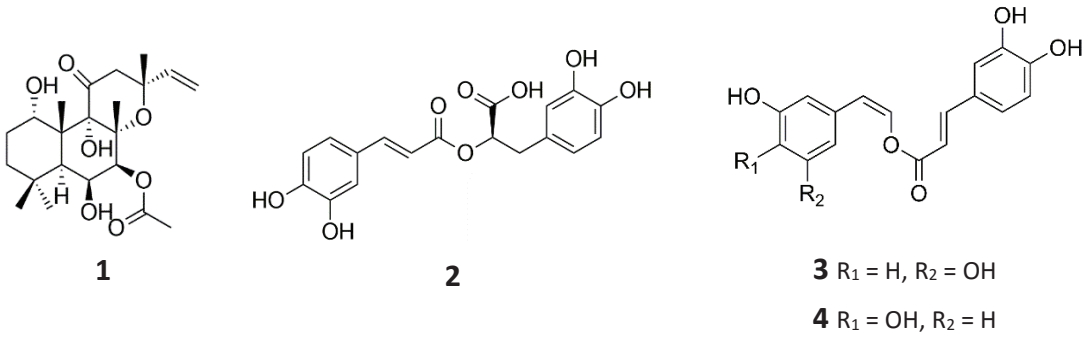

Figure 2. Structures of the bioactive diterpene forskolin (**1**) from *P. barbatus* and of the chemotaxonomic relevant caffeic acid esters rosmarinic acid (**2**) and the nepetoidins A (**3**) and B (**4**).

The phytochemical study of the *Plectranthus* genus have been focused in two main objectives: a chemotaxonomic approach as represented by the studies of the Eugster group in the 1970s and 1980s[56,59–63]; and a bioactivity guided approach as verified in the studies from the Simões group in the 2000s and 2010s[39,40,64–66].

Presently, the only review of the *Plectranthus* genus phytochemistry, compiled by Abdel-Mogib and its collaborators[55], have focused on the identified compounds up to 1999. The major constituents obtained in this genus were essential oils, phenolics and a high variety of diterpenes. The more frequently found diterpenic structures were abietenoids as royleanones, spirocoleons, vinylogous quinones, quinone methides, acylhydroquinones, 1,4-phenanthraquinones (Figure **3**) along with some miscellaneous phenolic abietanoids, dimeric abietanoids and secoabietanoids[55].

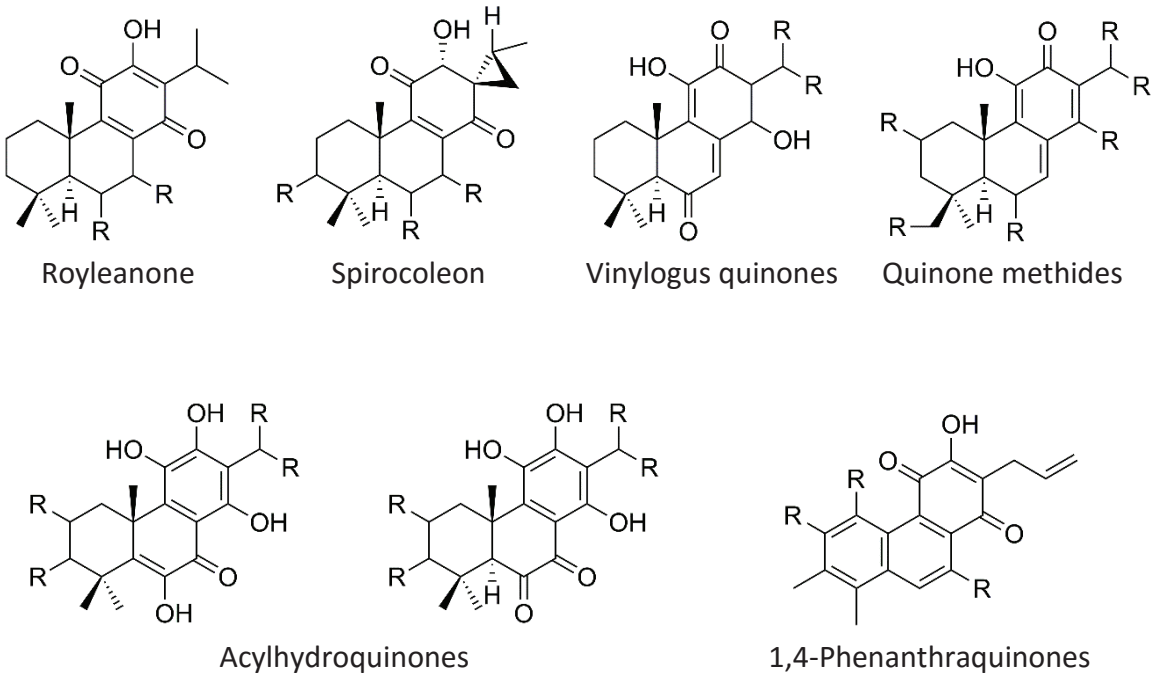

Figure 3. Representative structures and more frequent patterns of substitution of the major abietenoid classes obtained from the *Plectranthus* genus as described by Abdel-Mogib, Albar and Batterjee in 2002[55].

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Over the 2000-2015 period, phytochemical studies in the *Plectranthus* genus have led to the identification of 166 secondary metabolites. Those discoveries highlighted some chemotaxonomic trends, namely, the presence of the hydroxycinnamic acids, rosmarinic acid and the nepetoidins A and B (table **1**) as chemical markers of the *Plectranthus* genus plants[29,54].

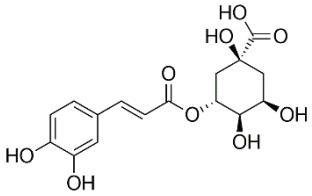
The presence of flavonoids was thought to be rare in the genus, however in the last 15 years, 17 flavonoids were found in the genus, especially in *P. amboinicus* (11 flavonoids from whole plant extracts) (Table **2**).

The abundance of diterpenic structures was confirmed in the genus, especially from abietane and labdane type, but also some kaurane, pimarane, halimane an beyranes were found (Tables **3** to **14**). Abdel-Mogib suggested that the absence of clerodane diterpenoids could be a distinguish character between *Plectranthus* and *Salvia* genus[67], however, four clerodanes were recently found for the first time in *Plectranthus* plants (Table **11**).

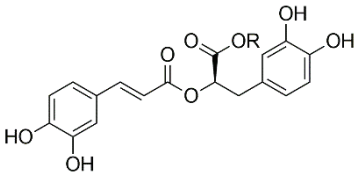
Triterpenic fractions in the *Plectranthus* genus remain poor studied, however, 9 triterpenes were found in recent years (Table **15**). Also, some acetophenones, sesquiterpenes and other miscellaneous compounds were found in the genus (Table **16**).

Hydroxycinnamic acids

H1

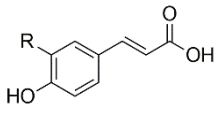


H4



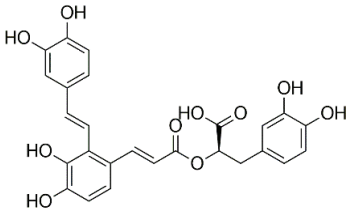
H5 R = H

H6 R = CH_3

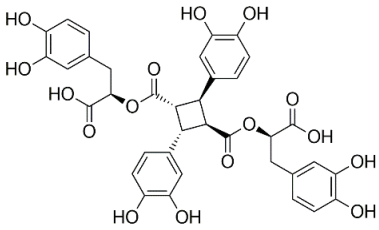


H2 R = H

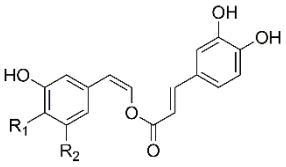
H3 R = OH



H7

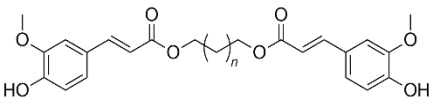


H8



H9 R_1 = H, R_2 = OH

H10 R_1 = OH, R_2 = H



H11 *n* = 26

H12 *n* = 28

Table 1. Hydroxycinnamic acids found in the *Plectranthus* plants over the period 2000-2015.

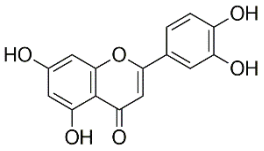
	Compound	*Plectranthus* species (section, solvent)	Ref
H1	Gallic acid	*P. amboinicus* (St, MeOH)	53,68
H2	Caffeic acid	*P. amboinicus* (WP, MeOH); *P. forsteri 'marginatus'* (Lf, MeOH)	53,68–70
H3	*p*-Coumaric acid	*P. amboinicus* (WP, MeOH, H_2O/EtAc)	53,68
H4	Chlorogenic acid	*P. amboinicus* (Lf, H_2O/EtAc)	70
H5	Rosmarinic acid	*P. amboinicus* (WP, MeOH, EtAc); *P. barbatus* (AP, H_2O); *P. forsteri 'marginatus'* (Lf, MeOH); *P. madagascariensis* (AP, MeOH)	36,53,68–72
H6	Methyl rosmarinate	*P. barbatus* (*in vitro* culture, MeOH)	73
H7	Salvianolic acid A	*P. amboinicus* (AP, H_2O)	53,71

Table 1 (cont.). Hydroxycinnamic acids found in the *Plectranthus* plants over the period 2000-2015.

H8	Shimobashiric acid	*P. amboinicus* (AP, H_2O)	53,71
H9	Nepetoidin A	*P. ambiguous; P. argentatus; P. argentifolius; P. asirensis; P. barbatus; P. buchananii; P. ciliates; P. coeruleus; P. comosus; P. crassus; P. aff. cyaneus; P. cylindraceus; P. ecklonii; P. elegans; P. ernstii; P. forsteri 'marginatus'; P. frederici; P. gracilis; P. grandis; P. hadiensis; P. hilliardiae; P. hyemalis; P. igniarius; P. kivuensis; P. lanuginosus; P. madagascariensis; P. mutabilis; P. neochilus; P. njassae; P. oertendahlii; P. ovatus; P. parviflorus; P. petiolaris; P. pseudomarrubioides; P. purpuratus; P. saccatus; P. sanguineus; P. aff. spicatus; P. strigosus; P. tenuiflorus; P. xerophilus; P. zuluensis* (not specified)	54,69
H10	Nepetoidin B	*P. ambiguous; P. argentatus; P. argentifolius; P. asirensis; P. barbatus; P. buchananii; P. ciliates; P. coeruleus; P. comosus; P. crassus; P. aff. cyaneus; P. cylindraceus; P. ecklonii; P. elegans; P. ernstii; P. forsteri 'marginatus'; P. frederici; P. gracilis; P. grandis; P. hadiensis; P. hilliardiae; P. hyemalis; P. igniarius; P. kivuensis; P. lanuginosus; P. madagascariensis; P. mutabilis; P. neochilus; P. njassae; P. oertendahlii; P. ovatus; P. parviflorus; P. petiolaris; P. pseudomarrubioides; P. purpuratus; P. saccatus; P. sanguineus; P. aff. spicatus; P. strigosus; P. tenuiflorus; P. xerophilus; P. zuluensis* (not specified)	54,69
H11	hexacosan-1,26-diol diester	*P. strigosus* (WP, Acet)	64
H12	octacosan-1,28-diol ferulate diester	*P. strigosus* (WP, Acet)	64

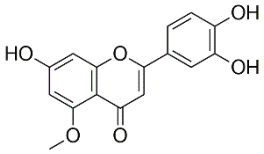
Lf – leaf; AP – aerial parts; St – steam; WP – whole plant. Acet – acetone; EtAc – ethyl acetate; MeOH – methanol H_2O – water.

Flavonoids



F1, R = H

F2, R = OH



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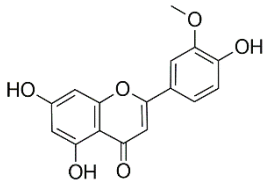

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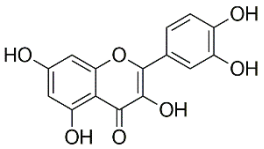

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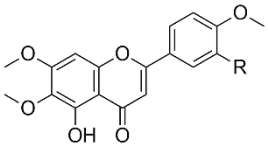
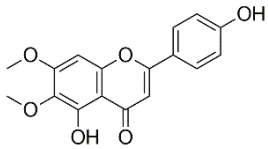


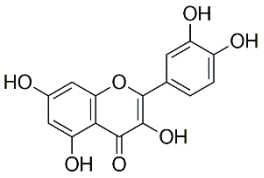
F6, R = H

F7, R = OH

F8, R = OCH_3



F9



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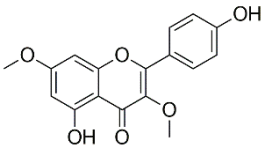

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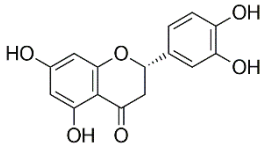

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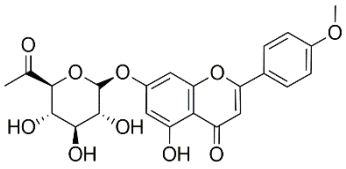

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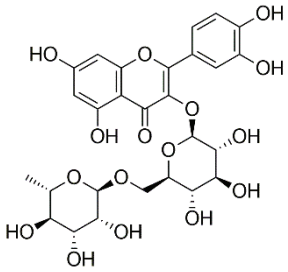

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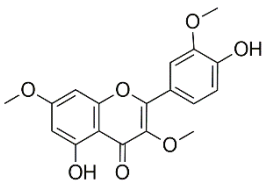
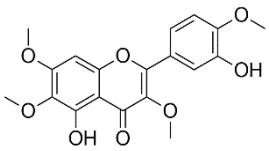
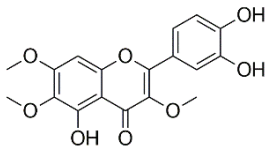


F15



F16



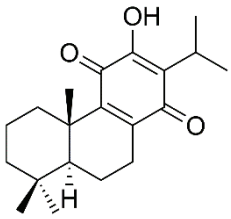
F17

Table 2. Flavonoids found in the *Plectranthus* plants over the period 2000-2015.

	Compound	*Plectranthus* species (section, solvent)	Ref
F1	Apigenin	*P. amboinicus* (WP, EtAc)	53
F2	Luteolin	*P. amboinicus* (WP, EtAc)	53
F3	5-O-Methyl-luteolin	*P. amboinicus* (WP, EtAc)	53
F4	Chrysoeriol	*P. amboinicus* (WP, TCM or H_2O/EtAc)	53
F5	Quercetin	*P. amboinicus* (WP, H_2O/EtAc)	53,68
F6	Salvigenin	*P. amboinicus* (Lf, TCM); *P. cyaneus* (Lf, EtOH); *P. strigosus* (WP, Acet)	53,64,74
F7	Eupatorin	*P. mollis* (Lf, Acet)	75
F8	3'-O-Methyleupatorin	*P. mollis* (Lf, Acet)	75
F9	Cirsimaritin	*P. amboinicus* (Lf, TCM)	53
F10	3,5,7,3',4'-Pentahydroxy flavanone	*P. amboinicus* (WP, EtAc)	53
F11	5,4'-Dihydroxy-3,7-dimethoxy flavone	*P. amboinicus* (WP, EtAc)	53
F12	Eriodictyol	*P. amboinicus* (WP, EtAc)	53
F13	Scutellarein 4'-O-methyl ether 7-O-glucuronide	*P. barbatus* (AP, H_2O)	36
F14	Rutin	*P. amboinicus* (St, MeOH)	53,68
F15	Pachypodol	*P. cylindraceus* (AP, EtOH)	76
F16	Casticin	*P. cylindraceus* (AP, EtOH)	76
F17	Chrysosplenol D	*P. cylindraceus* (AP, EtOH)	76

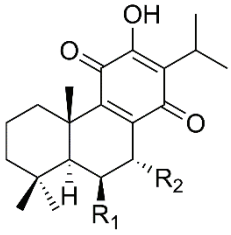
Lf – leaf; AP – aerial parts; St – steam; WP – whole plant. Acet – acetone; EtAc – ethyl acetate; EtOH – ethanol; MeOH – methanol; TCM – chloroform.

Diterpenoids: Abietane Royleanones



D1

D2 Δ6,7



D3 R_1 = H, R_2 = OH

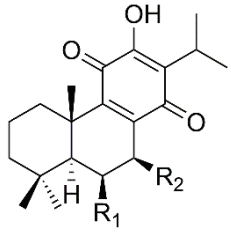
D4 R_1 = OH, R_2 = OH

D5 R_1 = OH, R_2 = OCH_3

D6 R_1 = OH, R_2 = OCHO

D7 R_1 = OH, R_2 = OAc

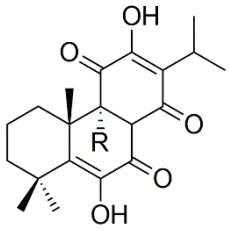
D8 R_1 = OH, R_2 = fatty acid carboxylate



D9 R_1 = H, R_2 = OH

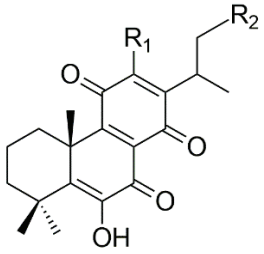
D10 R_1 = OH, R_2 = OH

D11 R_1 = OH, R_2 = OAc



D12 R = CH_2COCH_3

D13 8α, 9α-epoxide



D14 R_1 = OH, R_2 = H

D15 R_1 = OAc, R_2 = OAc

Table 3. Abietane Royleanones found in the *Plectranthus* plants over the period 2000-2015.

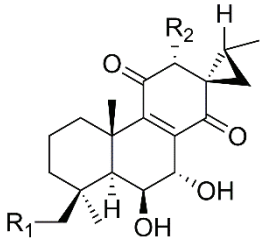
	Compound	*Plectranthus* species (section, solvent)	Ref
D1	Royleanone	*P. grandidentatus* (AP, Acet)	77,78
D2	6,7-dehydroroyleanone	*P. bishopianus* (Lf, MeOH); *P. grandidentatus* (AP, Acet)	77–79
D3	Horminone	*P. grandidentatus* (AP, Acet)	77,78
D4	6β,7α-dihydroxyroyleanone	*P. bishopianus* (Lf, MeOH)	79
D5	7α-methoxy-6β-hydroxyroyleanone	*P. bishopianus* (Lf, MeOH)	79

Table 3 (cont.). Abietane Royleanones found in the *Plectranthus* plants over the period 2000-2015.

D6	7α-formyloxy-6β-hydroxyroyleanone	*P. hadiensis* (Lf, DCM)	80
D7	7α-acetoxy-6β-hydroxyroyleanone	*P. hadiensis* (Lf, DCM); *P. grandidentatus* (AP, Acet)	77,78,80
D8	7-Fatty acid esters of 6,7-dihydroxyroyleanone	*P. grandidentatus* (AP, Acet)	77,78
D9	6β-hydroxyroyleanone	*P. grandidentatus* (AP, Acet)	77,78
D10	7β,6β-dihydroxyroyleanone	*P. forsteri 'marginatus'* (Lf, MeOH); *P. madagascariensis* (AP, MeOH)	69,72
D11	7β-acetoxy-6β-hydroxyroyleanone	*P. forsteri 'marginatus'* (Lf, MeOH); *P. madagascariensis* (AP, MeOH)	69,72
D12	9α-(2-oxopropyl)abietane derivative	*P. grandidentatus* (AP, Acet)	81
D13	8α,9α-epoxycoleon U-quinone	*P. xanthanthus* (AP, Acet 70%)	82
D14	Coleon U-quinone	*P. forsteri* (Lf, Acet); *P. madagascariensis* (AP, MeOH); *P. xanthanthus* (AP, Acet 70%)	72,82,83
D15	Xanthanthusin G	*P. xanthanthus* (AP, Acet 70%)	82

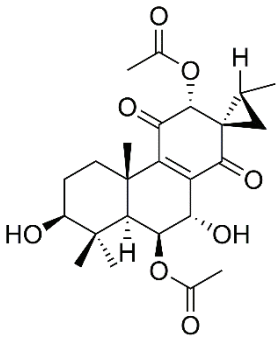
Lf – leaf; AP – aerial parts; Acet – acetone; DCM – dichloromethane; MeOH – methanol.

Diterpenes: Abietane spirocoleons

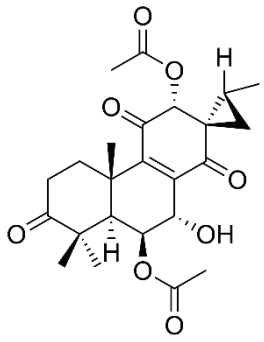


D16 $R_1 = CH_2OH$, $R_2 = OH$

D17 $R_1 = H$, $R_2 = OAc$



D18

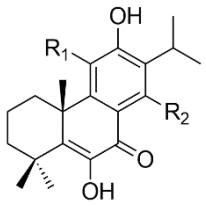


D19

Table 4. Spirocoleons found in the *Plectranthus* plants over the period 2000-2015.

	Compound	*Plectranthus* species (section, solvent)	Ref
D16	(13S,15S)-6β,7α,12α,19-tetrahydroxy-13β,16-cyclo-8-abietene-11,14-dione	*P. porcatus* (AP, Acet)	35
D17	Coleone P	*P. zeylanicus* (WP, Hex)	84
D18	3β-hydroxy-3-deoxibarbatusin	*P. barbatus* var. *grandis* (Lf, TCM)	85
D19	Barbatusin	*P. barbatus* var. *grandis* (Lf, Hex)	85

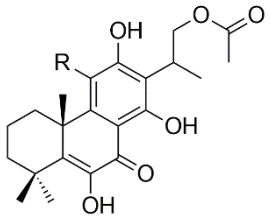
Diterpenes: Abietane Acylhydroquinones



D20 R_1 = OH, R_2 = OH

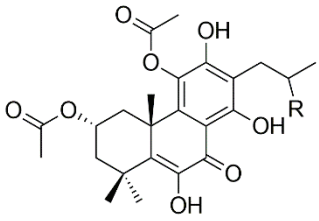
D21 R_1 = $OCOCH_3$, R_2 = OH

D22 R_1 = OH, R_2 = OAc



D23 R = OH

D24 R = OAc



D23 R = OH

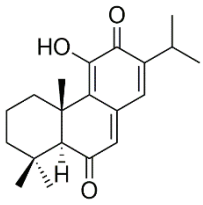
D24 R = $OCH(CH_3)_2$

Table 5. Abietane Acylhydroquinones found in the *Plectranthus* plants over the period 2000-2015.

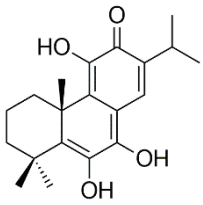
	Compound	*Plectranthus* species (section, solvent)	Ref
D20	Coleon U	*P. grandidentatus* (AP, Acet); *P. forsteri* (Lf, Acet); *P. xanthanthus* (AP, Acet 70%)	77,82,83
D21	Coleon U 11-acetate	*P. xanthanthus* (AP, Acet 70%)	82
D22	14-O-Acetylcoleon U	*P. grandidentatus* (AP, Acet)	86
D23	16-O-Acetylcoleon C	*P. xanthanthus* (AP, Acet 70%)	82
D24	16-Acetoxycoleon U 11-acetate	*P. xanthanthus* (AP, Acet 70%)	82
D25	Xanthanthusin F	*P. xanthanthus* (AP, Acet 70%)	82
D26	Xanthanthusin G	*P. xanthanthus* (AP, Acet 70%)	82

Lf – leaf; AP – aerial parts. Acet – acetone.

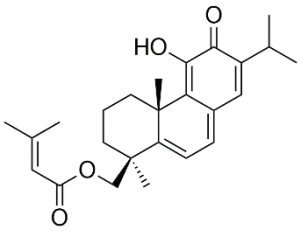
Diterpenes: Abietane Quinone Methides



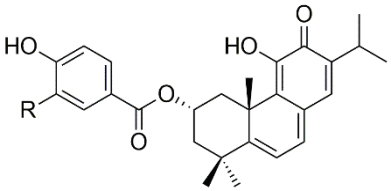
D27



D28

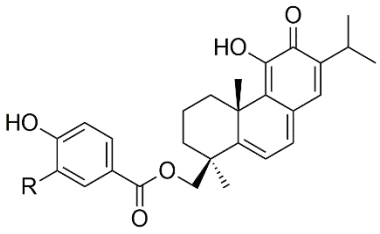


D29



D30 R = H

D31 R = OH



D32 R = H

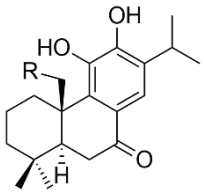
D33 R = OH

Table 6. Abietane Quinone Methides found in the Plectranthus plants over the period 2000-2015.

	Compound	*Plectranthus* species (section, solvent)	Ref
D27	Taxodione	*P. barbatus* (AP, EtOH 70%)	87
D28	5,6-didehydro-7-hydroxytaxodone	*P. barbatus* (AP, EtOH 70%)	87
D29	11-hydroxy-19-(methyl-buten-2-oyloxy)-abieta-5,7,9 (11),13-tetraene-12-one	*P. lucidus* (Lf, DCM); *P. purpuratus* (Lf, DCM)	80
D30	Parviflorone D	*P. ecklonii* (Lf, DCM), (WP, Acet); *P. lucidus* (Lf, DCM); *P. strigosus* (WP, Acet)	41,64,80
D31	Parviflorone F	*P. ecklonii* (Lf, DCM); *P. nummularius* (Lf, Acet); *P. strigosus* (WP, Acet)	64,80,88
D32	Parviflorone C	*P. purpuratus* subsp. *tongaensis* (Lf, DCM)	80
D33	Parviflorone E	*P. nummularius* (Lf, Acet); *P. purpuratus* subsp. *tongaensis* (Lf, DCM)	80,88

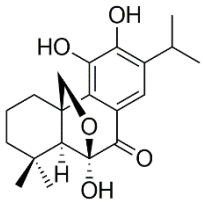
Lf – leaf; AP – aerial parts; WP – whole plant. Acet – acetone; DCM – dichloromethane; EtOH – ethanol.

Diterpenes: Phenolic abietenoids

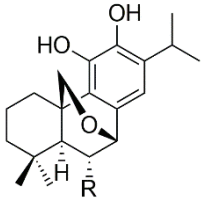


D34 R = H

D35 R = OH

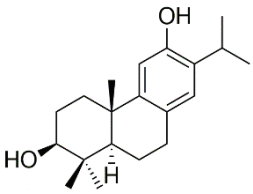


D36

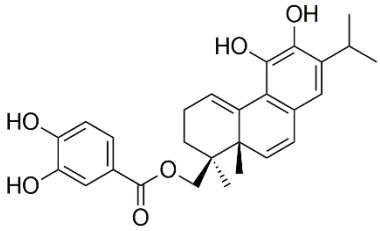


D37 R = H

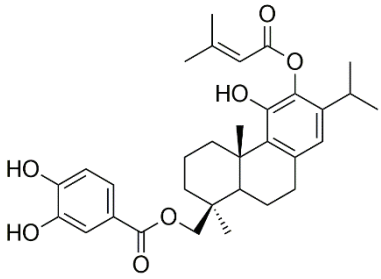
D38 R = OH



D39



D40



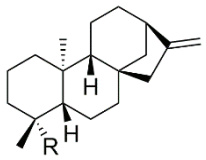
D41

Table 7. Phenolic abietenoids found in the *Plectranthus* plants over the period 2000-2015.

	Compound	*Plectranthus* species (section, solvent)	Ref
D34	11-Hydroxysugiol	*P. cyaneus* (Lf, EtOH)	74
D35	11,20-dihydroxysugiol	*P. cyaneus* (Lf, EtOH)	74
D36	Carnosolon	*P. cyaneus* (Lf, EtOH)	74
D37	20-deoxocarnosol	*P. barbatus* (AP, EtOH 70%)	87
D38	6α,11,12,-trihydroxy-7b,20-epoxy-8,11,13-abietatriene	*P. barbatus* (AP, EtOH 70%)	87
D39	Hinokiol	*P. strigosus* (WP, Acet)	64
D40	Plectranthol A	*P. nummularius* (Lf, Acet)	88
D41	Plectranthol B	*P. nummularius* (Lf, Acet)	88

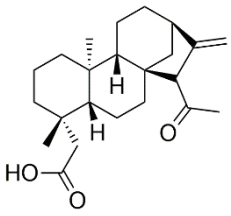
Lf – leaf; AP – aerial parts; WP – whole plant. Acet – acetone; EtOH – ethanol.

Diterpenes: Kaurenes

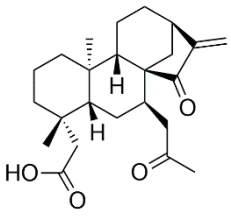


D42 R = CH_2OH

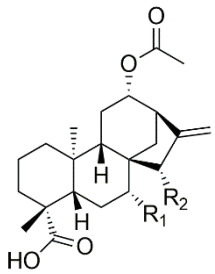
D43 R = COOH



D44

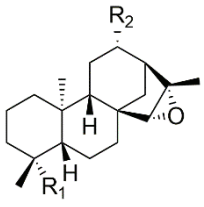


D45



D46 R_1 = H, R_2 = OH

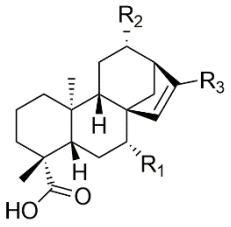
D47 R_1 = OH, R_2 = H



D48 R_1 = COOH, R_2 = OAc

D49 R_1 = COOH, R_2 = H

D50 R_1 = CH_2OH, R_2 = H



D51 R_1 = OH, R_2 = H, R_3 = CH_3

D52 R_1 = OH, R_2 = OAc, R_3 = CH_3

D53 R_1 = H, R_2 = OAc, R_3 = CHO

D54 R_1 = H, R_2 = H, R_3 = CHO

Table 8. Kaurenes found in the *Plectranthus* plants over the period 2000-2015.

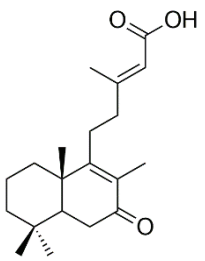
	Compound	*Plectranthus* species (section, solvent)	Ref
D42	*ent*-16-Kauren-19-ol	*P. strigosus* (WP, Acet)	64
D43	*ent*-16-Kauren-19-oic	*P. strigosus* (WP, Acet)	64
D44	Xylopic acid	*P. strigosus* (WP, Acet)	64
D45	Xylopinic acid	*P. strigosus* (WP, Acet)	64
D46	*ent*-12-Acetoxy-15-hydroxykaur-16-en-19-oic acid	*P. fruticosus* (AP, Acet)	89
D47	*ent*-12-Acetoxy-7-hydroxykaur-16-en-19-oic acid	*P. fruticosus* (AP, Acet)	89

Table 8 (cont.). Kaurenes found in the *Plectranthus* plants over the period 2000-2015.

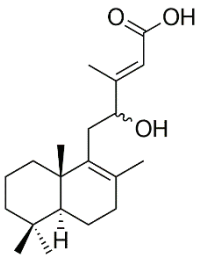
D48	*ent*-12-acetoxy-15,16-epoxykauran-19-oate	*P. fruticosus* (AP, Acet)	89
D49	*ent*-15β,16β-epoxykauran-19-oic acid	*P. fruticosus* (AP, Acet)	90
D50	*ent*-15β,16β-epoxykauran-19-ol	*P. fruticosus* (AP, Acet)	90
D51	*ent*-7-Hydroxykaur-15,16-en-19-oic acid	*P. coesta* (Lf, MeOH); *P. fruticosus* (AP, Acet)	89,91
D52	*ent*-12-Acetoxy-17-oxokaur-15-en-19-oic acid	*P. fruticosus* (AP, Acet)	89
D53	*ent*-17-oxokaur-15,16-en-19-oic acid	*P. coesta* (Lf, MeOH)	91
D54	*ent*-7-Hydroxy-15,16-epoxykauran-19-oic acid	*P. coesta* (Lf, MeOH); *P. fruticosus* (AP, Acet)	89,91

Lf – leaf; AP – aerial parts; WP – whole plant. Acet – acetone; MeOH – methanol.

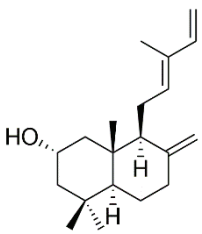
Diterpenes: Labdanes



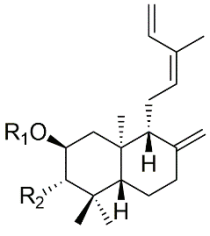
D55



D56



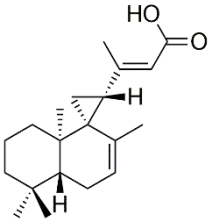
D57



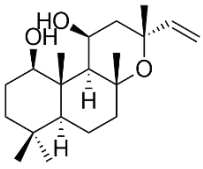
D58 $R_1 = R_2 = H$

D59 $R_1 = COCH_3$, $R_2 = OH$

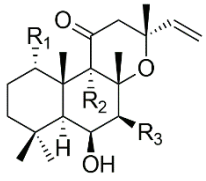
D60 $R_1 = H$, $R_2 = OAc$



D61



D62



D63 $R_1 = R_2 = R_3 = H$

D64 $R_1 = R_2 = H$, $R_3 = OH$

D65 $R_1 = R_2 = H$, $R_3 = OAc$

D66 $R_1 = H$, $R_2 = R_3 = OH$

D67 $R_1 = H$, $R_2 = OH$, $R_3 = OAc$

D68 $R_1 = R_2 = R_3 = OH$

D69 $R_1 = OH$, $R_2 = H$, $R_3 = OAc$

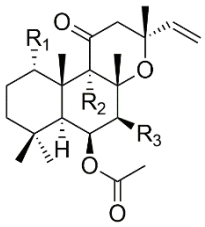
D70 $R_1 = R_2 = OH$, $R_3 = OAc$

D71 $R_1 = OAc$, $R_2 = OH$, $R_3 = OH$

D72 $R_1 = OAc$, $R_2 = OH$, $R_3 = H$

D73 $R_1 = OAc$, $R_2 = OH$, $R_3 = OAc$

D74 $R_1 = OAc$, $R_2 = H$, $R_3 = OAc$



D75 $R_1 = H$, $R_2 = OH$, $R_3 = OH$

D76 $R_1 = H$, $R_2 = OH$, $R_3 = OAc$

D77 $R_1 = R_2 = H$, $R_3 = OH$

D78 $R_1 = R_2 = H$, $R_3 = OAc$

D79 $R_1 = OH$, $R_2 = R_3 = H$

D80 $R_1 = R_2 = R_3 = OH$

D81 $R_1 = OH$, $R_2 = H$, $R_3 = OH$

D82 $R_1 = OH$, $R_2 = H$, $R_3 = OAc$

D83 $R_1 = R_2 = OH$, $R_3 = OAc$

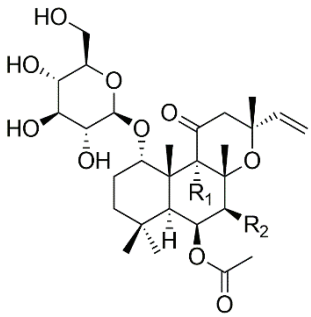
D84 $R_1 = R_2 = OH$, $R_3 = \alpha OAc$

D85 $R_1 = OAc$, $R_2 = R_3 = H$

D86 $R_1 = OAc$, $R_2 = R_3 = OH$

D87 $R_1 = OAc$, $R_2 = OH$, $R_3 = OAc$

D88 $R_1 = OAc$, $R_2 = H$, $R_3 = OAc$



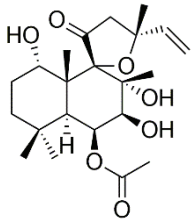
D89 $R_1 = R_2 = H$

D90 $R_1 = H$, $R_2 = OH$

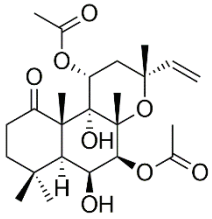
D91 $R_1 = H$, $R_2 = OAc$

D92 $R_1 = R_2 = OH$

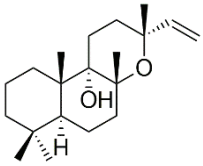
D93 $R_1 = OH$, $R_2 = OAc$



D94



D95



D96

Table 9. Labdanes found in the *Plectranthus* plants over the period 2000-2015.

	Compound	*Plectranthus* species (section, solvent)	Ref
D55	Rhinocerotinoic acid	*P. ornatus* (AP, Acet)	86
D56	12-hydroxy-8,13E-labdadien-15-oic acid	*P. barbatus* (WP, EtOH)	92

Table 9 (cont.). Labdanes found in the *Plectranthus* plants over the period 2000-2015.

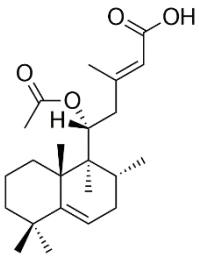
D57	3β-acetoxylabda-8(17),12E,14-trien-2r-ol	*P. fruticosus* (AP, Acet)	90
D58	*ent*-Labda-8(17),12Z,14-trien-2r-ol	*P. fruticosus* (AP, Acet)	90
D59	*ent*-2r-acetoxylabda-8(17),12Z,14-trien-3β-ol	*P. fruticosus* (AP, Acet)	90
D60	*ent*-3-Acetoxylabda-8(17),12Z,14-trien-2r-ol (2)	*P. fruticosus* (AP, Acet)	89
D61	Forskoditerpene A	*P. barbatus* (WP, EtOH)	93
D62	1R,11S-Dihydroxy-8R,13R-epoxylabd-14-ene	*P. ernstii* (WP, Hex)	94
D63	6b-hydroxy-8,13-epoxy-labd-14-ene-11-one	*P. barbatus* (*in vitro* culture, MeOH)	73
D64	1,9-dideoxy-deacetylforskolin (deacetylforskolin)	*P. barbatus* (*in vitro* culture, MeOH)	73
D65	1,9-dideoxyforskolin	*P. barbatus* (*in vitro* culture, MeOH)	73
D66	1-deoxy-deacetylforskolin	*P. barbatus* (*in vitro* culture, MeOH)	73
D67	1-deoxyforskolin	*P. barbatus* (*in vitro* culture, MeOH)	73
D68	Forskolin D	*P. barbatus* (WP, EtAc)	95
D69	Deoxycoleonol (9-deoxy-deacetylforskolin)	*P. barbatus* (WP, EtAc), (*in vitro* culture, MeOH)	73,95
D70	Forskolin	*P. barbatus* (WP, EtAc), (*in vitro* culture, MeOH)	73,95
D71	1-Acetyl-7-deacetylforskolin	*P. barbatus* (WP, EtAc)	95
D72	1-Acetoxycoleosol	*P. barbatus* (WP, EtAc)	95
D73	Forskolin B	*P. barbatus* (WP, EtAc)	95
D74	Forskolin E	*P. barbatus* (WP, EtAc)	95
D75	1-Deoxycoleonol B	*P. barbatus* (*in vitro* culture, MeOH)	73
D76	6-Acetyl-1-deoxyforskolin	*P. barbatus* (WP, EtOH/EtAc)	92,95

Table 9 (cont.). Labdanes found in the *Plectranthus* plants over the period 2000-2015.

D77	1,9-Dideoxycoleonol B	*P. barbatus* (*in vitro* culture, MeOH)	73
D78	6-acetyl-1,9-dideoxyforskolin	*P. barbatus* (WP, EtOH)	92
D79	Plectrornatin B	*P. ornatus* (AP, Acet)	96
D80	Isoforskolin (Coleonol B)	*P. barbatus* (WP, EtOH) (*in vitro* culture, MeOH)	73,95,97
D81	9-deoxyc-oleonol B	*P. barbatus* (*in vitro* culture, MeOH)	73
D82	Forskolin G	*P. barbatus* (WP, EtOH/EtAc)	95,97
D83	6-Acetylforskolin	*P. barbatus* (Rt, EtOH), (WP, EtAc); *P. ornatus* (AP, Acet)	95,98,99
D84	Forskolin I	*P. barbatus* (Rt/WP, EtOH)	97,98
D84	Plectrornatin C (Coleolin)	*P. barbatus* (*in vitro* culture, MeOH); *P. ornatus* (AP, Acet)	73,86,96
D85	1,6-Diacetyl-7-deacetylforskolin	*P. barbatus* (WP, EtAc)	95
D87	Forskolin A (1,6-di-O-acetylforskolin)	*P. barbatus* (WP, EtAc), *P. ornatus* (AP, Acet), (AP, Hex)	95,99,100
D88	1,6-di-O-acetyl-9-deoxyforskolin	*P. neochilus* (AP, toluene), *P. ornatus* (AP, Acet), (AP, Hex)	99–101
D89	Forskoditerpenoside E	*P. barbatus* (WP, EtOH)	93
D90	Forskoditerpenoside C	*P. barbatus* (WP, EtOH)	93
D91	Forskoditerpenoside D	*P. barbatus* (WP, EtOH)	93
D92	Forskoditerpenoside A	*P. barbatus* (WP, EtOH)	102
D93	Forskoditerpenoside B	*P. barbatus* (WP, EtOH)	102
D94	Spirocoleonol B	*P. barbatus* (*in vitro* culture, MeOH)	73
D95	Colroforskolin	*P. barbatus* (*in vitro* culture, MeOH)	73
D96	Coleorol	*P. barbatus* (*in vitro* culture, MeOH)	73

Lf – leaf; Rt – root; AP – aerial parts; WP – whole plant. Acet – acetone; DCM – dichloromethane; EtAc – ethyl acetate; EtOH – ethanol; Hex – *n*-hexane; MeOH – methanol.

Diterpenes: Halimane



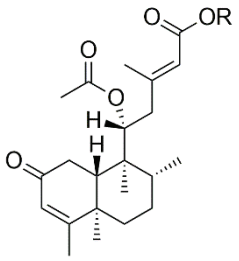
D97

Table 10. Halimanes found in the *Plectranthus* plants over the period 2000-2015.

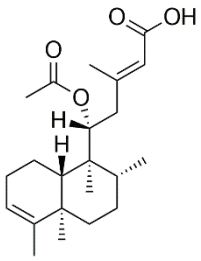
	Compound	*Plectranthus* species (section, solvent)	Ref
D97	11-acetoxyhalima-5,13E-dien-15-oic acid	*P. ornatus* (AP, Acet)	86

AP – aerial parts; Acet – acetone.

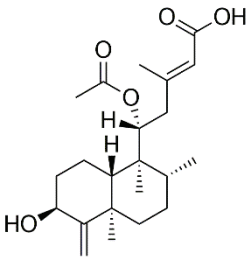
Diterpenes: Clerodanes



D98 R = H **D99** R = CH_3



D100



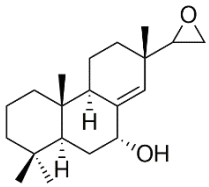
D101

Table 11. Clerodanes found in the *Plectranthus* plants over the period 2000-2015.

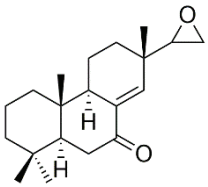
	Compound	*Plectranthus* species (section, solvent)	Ref
D98	11-acetoxy-2-oxo-neocleroda-3,13E-dien-15-oic acid	*P. ornatus* (AP, Hex)	100
D99	Plectrornatin A	*P. ornatus* (AP, Acet)	96
D100	11-acetoxyneocleroda-3,13E-dien-15-oic acid	*P. ornatus* (AP, Hex)	100
D101	11-acetoxy-3β-hydroxyneocleroda-4(18),13E-dien-15-oic acid	*P. ornatus* (AP, Hex)	100

Lf – leaf; AP – aerial parts; Acet – acetone; Hex – *n*-hexane.

Diterpenes: Pimaranes



D102



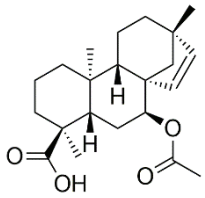
D103

Table 12. Pimaranes found in the *Plectranthus* plants over the period 2000-2015.

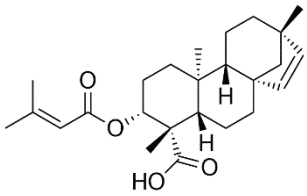
	Compound	*Plectranthus* species (section, solvent)	Ref
D102	rel-15(ζ),16-Epoxy-7α-hydroxypimar-8,14-ene	*P. ernstii* (WP, Hex)	94
D103	rel-15(ζ),16-Epoxy-7-oxopimar-8,14-ene	*P. ernstii* (WP, Hex)	94

WP – whole plant. Hex – *n*-hexane.

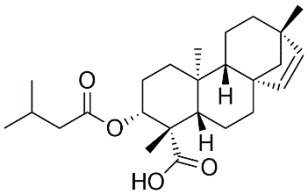
Diterpenes: Beyranes



D104



D105



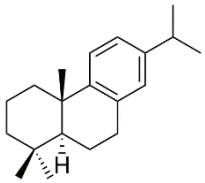
D106

Table 13. Beyranes found in the *Plectranthus* plants over the period 2000-2015.

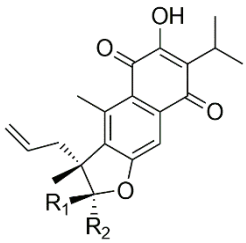
	Compound	*Plectranthus* species (section, solvent)	Ref
D104	*ent*-7α-acetoxy-15-beyeren-18-oic acid	*P. saccatus* (AP, Acet)	35
D105	*ent*-3β-(3-methyl-2-butenoyl)oxy-15-beyeren-19-oic acid	*P. saccatus* (Lf, Acet)	83
D106	*ent*-3β-(3-methylbutanoyl) oxy-15-beyeren-19-oic acid	*P. saccatus* (Lf, Acet)	83

Lf – leaf; AP – aerial parts; Acet – acetone.

Miscellaneous diterpenoids



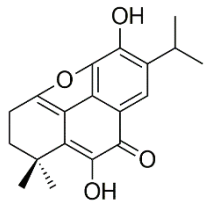
D106



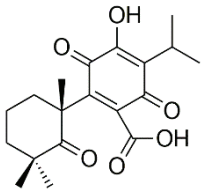
D107 R_1 = OH, R_2 = H

D108 R_1 = H, R_2 = OH

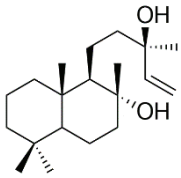
D109 R_1 = R_2 = O



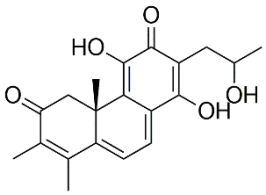
D110



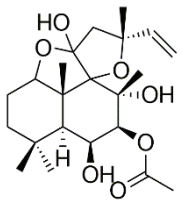
D111



D112



D113



D114

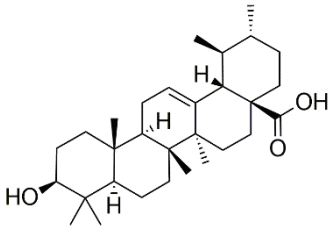
Table 14. Miscellaneous diterpenoids found in the *Plectranthus* plants over the period 2000-2015.

	Compound	*Plectranthus* species (section, solvent)	Ref
D107	Dehydroabietane	*P. barbatus* (AP, EtOH 70%)	87
D108	(4R,19R) Coleon A	*P. aff. puberulentus* (Lf, Acet)	83
D109	(4R,19S) Coleon A	*P. aff. puberulentus* (Lf, Acet)	83
D110	Coleon A-lactone	*P. barbatus* (Lf, TCM), *P. puberulentus* (Lf, Acet)	83,103

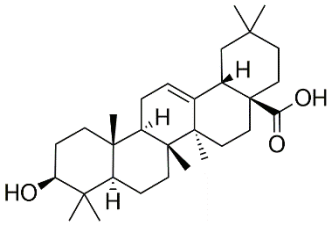
Table 14 (cont.). Miscellaneous diterpenoids found in the *Plectranthus* plants over the period 2000-2015.			
D111	1,11-epoxy-20-norabietanoid	*P. cyaneus* (Lf, EtOH)	74
D112	xanthanthusin E	*P. xanthanthus* (AP, Acet 70%)	82
D113	13-epi-sclareol	*P. barbatus* (Rt, EtOH)	104
D114	(16S)-coleon E	*P. barbatus* (AP, H_2O)	36
D115	Coelusin factor	*P. barbatus* (Rt, Acet)	105,106

Lf – leaf; Rt – root; AP – aerial parts; Acet – acetone; EtOH – ethanol; H_2O – water; TCM – Chloroform.

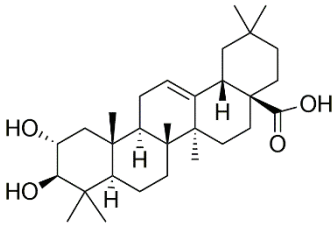
Triterpenoids



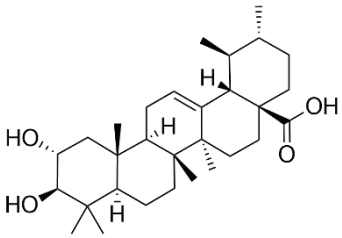
T1



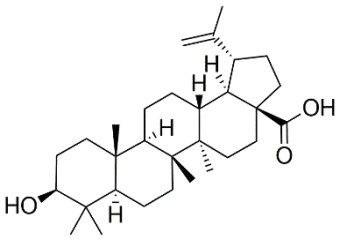
T2



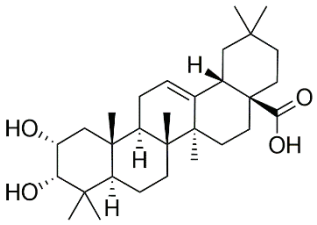
T3



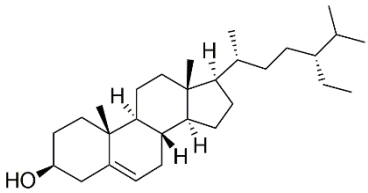
T4



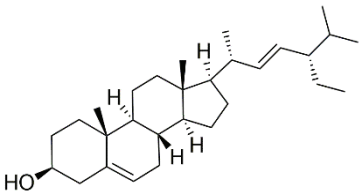
T5



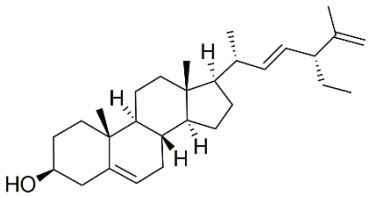
T6



T7



T8



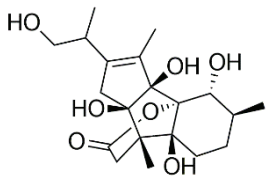
T9

Table 15. Triterpenoids found in the *Plectranthus* plants over the period 2000-2015.

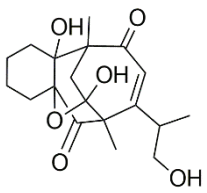
	Compound	*Plectranthus* species (section, solvent)	Ref
T1	Ursolic acid	*P. mollis* (Lf, Acet); *P. ornatus* (AP, Acet); *P. rotundifolius* (tubers peel and flesh, EtOH); *P. strigosus* (WP, Acet)	64,75,99,107
T2	Oleanolic acid	*P. bishopianus* (Lf, MeOH); *P. ornatus* (AP, Acet); *P. rotundifolius* (tubers peel and flesh, EtOH)	79,99,107
T3	Maslinic acid	*P. rotundifolius* (tubers peel and flesh, EtOH)	108
T4	Corosolic acid	*P. mollis* (Lf, Acet)	75
T5	Betulinic acid	*P. barbatus* (*in vitro* culture, MeOH)	73
T6	3-*epi*-Maslinic acid	*P. barbatus* (*in vitro* culture, MeOH)	73
T7	β-Sitosterol	*P. bishopianus* (Lf, MeOH)	79
T8	Stigmasterol	*P. bishopianus* (Lf, MeOH); *P. mollis* (Lf, Acet); *P. zeylanicus* (WP, Hex)	75,79,84
T9	Stigmaste-5,22,25-trien-3-β-ol	*P. zeylanicus* (WP, Hex)	84

Lf – leaf; Rt – root; AP – aerial parts; WP – whole plant. Acet – acetone; DCM – dichloromethane; EtOH – ethanol; Hex – *n*-hexane; MeOH – methanol.

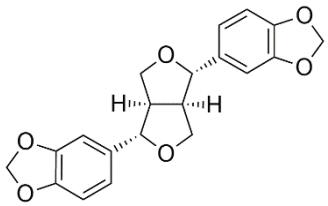
Miscellaneous compounds



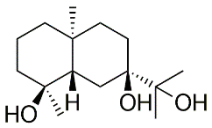
M1



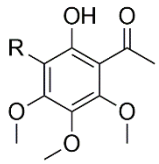
M2



M3

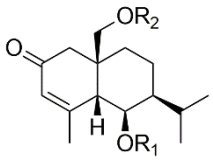


M4



M5 R = OCH_3

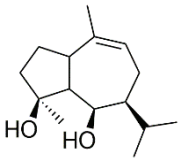
M6 R = H



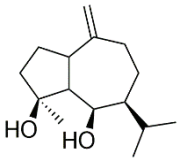
M7 R_1 = $COCH_3$, R_2 = $COCH_3$

M8 R_1 = $COCH_3$, R_2 = H

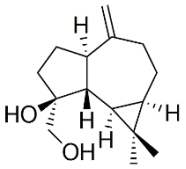
M9 R_1 = H, R_2 = $COCH_3$



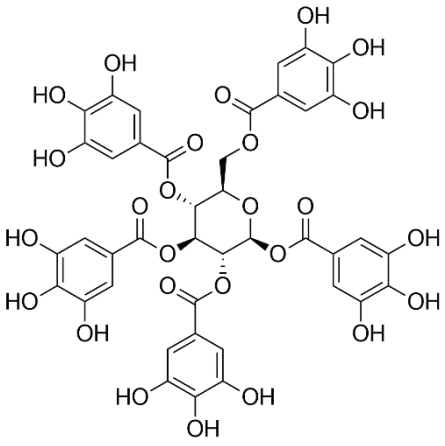
M10



M11



M12



M13

Table 16. Miscellaneous diterpenoids found in the *Plectranthus* plants over the period 2000-2015.

	Compound	*Plectranthus* species (section, solvent)	Ref
M1	Cinncassiol A	*P. zeylanicus* (WP, Hex)	84
M2	Cinncassiol C	*P. zeylanicus* (WP, Hex)	84
M3	(+)-Sesamin	*P. mollis* (Lf, Acet)	75
M4	4α,7α,11-enantioeudesmantriol	*P. barbatus* (WP, EtOH)	102
M5	2-hydroxy-3,4,5,6-tetramethoxy-acetophenone	*P. venteri* (Lf, DCM)	109
M6	2-hydroxy-4,5,6-trimethoxy-acetophe-none	*P. venteri* (Lf, DCM)	109
M7	Plectranthone	*P. cylindraceus* (AP, EtOH)	76
M8	Desacetylplectranthone	*P. cylindraceus* (AP, EtOH)	76
M9	Isodesacetylplectranthone	*P. cylindraceus* (AP, EtOH)	76
M10	4,6-dihydroxy-1,5(H)-guai-9-ene	*P. strigosus* (WP, Acet)	64
M11	4,6-dihydroxy-1,5(H)-guai-10(14)-ene	*P. strigosus* (WP, Acet)	64
M12	10(14)-Aromadendrene-4β,15-diol	*P. fruticosus* (AP, Acet)	89
M13	1,2,3,4,6-penta-O-galloyl-b-D-glucose	*P. barbatus* (Lf, MeOH)	110

Lf – leaf; Rt – root; AP – aerial parts; WP – whole plant; Acet – acetone; DCM – dichloromethane; EtOH – ethanol; Hex – *n*-hexane.

From the abundant diterpenic structures, royleanones are some of the most widespread, not only in the *Plectranthus* genus, but in all Lamiaceae family and associated to diverse pharmacologic activities including antimicrobial[33,34], antimycobacterial[65] and antitumoral[39,40] activities. Chemically those diterpenes are hydroquinonic abietanes with a 12-hydroxy-11,14-di-oxo-quinone moiety in ring C. The presence of a conjugated quinone system in such compounds exists in redox equilibrium between a diphenol (hydroquinone) and diketone (quinone) forms (Figure **4**). Those systems possess several physiologic examples as the case of coenzyme Q in electron transport systems, vitamin K in blood antihemorrhagic system along with several "quinoenzymes" whose action is dependent on hydroxylated amino acids[111]. Such compounds are also a source of stable free radicals and were able to bind irreversible to

some amino acids and proteins, acting as Michael acceptors, often leading damage and/or protein loss of function. Those properties could be in the source of antimicrobial or cytotoxic activities of such natural compounds[112,113].

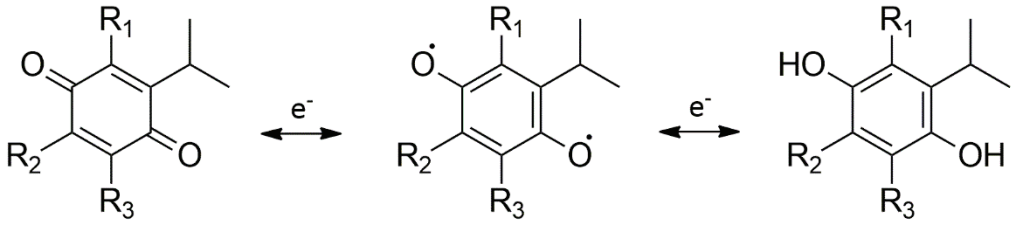


Figure 4. Simplified representation of the reduction and oxidation reactions between diketone and diphenol forms (illustration adapted from de Melo, 2016 [114]).

1.3. Microbial resistance and antimicrobials from *Plectranthus* genus

Since the discovery of penicillin by Alexander Fleming in 1928 there was a revolution in antibiotherapy with the development of the main classes of clinical used antibiotics in the following 30 years[115]. Although from the 1970s there has been a dearth of new compounds. After the initial success of the golden age of antibiotics, drug resistance began to emerge, and some molecules initially active against many bacterial strains were no longer effective[116]. Bacterial resistance is often driven by decreased susceptibility to antibacterial agents originated by halted division, genetic alteration, and over-expression of efflux pumps[117] (Figure **5**). Such targets could be modulated by natural products with appropriate molecule shape, aromatic ring count and the presence of some polar atoms[118].

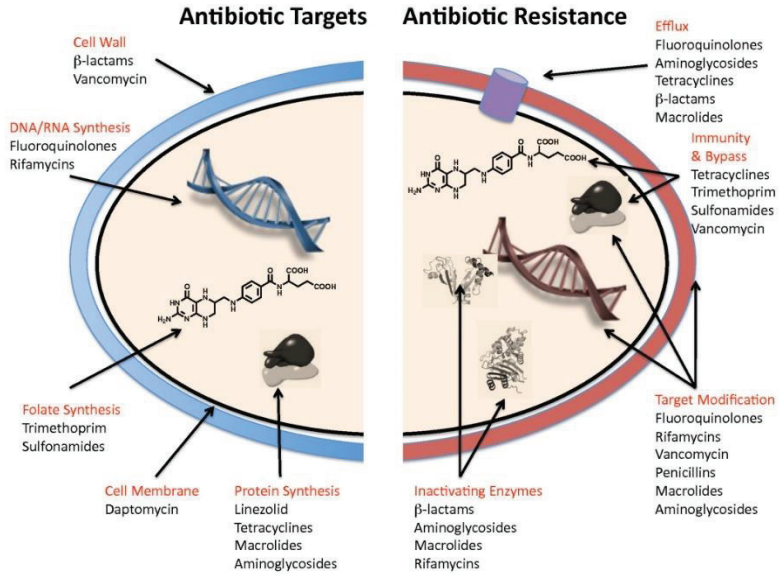


Figure 5. Antibiotic targets and identified mechanisms of antibiotic resistance[117].

The World Health Organization (WHO) considered the spreading of multidrug resistant (MDR) organisms a public health problem and emphasised the surge for new antibacterial agents, with new modes of action[119]. While combinatory chemistry approaches have not been particularly effective in the development of new antimicrobial agents[120], the neglected natural products have been once again in focus of antimicrobial screenings. Natural antimicrobial products generally possess complex architectural scaffolds and densely deployed functional groups, affording the maximal number of interactions with molecular targets, often leading to exquisite selectivity for pathogens versus the host[112,121].

1.3.1. Antimicrobial compounds from *Plectranthus* spp.

From the *Plectranthus* genus, relevant antimicrobial metabolites have been obtained (Figure **6**). Rijo et al. reviewed in 2013 the antimicrobial properties of many diterpenic compounds obtained from *Plectranthus* plants[33]. Among the antimicrobial diterpenes widespread in *Plectranthus* genus, interesting antibacterial acitivites have been found in pimarane (15,16-epoxy-7α-hydroxypimar-8,14-ene and 15,16-epoxy-7-oxopimar-8,14-ene[94]) (**5**, **6**), neoclerodane (plectrornatin A[96]) (**7**), labdane (1,11-dihydroxy-8,13-

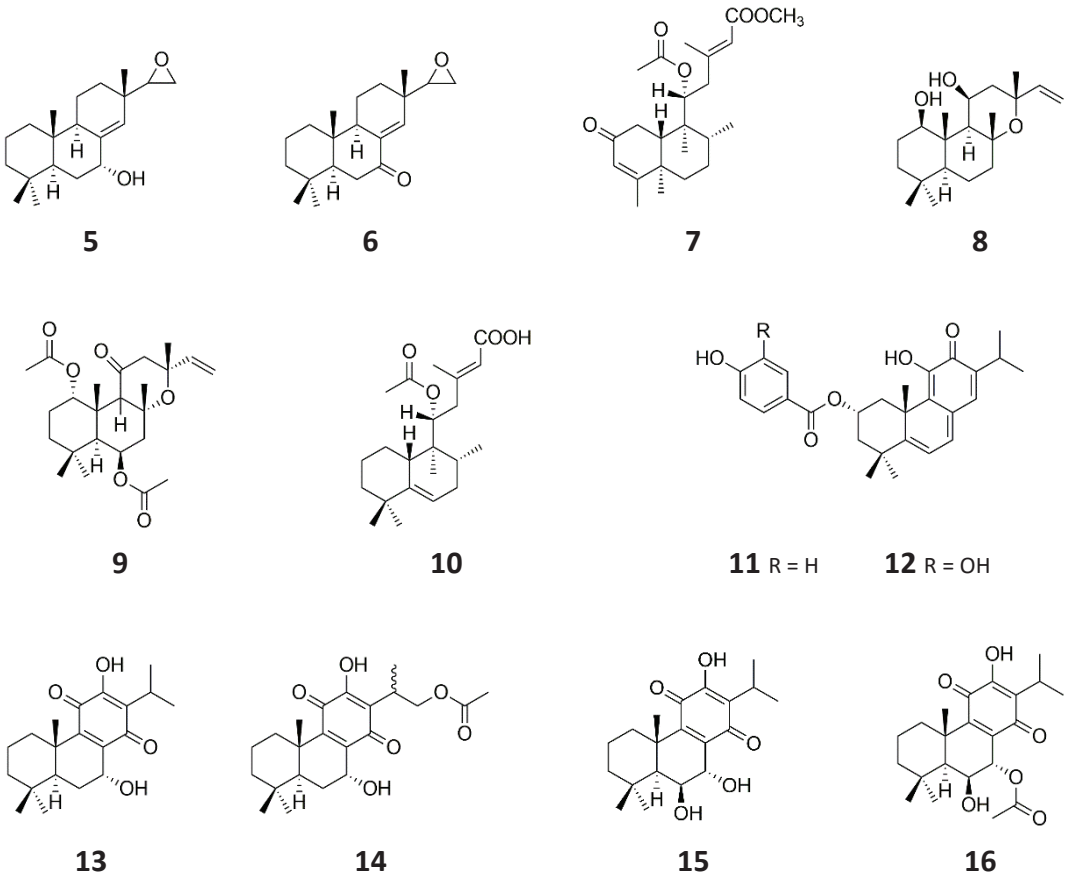


Figure 6. Antimicrobial compounds obtained from *Plectranthus* species.

epoxylabd-14-ene[94] and plectrornatin C[96]) (**8**, **9**) and halimane (11-acetoxyhalima-5,13-dien-15-oic acid[66,86]) (**10**) diterpenes. Abietane diterpenes as Parvifloron D (**11**) and F (**12**) were obtained from the ethyl acetate extract of *P. ecklonii* and were both active against *Listeria monocytogenes*[122], but only **11** showed relevant antimicrobial activity against *Staphylococcus*, *Enterococcus*[123] and *Mycobacterium* strains[122]. Royleanone type abietane diterpenes are antimicrobial secondary metabolites, frequently obtained from Lamiaceae plants, and widespread in the *Plectranthus* genus. Horminone[34] (**13**) along with its 16-*O*-acetoxy derivative[34] (**14**) and their related 7α,6β-dihydroxyroyleanone (**15**) and 7α-acetoxy-6β-hydroxyroyleanone (**16**) were some of the most frequently found royleanone-type antimicrobial diterpenes.

The 7α-acetoxy-6β-hydroxyroyleanone (**16**) have been obtained in fair amounts from *P. grandidentatus*[78], *P. hereroensis*[34] and *P. sanguineus*[124]. Considering its broad range of activities (Gram positive, Gram negative and Mycobacterium strains) and low MIC values (3.12-15.6 µg/mL), this compound was selected as antimicrobial lead compound. From the royleanone **16**, a library of 12-O-ester (**17-19**), 6β,12-O-diester (**26-23**) and 6β-O-ester (**24-27**) derivatives was synthetized, delivering new insights on the structure activity relationships (SAR) of the antimicrobial diterpenoids[65,125] (Figure **7**). The lipophilic framework composed by the three 6-membered rings (A, B and C) was

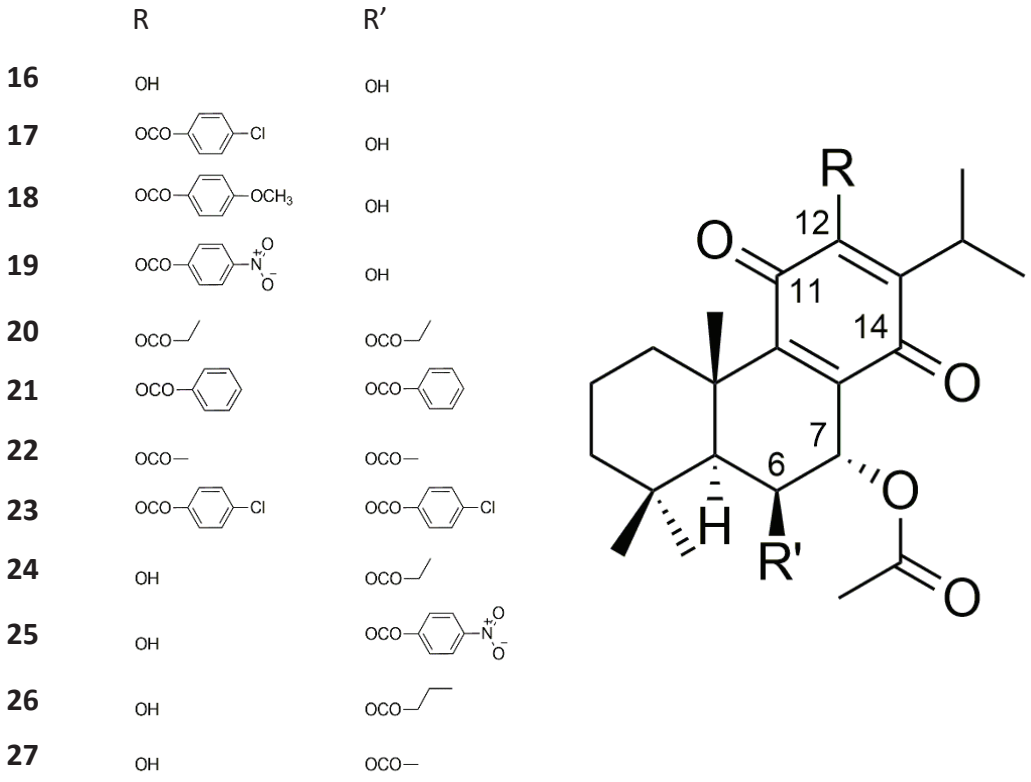


Figure 7. 12-O-ester (**17**-**19**), 6β,12-O-diester (**20**-**23**) and 6β-O-ester (**24**-**27**) derivatives of the prototype antimicrobial 7α-acetoxy-6β-hydroxyroyleanone (**16**).

required for the insertion into the bacterial cell membrane while the oxygenated substituents (hydroxyl, carbonyl and esters) act as hydrogen-bond-donor/acceptor group and interact with other hydrogen-bond-acceptor/donor groups in the prokaryotic membrane as peptidoglycans and lipoteichoic acid[33,126]. The esterification of the compound **16** in the position 6 and 12 increased the lipophilicity which in most derivatives originated an increase of its antimicrobial activity.

Enthusiastic results of this derivatization were verified for the 12-chlorobenzoyl (**18**), 12-methoxybenzoyl (**19**) and 12-nitrobenzoyl (**20**) esters along with the 6,12-dibenzoyl ester (**21**) with activity improvement against a multidrug resistant (MDR) *Mycobacterium tuberculosis* (TB) strain. The obtained anti-MDR-TB activity was even superior to the first line antituberculostatic agents isoniazid and rifampicin[65]. The royleanone derivative **18** presented potent activity with acceptable cytotoxicity in a Vero cell model and could be considered an improved antimycobacterial prototype in comparison to the royleanone **16**[65].

Royleanone derivatives **17**, **18** and **27** showed improved antibacterial activity in Gram positive human pathogens over the prototype royleanone **16**[125]. A trend for a diminished antimicrobial activity for the 6β,12-O-diester derivatives was also verified. Those correspond to the more lipophilic derivatives which could display an unfavourable shape and/or steric hindrance, and an incorrect spatial distribution of the hydrophobic moieties wall and the loss of some important hydrogen donor/receptor interactions that were maintained in the monoester derivatives. Those observations were in concordance with other works in which the excess increase in lipophilicity led to a decrease in the antimicrobial activity[112,127,128].

The antimicrobial mechanism of action of diterpenoid compounds remain unclear. The only diterpenic compound in which a tentative of antibacterial action mechanism establishment was performed is horminone (**13**). This compound possesses a negative site, between the C_7 hydroxyl and the C_{14} carbonyl, which was showed to be favourable for the binding of a positive ion like Mg^{2+} (and eventually Ca^{2+}). The horminone-Mg^{2+} complex was suggested to play an important role in the antimicrobial activity, being able to cross its membrane and at the cytosol level interacting with ribosomal ribonucleic acid and thus inhibiting the protein synthesis in bacteria[129,130]. This mechanism was proposed to be also responsible for the cytotoxicity verified for compound **13** in some mammalian cell lines[129,130]. Also the presence of carbonyl and hydroxyl groups at the 7 position of B ring and at the 11, 12 and 14 positions of the ring C was discussed to play a significant role in the biologic activities of the abietane diterpenoids[129,130]. The proposed mechanism of cell toxicity for horminone could not be extrapolated to many abietane diterpenes as some potent antimicrobials (i.e. **16**-**27**) present a decreased basic character of the oxygen atom of the acetyl group at position 7 when compared with the Lewis basic character of the hydroxyl at position 7 of compound **13**. Such chemical features were not the optimal for the coordination of the Mg^{2+} cation and such mechanism should only take place if hydrolysis of the acetyl group occurs[37,125]. Although some indications of membrane interaction and disruption effects were in study by our

group[131]. Interestingly many diterpenes showed an effective antimicrobial effect against drug resistant pathogens[34]. Also, some diterpenes as abietic acid, isopimarinic acid and totarol were known to inhibit the action of efflux pumps and thus reverting the resistant of some drug resistant strains[132,133]. Considering those effects played by some diterpenes, it is possible that other abietane diterpenes, as the case of royleanones, possess such action mechanism, but further studies are needed to explore this hypothesis.

The *Plectranthus* genus constitutes an important source of antimicrobial secondary metabolites that due to its interesting potency could correspond to infectious diseases drug candidates. The improvement of compound efficacy and the elucidation of the action mechanism of such compounds would correspond to future lines of the work with antimicrobial *Plectranthus* derived compounds.

1.3. Cancer biology and antiproliferative potential of *Plectranthus* spp.

Cancer is a group of diseases occurring in higher multicellular organisms. It is associated with alterations in gene expression leading to dysregulated balance of the cell proliferation and programmed death and originating a tumoural cell population ultimately able to invade tissues and metastasize in distant sites[134]. When a malignant cancer is present, the host suffer significant morbidity, and it could be lethal if the condition remains untreated. Cancer constitutes the fourth world cause of death and its prevalence is estimated to increase in following years[135]. However, the reason why cancer accounts for a major proportion of deaths nowadays is because of a much higher life expectancy which nearly duplicated in the last 100 years[136]. This assume special relevance due to the slow carcinogenesis process, in which 10 to 20 years may pass from the initial growth of a neoplasm to the formation of a clinical detectable tumor[135]. The causes of cancer are still unclearly defined, but both internal (genetic predisposition, gene expression alterations, immune system failure, etc.) and external (virus, chemicals, radiation, etc.) are involved. Those factors may also act together to initiate or promote cancer development[134].

The developments in genetics and molecular biology contributed substantially for the better understanding of the cancer cell biology and its interactions with the surrounding medium. Such techniques led to the reinterpretation of the tumour, from a group of rapidly dividing cancerous cells, to complex mixtures of several cell types that collaborate to create malignant growth: the tumour microenvironment[134,137,138]. By 2000 it was proposed that virtually all cancers possess six hallmark capabilities that favour its growth and metastatic dissemination: sustaining proliferative signalling, insensitivity to growth suppressors, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis and activating tissue invasion and metastasis[137] (Figure **8**). The 2010 update on the cancer hallmarks added additional capabilities: reprogramming of energy metabolism and evading immune destruction[138] (Figure **8**). Those capabilities were potentiated in the presence of enabling

characteristics as genome instability, which contributes to tumour cell variability, and inflammation, which contribute to the establishment of multiple hallmark functions. The knowledge of such mechanisms of tumorigenesis was related to the uprising of many new cancer therapeutics.

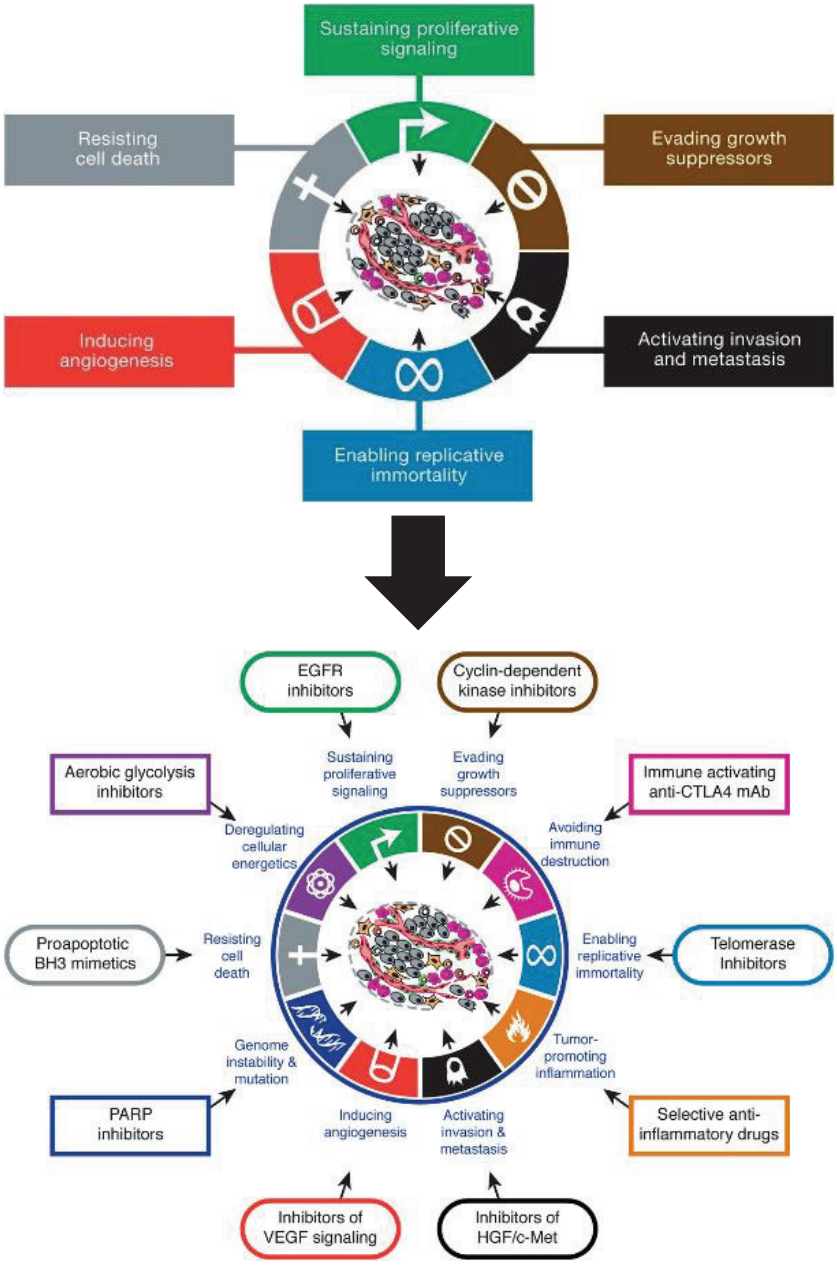


Figure 8. Cancer hallmarks evolution from 2000 to 2010[137,138].

The cancer treatment can be archived by three main strategies: chirurgic, radiotherapy or chemotherapy. In most cancers, it is not possible (or advisable) to use a

single treatment option for the total resolution of the disease[134]. Classic cancer chemotherapy uses small molecules or biologics to destroy rapidly dividing cells. This treatment modality can treat the entire body, including cells that may have escaped from the primary tumours. However, normal cells that divide quickly (i.e. bone marrow, reproductive system or hair follicles) are also affected by the chemotherapeutic agents. Also, the low specificity of many chemotherapeutic agents for cancer cells could result in low efficacy (the drug don't reach the tumour site) and severe toxicity (systemic side effects). Thus, there is a current need for the development of alternative anticancer drugs with improved specificity and minimal side-effects[134]. Natural products have been a reliable source of new drug scaffolds, namely anti-cancer drugs[5]. Almost half of all the approved anti-cancer drugs in Europe, North America and Japan are natural products (14%), their semi-synthetic derivatives (28%) or inspired by natural products (5%)[139].

1.3.1. Cytotoxic and antiproliferative activities in the *Plectranthus* genus

Plants from the *Plectranthus* genus have showed in many studies the potential for anticancer applications based on its ethnopharmacologic indications (as reviewed by Lukhoba et al., 2006[27]) and cytotoxicity screenings of both extract and isolated compounds.

Several studies have focused on cytotoxicity screenings of medicinal plants including members of the genus *Plectranthus*. The screening of 67 Lamiaceae species from Australian flora included 25 *Plectranthus* species[140]. In this study, the most potent cytotoxic effects were verified for *P. fasciculatus* (IC_{50} of 5.50 µg/mL in D.mel-II *Drosophila* cell model), although 6 other *Plectranthus* species showed relevant cytotoxicity[140]. Saeed et al.[141] screened 26 South-African medicinal plants traditionally used in the treatment or prevention of cancer. From those, the extracts of *P. barbatus* and *P. ciliates* inhibited the growth of two leukaemia cell lines (the drug sensitive CCRF-CEM and the drug resistant CEM/ADR5000)[141]. The screening of 7 *Plectranthus* species (*P. ornatus*, *P. amboinicus*, *P. argentatus*, *P. cilatus*, *P. hadiensis*, *P. zuluenesis* and *P. fructicosus*) indicated that the most potent cytotoxic effect was present in acetone extracts of *P. cilatus*[142].

Different studies revealed considerable cytotoxicity in *P. amboinicus* extracts[143–145]. The extract obtained with ethyl acetate inhibited considerably the growth of the breast cancer cell line MCF-7[143]. The ethanolic extract from the same plant presented relevant cytotoxicity towards a human lung cancer cell line (A549) with fifth percent growth inhibition of 31.2 µg/ml (MTT, 48h) while a lower growth inhibition effect was verified in Vero cells, which could represent some selectivity[144]. The hydroethanolic extract from *P. amboinicus*, showed interesting *in vivo* antitumor effects in mice inoculated with Sarcoma-180 and Ehrlich carcinoma with the higher doses reducing the tumour growth up to 66%[145].

The extracts from *P. hadiensis* were screened by two different groups, being verified moderate cytotoxicity in FL (IC_{50} of 150 µg/mL) and HeLa (IC_{50} = 141.3 µg/mL) cervical cancer lines.

The ethanolic extract from aerial parts of *P. neochilus* of was showed some cytotoxicity in *Artemia salina* model with LC_{50} of 210.31 µg/mL[146], and more recently, an hexane extract showed growth inhibitory effects in head and neck squamous cell carcinoma cell lines[147].

The promising results of plant extract screening could be followed by the bioguided isolation of the active compounds. In the *Plectranthus* genus this strategy conducted to the elucidation of some promising anticancer lead compounds, mainly labdane and abietane diterpenes, as described therefore.

The labdane diterpene forskolin (**1**) (Figure **3**), isolated from the roots of *P. barbatus*, was one of the first *Plectranthus* isolated compounds with promising anticancer activities. In the late 1980s was considered a promising antimetastatic agent due to its ability to limit metastasis formation in mice model[148]. This compound is a adenylyl cyclase activator, leading to an increase of cAMP and protein kinase A (PKA) intracellular level[50]. Some oncogenic pathways are influenced by the modulation of cAMP and therefore it was expected a favourable outcome for the treatment of such cancer forms with forskolin[149]. In concordance, this compound showed to be a potent inhibitor of the growth of the "low cAMP addicted" KM12C colon cancer cell line with induction of cycle arrest at G1 phase and further apoptosis[150].

Coleusin factor (**28**) (Figure **9**) is a forskolin related labdane also obtained from the *P. barbatus* root that showed antiproliferative effects in hepatoma[151], gastric cancer[106] and rat osteoscarcoma cell lines[152]. This compound induce G0/G1 cycle arrest and apoptosis, both *in vivo* and *in vitro*[106] models. The occurrence of cycle arrest was related to the increase of p27Kip1 and decrease of cyclin D1 in a p53-dependent p21 pathway[153] while the apoptosis was related to caspase activation and dissipation of mitochondria membrane potential with cytochrome C release into cytosol[106]. More recently coleusin factor was showed to restore differentiation in osteoscarcoma cells by BMP-2 induction and the expression of transcription factor RUNX2 in absence of apoptosis[105].

Other compounds, also obtained from *P. barbatus*, also have could have potential anticancer applications. Coleon A lactone (**29**), also obtained from *P. puberulentus*[62,83], present potent anti-angiogenic effects in zebrafish embryo model along with inhibition of mouse and bovine aortic endothelial cell lines with potency similar to those of a known vascular endothelial growth factor (VEGF) receptor inhibitor and two phosphoinositide 3-kinase (PI3K) inhibitors[103]. 13-epi-sclareol (**30**) showed a cell proliferation inhibition similar to the verified for tamoxifen in MCF-7 breast cancer model but significantly superior to tamoxifen in Ishikawa uterine cancer model with low cytotoxicity in Vero model, which could represent some selectivity to cancer cells[154]. Coleon C (**31**) appears to exerts a direct inhibitory effect on tumour proliferation *in vitro* and also *in vivo* (Lewis lung carcinoma in mouse) with some selectivity to human melanoma (A375) and human acute myeloid leukaemia (HL60) cancer cells over non-cancer cells. The mechanism of its effects seems to be related to the induction of apoptosis in the sub-G0/G1 cycle phase[155].

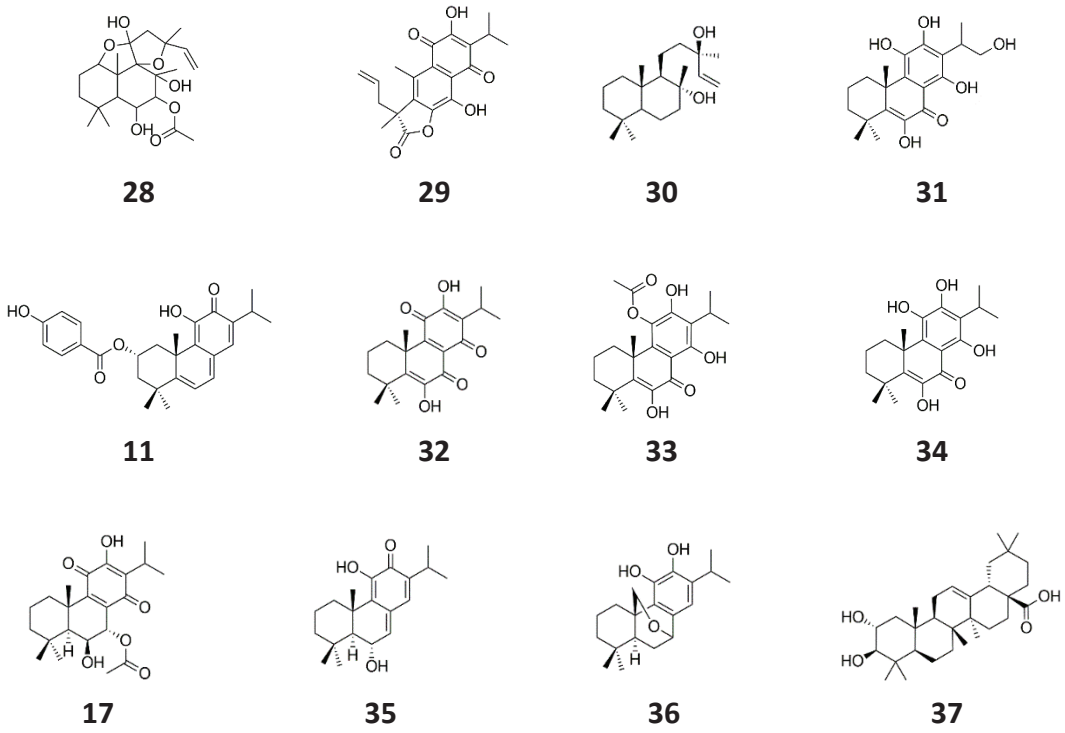


Figure 9. Antiproliferative compounds obtained from *Plectranthus* species.

Abietane diterpenes possessing conjugated quinone systems, such as parvifloron D (**11**) obtained from *Plectranthus ecklonii*[41] or coleon U-quinone (**32**) and coleon U 11-acetate (**33**), obtained from *P. xanthanthus*[82], were described as potent cytotoxic against human leukaemia cancer cells. The related compounds coleon U (**34**) and 7α-acetoxy-6β-hydroxyroyleanone (**17**), obtained from *P. grandidentatus*[39], and their hemisynthetic derivatives also showed transversal cytotoxic activity to several cancer cell lines[39,40]. Moreover, coleon U (**34**) was described as a potent and selective activator of the pro-apoptotic protein kinase C delta (PKCδ) which could explain its reported anti-tumour action[156] (discussed in more detail in **1.3.2.** sub-section).

At last, some compounds rarely found in the *Plectranthus* genus were also found to have cytotoxic effects. Taxodione (**35**) was originally obtained from *Taxodium distichum* Rich (Cupressaceae)[157] but also present in *P. barbatus*[87]. This compound was found to be one of the main cytotoxic compounds present in some Lamiaceae species including *Rosmarinus officinalis*[158], *Salvia chorassanica*[159] and *Salvia staminea*[160] and tested into a comprehensive panel of mammal cancer cell lines (IC_{50} 0.3-60.32 μg/mL). Other cytotoxic compound frequently found in other Lamiaceae was 20-deoxocarnosol (**36**), which was also found in *P. barbatus*, and possessing growth inhibition of several cancer cell lines (IC_{50} 4.6 to 32 μM)[87,161]. Maslinic acid (**36**) is a pentacyclic triterpenic compound

found in olive skin (*Olea europaea*) but also obtained from tubercles of *P. rotundifolius* (formerly *Coleus tuberosus*) which showed potent antiproliferative activity against HT29 and Caco2 colon cancer cell lines[107,108,162]. Cycle arrest at G0 phase and apoptosis thought caspase-3 activation in a p53 dependent pathway were observed for maslinic acid[108,162,163].

The overall studies on *Plectranthus* derived antiproliferative and cytotoxic extracts and compounds indicate that this genus is a source of potential anticancer lead compounds. Further studies, including bioassay guided isolation of pure compounds from active extracts, derivatization of known compounds with establishment of structure-activity relationships (SAR) and the detailed characterization of the underlying action mechanisms in both *in vitro* and *in vitro* models were needed for the establishment of potential new anticancer therapies.

1.3.2. PKC as anticancer natural product target

The protein kinase C (PKC) family consists of ten serine/threonine protein kinases classified based on their regulatory domain structure and cofactor requirements for activation. Three isoforms subfamilies could be considered: classical (or conventional) PKC including α, βI, βII, and γ; novel PKCs including δ, ε, η and θ; and atypical PKCs including ζ and λ\ι[164]. All PKC family members share a common structure composed by a cell membrane targeting NH_2-terminal regulatory domain and a COOH-terminal catalytic domain, with four conserved (C1-C4) and five variable (V1-V5) regions (Figure **11**). These two major domains are linked by a flexible hinge region (V3), which is the site of caspase proteolytic cleavage, protein-protein interactions and tyrosine phosphorylations[165].

The classical and novel isoforms contain a C1 domain with two cysteine-rich motifs (C1a and C1b), which is the binding site of diacylglycerol (DAG) and also the competitive binding site to tumour-promoting phorbol esters (i.e. TPA, **41**)[166]. The C2 domain differs from classic PKC to novel PKC. Whereas the classical C2 domain binds to PS of membranes in a Ca^{2+}-dependent manner, novel PKC contain a variant (C2-like) that binds to phospholipids in a Ca^{2+}-independent manner[165]. Atypical PKC isoforms contain a modified C1 domain, termed 'atypical' C1 domain that retains the ability to bind anionic phospholipids, although with lower affinity. Additionally, these isoforms present a Phox/Bem1 (PB1) domain responsible for their interaction with other PB1-containing proteins[164,165]. The regulatory domain of PKCs contains an auto-inhibitory pseudosubstrate sequence that retains the kinase in an inactive state by occupation of the substrate binding pocket in the catalytic domain (Figure **10**). The catalytic domain of PKCs is a highly conserved region among the distinct PKC isoforms and contains the ATP binding site (C3) and the substrate binding site (C4)[164,165].

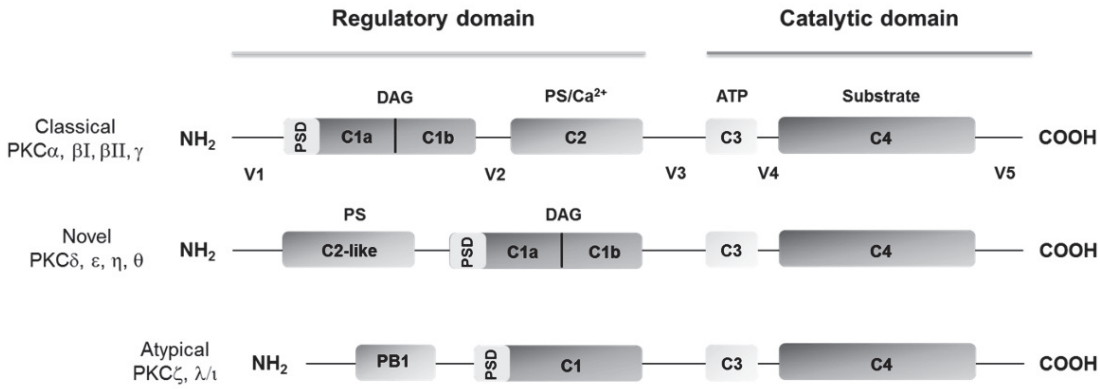


Figure 10. PKC families regulatory and catalytic domains.

This multifamily of structurally related kinases has a crucial role in cellular signalling transduction, being their members involved in the regulation of several biological processes, including proliferation, apoptosis, differentiation, survival, and migration. In fact, dysregulation of PKCs, in terms of both expression levels and activity, is frequently associated with distinct human diseases, including cancer[164]. Among the several PKC isoforms, PKCα, δ, ε and ζ have deserved particular attention in cancer research. PKCα has been predominantly linked to increased proliferation and/or survival, being commonly recognized as a tumour promoter. For instance, in U87 glioblastoma cell line, PKCα increased the resistance to apoptosis in response to radiation and chemotherapy[167,168]. Nevertheless, depending on the cellular background, PKCα can also behave as a tumour suppressor. For example, in LNCaP cells, the activation of PKCα by phorbol esters induced apoptosis, an effect abrogated by the expression of a PKCα kinase-dead mutant upon PMA treatment and radiation[169]. In contrast, PKCδ is frequently associated with pro-apoptotic functions. In fact, PKCδ has been broadly implicated as a death mediator of chemotherapeutic agents and radiotherapy. Ectopic overexpression of PKCδ induced growth inhibition in NIH 3T3 cells[170], an effect also observed for other cell lines. However, pro-survival properties of PKCδ in a number of tumour models, including breast, lung, pancreatic and liver cancer were also described[171]. PKCε has been described as an oncogenic isoform. It is frequently overexpressed in a large number of cancers, namely in breast[172], prostate[173], and in primary NSCLC cancers[174]. PKCε has a prominent anti-apoptotic function, promoting survival in several tumour cells, what has been intimately related with the modulation of caspases and Bcl-2 family proteins. Actually, it was reported that PKCε has the potential to enhance the progression and to confer resistance to apoptosis of prostate cancer[175,176]. Regarding PKCζ, both up- and down-regulation of this isoform have been reported in human cancers. Also for this isoform much controversy exists around its role in cancer development[177]. Many studies have reported a tumour suppression function for PKCζ, which mainly occurs through down-regulation of Ras-induced interleukin-6 production by PKCζ[178]. It was also revealed a link with c-Myc, which contributes to the more aggressive phenotype associated with PKCζ loss[179]. However, there are several

reports highlighting a pro-survival role for PKCζ[180,181]. The proliferation and anti-apoptotic activities of PKCζ seem to involve ERK and NF-kB/IkB pathways[182,183].

Some compound classes obtained from Lamiaceae family plants were able to modulate the PKC activity. Carnosol (**38**) (Figure **11**) is an abietane diterpene with an *o*-diphenolic C-ring and a lactone moiety between C_7 and C_{20} atoms and was first isolated from *Salvia carnosa*[184]. This compound is present in high yields in *Rosmarinus officinalis* leafs[185] and showed promising antiproliferative activity in prostate, breast, skin, leukaemia, and colon cancer cell models[186]. The cytotoxicity of carnosol seems to be linked to the modulation of multiple deregulated pathways including nuclear factor kappa B (NF-kB), PI3K and PKC[187]. In addition, the concomitant administration of carnosol with other cytotoxic agents promoted a synergistic effect in reducing cancer cell viability[188]. Coleon U (**34**), has been isolated from several *Plectranthus* species such as *P. forsteri*[83], *P. grandidentatus*[39], *P. madagascariensis*[189] and *P. myrianthus*[56]. This diterpene is a quinone methide abietane exhibiting potent cytotoxic effects transversal to several cancer cell lines including breast[39,40], leukemia[40,190] and melanoma[40,190]. The mechanism by which coleon U induces its cytotoxic effect may be related with *in vitro* selective activation of novel PKCs namely PKCδ and ε. This effect, originates a nucleus translocation of activated PKC isoforms and the subsequent apoptosis mediated by metacaspases in the yeast model[156]. However, coleon U is easily degraded to its oxidized form coleon U-quinone that owns also cytotoxic effects[82,83]. Whether this derivative is also a PKC activator is still unknown. These abietane diterpenoids coleon U and carnosol may be lead compounds for anticancer treatment particularly as PKC modulators. However, the degradation profiles of carnosol[191] and coleon U[83] highlight the need of more stable derivatives for further potential clinical use.

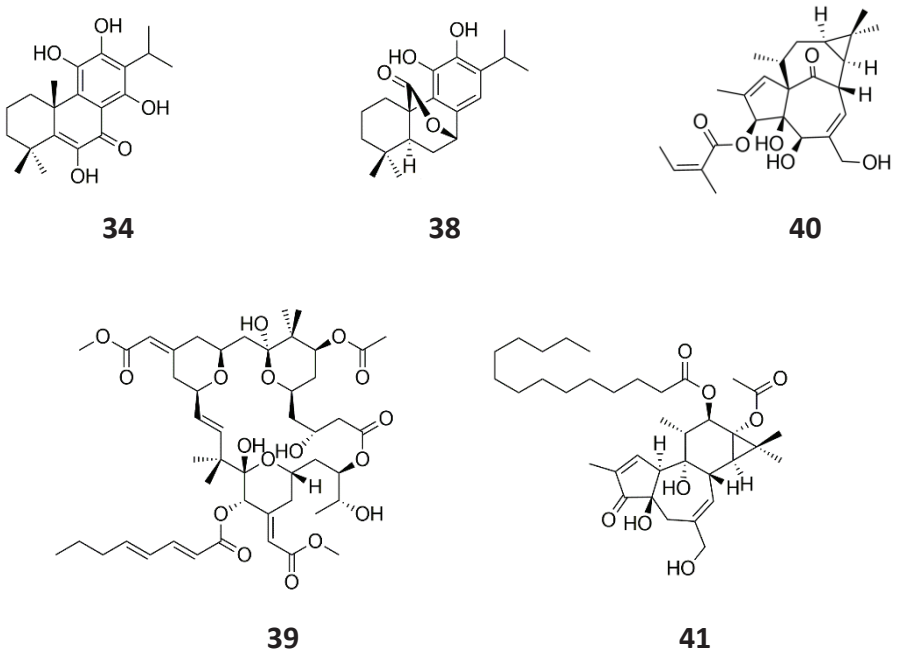


Figure 11. Chemical structure of some natural products that act as PKC isoforms modulators.

Other natural products have also found to target PKC isoforms. The most clinically relevant were the marine origin polioxygenated macrocyclic lactones known as bryostatins[192,193]. This class of compounds showed antiproliferative activity against a wide range of cancer cell lines such as P388 leukemia[194], ovarian sarcoma[195], B16 melanoma[195,196] or M5076 reticulum cell sarcoma[195]. Subsequently, the bryostatin 1 (**39**) was submitted to more than thirty-five human clinical trials (phase I/II). Nevertheless, the efficacy of bryostatin 1 used as a single drug, for example against melanoma, colorectal cancer and gastric carcinoma, has been variable and with some disappointing results. However, when combined with conventional chemotherapeutic agents, such as paclitaxel[197] and gemcitabine[198], significant synergistic effects were observed. These results indicated that bryostatin 1 could be a useful enhancer of the cytotoxic activity of some therapeutic regimens, rather than an anticancer agent. Consequently, bryostatin 1 has been under different clinical studies in combination with diverse therapeutics. Food and Drug Administration (FDA) has approved the bryostatin 1 as an Orphan Drug in combination with paclitaxel against the oesophageal carcinoma[199]. Synthetic analogues have been developed by different strategies mainly by Wender[200,201] and Keck[202] groups but more recently a function oriented strategy was able to produce simplified analogues with a nanomolar binding affinity to PKC[203].

Another group of clinical importance were the ingenanes obtained from the milky latex of *Euphorbia* species such as *E. antiquorum*, *E. drummondii*, *E. helioscopia, E. hirta* and *E. paralias*[204,205]. Extracts from Euphorbiaceae plants have been used in traditional medicine for centuries in the treatment of some skin conditions as warts, keratosis and cancers[206]. The *E. peplus* phytochemical study of these extracts yielded several macrocyclic diterpenes, being the ingenol mebutate (**40**) the most active component[207,204,208,209], including antitumour activity in several cancer cell lines including breast, colon, lung and melanoma[210,211,212]. The mechanism of action of ingenol mebutate is, at least, partially related to the activation of PKC to which has a potent binding affinity. *In vitro*, low isozyme selectivity was verified with a K_i ranging from 0.105 - 0.376 nM[213]. These results supported the preclinical and clinical trials for the topical treatment of actinic keratosis (AK) carried out by both FDA and EMA (European Medicines Agency)[214]. Recent studies indicated that both a dual proapoptotic and an immunostimulatory effects occurred in the leukaemia disease[215].

The role of PKCs in carcinogenesis is known since the late 1980s. Nevertheless, the development of PKC targeting drugs has not been an easy task. The PKCs isoforms are the target of many natural products, although very few are selective to solely one isoform, which is not suitable to clinical use. The natural products arise as useful compounds for the study of bio-molecular complex interactions involved in the carcinogenesis process. Furthermore, some natural products or their closely related analogues are under clinical trials. Two approved compounds for specific cancer types are bryostatin 1 (**39**) in combination with paclitaxel and ingenol mebutate (**40**). The

search for more selective PKC modulators remains a promising strategy for future anticancer treatment.

1.4. Natural product delivery systems

Besides the importance of natural products in the development of new drugs, intrinsic physical and chemical characteristics of the natural drugs stand for a poor pharmacokinetic profile. For adequate bioavailability, drugs should present a good balance between hydrophilicity (ability to dissolve in water mediums as gastrointestinal fluids and blood) and lipophilicity (ability to cross lipidic mediums as the case of biologic membranes). However, many plant secondary metabolites, from the most prevalent classes, are either low fat soluble (i.e. flavonoids, polyphenols, etc.), low water soluble (i.e. terpenes) or present high molecular weight (i.e. saponins, tannins) which also limits their oral bioavailability. Also, the stability of natural products was frequently impaired in the gastro-intestinal environment or can be either metabolized by gut bacteria or suffer rapid liver metabolism.

Many strategies have been applied to overcome those limitations. The most used were the chemical derivatization or the novel drug delivery systems (NDDS). The first implies a chemical modification of the compound structure with possible implications on its original activity. Also, this strategy could only be applied to a pure compound and not to a standardized extract. It is somewhat frequent that the isolation of the most active compound in an extract not always led to an improvement of the activity, which could be explained by the existence of a synergic potential between natural components. Those factors indicated that chemical derivatization was not always applicable. On the other hand, NDDS have been in focus in recent years with the development and improvement of many drug delivery systems including microparticles and nanoparticles, among others[216,217]. Those systems intend to improve the therapeutic outcome with minimized adverse or toxic effects and improved patient compliance due to an improved drug dosage, compatibility with physiologic mediums, targeted delivery and favourable release profile, without alterations on the molecule structure[216].

The discovery that some dietary components, containing phospholipids, improve the absorption of low bioavailable drugs led to the development of lipidic based-systems[216,218]. Those can assume diverse forms as liposomes, nanosomes, niosomes, ethosomes and, the more interesting for natural product delivery: phytosomes. Phytosome is the name of a patented technology developed by the Italian pharmaceutical company Indena. The name derived from the conjugation of the Greek words “phyto” meaning “from plant” and “some” which means “cell-like”. Other designations have been used for phytosomes including phyto-phospholipidic nanoparticles, planterosomes and herbosomes[216,219].

1.4.1. Phospholipids: structure, properties and complexation

The main constituents of every life form membrane are phospholipids. Those polar lipids were constituted by both a hydrophilic ("head") and two hydrophobic ("tail") portions which in conjugation confer substantial solubility in both aqueous and oily mediums. Phospholipids can be classified as glycerophospholipids (with a diacylglycerol backbone) and sphingomyelins (with a ceramide backbone). In the eukaryotic cell membrane the glycerophospholipids including phosphatidylcholine (PdC), phosphatidylethanolamine (PdE), phosphatidylinositol (PdI), phosphatidylserine (PdS) and phosphatidic acid (PdA) were the most abundant constituents[220]. Those molecules were classified and named based on the constitution of the head group and the length and saturation of hydrophobic side chains (Figure **12**).

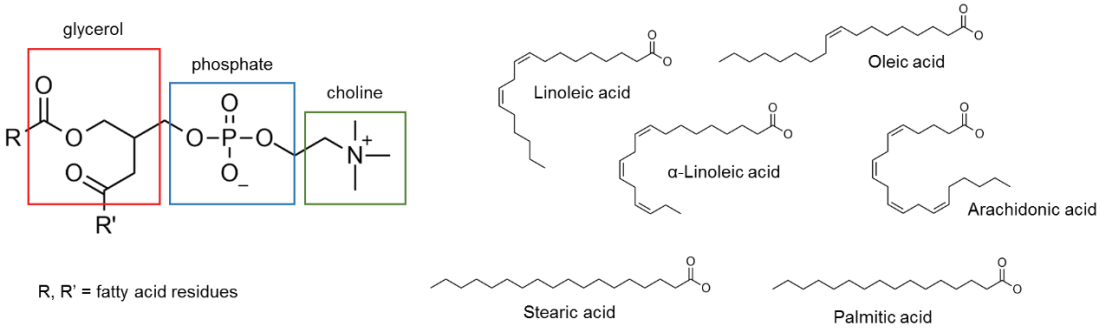


Figure 12. Main structure of the PdC, representative PdC groups and main natural fatty acid residues.

Animal tissues (egg yolk and bovine or swine brain) and vegetable oils (soybean, cotton seed, sunflower, etc.) are the most important natural sources of phospholipids. Naturally obtained phospholipids were composed by a saturated fatty acid (stearic or palmitic acid) in the glycerol carbon 1 and an unsaturated fatty acid (oleic, linoleic, α-linoleic or arachidonic acid) in the glycerol carbon 2 (Figure **12**). The more frequently used phospholipids in pharmaceutical applications were those obtained from soya bean (*Glycine max*) that contains about 70% of PdC with a high content of linoleic and oleic acids which offers excellent compatibility to the mammalian membrane[221].

In the human physiology, phospholipids were also present as emulsifiers at intestinal level, together with cholesterol and bile acids are able to form micelles that improve the absorption of both fat and water soluble substances[222]. Phospholipids can also be found as wetting agents in the pleura and alveoli of lungs, pericardium and joints[220]. As expected from physiologic relevant molecules they are biocompatible with no signs of carcinogenic, immunogenic or teratogenic effects even when administered at high dosages in clinical trials[222].

The conjugation phenomena of phytomedicines with phospholipids have been briefly studied. Research on the interaction between some flavonoids and cellular PdC demonstrated that most molecules possessing π electron systems are able to form different complexes with membrane phospholipids[223]. Those interactions were

confirmed in several papers by the use of spectroscopic, thermographic and molecular imaging techniques that demonstrated the formation of the phytophospholipidic complexes (PPC).

Thermal analysis, by differential scanning calorimetry (DSC), is a fast and reliable method to revealing the distribution patter and further interactions between phytocomponents and phospholipids in the phytosome matrix. The comparison of phase diagrams of natural compound, phospholipids, their physical mixture and the phytosome allow the estimation of the presence of an interaction by the elimination of endothermic peaks, appearance of new peaks, change in peak shape, area and/or melting points. This fact occurs presumably due to the formation of an interaction between the two molecules. Such interaction could be originated from the hydrogen bonding established between hydroxyl groups in the natural product (i.e. phenol moieties) and those in the polar head of the phospholipid (phosphate and/or ammonium groups)[224]. Van der Walls forces were also suggested occur between the two moieties[224,225]. Those strong interactions between the polar components allow the free turning of the hydrocarbon chains in the phospholipid which enwrap the polar head of the phospholipid containing the natural product[224–227].

The infrared spectroscopy and namely Fourier transform infrared spectroscopy (FT-IR) could confirm the complex formation by comparing the spectrum of the complex with individual components and their physical mixture. The FT-IR spectra of pure phospholipids is generally characterized for the presence of peaks at approximately 3500 cm^{-1} (hydroxyl stretching), 2900 cm^{-1} (C-H stretching at fatty acid residues), 1700 cm^{-1} (carbonyl stretching of the fatty acid ester), 1200 cm^{-1} (P=O stretching), 1100 cm^{-1} (P-O-C stretching) and 970 cm^{-1} (N-$(CH_3)_3$ stretching)[228,229]. Many natural products like polyphenols or flavonoids have in their structures one or more hydroxyl groups whose O-H stretching appear in the FT-IR spectra as sharp peaks in the 3800-3200 cm^{-1} range. Also some signals measured in the about 3500-3300 cm^{-1} range correspond to N−H stretching vibrations of natural products containing amine groups[229]. While analysing the FT-IR spectra of the PPC it could generally be observed a shift and broadening of the signal corresponding to the natural products hydroxyl O-H or amine N-H stretching along with the signal corresponding to the aliphatic phosphate (P=O stretching) and choline quaternary ammonium (N-$(CH_3)_3$ stretching) groups of the phospholipids with no alterations found in the bands of the aliphatic carbon chains of the fatty acids. These observations suggest that some weak physical interactions between free hydroxyl or amine of the natural components and the polar groups of phospholipids took place during complex formation[230].

Additional confirmatory assays could be performed using x-ray powder diffraction (XRD)[226,228,231] or ^{1}H, ^{13}C and ^{31}P nuclear magnetic resonance (NMR)[228]. Slight or no changes are verified in the side chain signals which indicate that the long aliphatic chains wrap around the polar head, containing the bonded phytocomponents, generating a lipophilic envelope[232]. Also UV absorbance could be used for complex analysis formation

but with rendering less useful information when compared to previous techniques[233–235].

The concomitant information retrieved from those thermographic and spectroscopic methods on the PPCs some key findings arise: the natural drugs and phospholipids establish weak (non-covalent) bindings as hydrogen or Van der Walls bonds. Those interactions occur mainly between the hydrogen atoms of hydroxyl or amine groups and the polar components of the phospholipid (phosphate and choline). The aliphatic chains of the phospholipid do not interact directly with the natural drug but form a lipophilic envelope around the natural drug-phospholipid complex (Figure **13**).

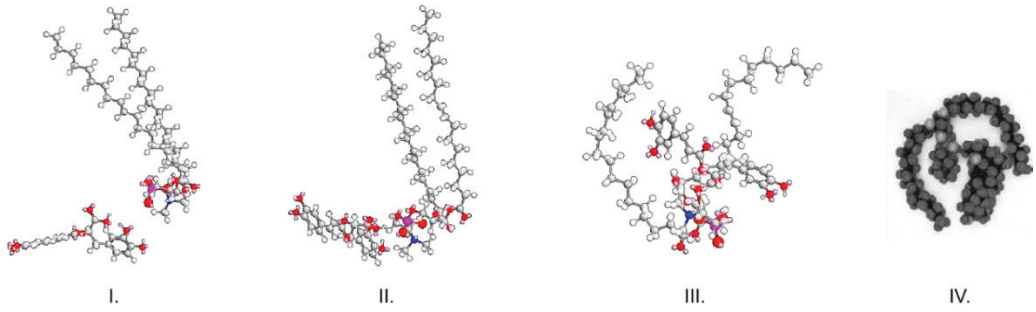


Figure 13. Illustration of the potential interactions during the formation of a phyto-phospholipidic complex. I. A phytochemical and PdC were combined and weak interactions occur between the polar moieties of the two molecules; II. A Van der Walls and hydrogen bonds were established between the phosphate group of the PdC and the phenolic hydroxyls of the phytochemical; III. The aliphatic side chains of the PdC involve the polar head where the phytochemical has been complexed. This corresponds to a lower energy form of the complex (ChemDraw 3D Pro 12.0). IV. Illustration of the structure of a phytophospholipid complex (adapted from Indena, SPA®).

1.4.2. Phytophospholipid complex optimization and preparation

Phytophospholipid complexes were obtained by reacting close to equimolar proportions of phytochemical and phospholipid for a certain amount of time in a suitable reacting medium being the recovery of phytosomes accomplished mostly by solvent evaporation or precipitation (Annex **1**). Reaction variables as the proportions of components, reaction time, temperature or solvent as long as the use of different strategies for the complex retrieval can influence the particle size, its dispersity and the entrapment efficiency were discussed in this section.

1.4.2.1. Optimization of drug to phospholipid proportions

The phytosome differ from other lipidic-based nanoparticles as the proportion of drug to phospholipid is close to equimolar. In systems as the liposomes, in which this proportion is much lower and being each drug molecule surrounded by hundreds of phospholipid molecules, exists limited drug interaction with the surrounding medium. Also a lower drug loading capacity is expected from liposomes when compared to phytosomes. The original patents on the phytophospholipidic complexation state that the complex ratio could range from 0.5 to 3 molar ratio[236]. A substantial proportion of

the recent papers and patents on phytosome preparation used a 1:1 molar proportion between its elements, however some experimental designs were focused on exploring other molar proportions (reviewed in Annex **1**).

1.4.2.1.1. Solvent selection

Original phytophospholipid complexation patents stated that the reaction should occur in an aprotic solvent such as acetone, dichloromethane, dioxane, ethyl acetate or tetrahydrofuran[233,237] but the use of protic solvents as ethanol have also been developed and patented by other groups[233,238]. Recalling the phytophospholipid complexation theory, in phospholipids, the nitrogen atom has a strong tendency to lose electrons while the oxygen atom of the phenolic hydroxyl group polyphenols tends to gain electrons, in order to make the complexation possible. This way, aprotic solvents were preferred, as do not interfere with the electrons exchange of the complexation process[239]. Song et al. (2008) have studied the feasibility to prepare the silybin-phospholipid complex using four solvents with low dielectric constant. The experience was failed when dichloromethane or ethyl acetate were used but it was successful using acetone and tetrahydrofuran (THF)[239]. The selected solvent should be able to dissolve both phospholipids and natural products for the complexation to take place. Although the markedly differences in the solubility of the complex components could not allow the use of a single solvent for their dissolution. The conjugation of two or more miscible solvents could then be applied. Also moderate heating or sonication could improve the components dissolution[240]. However, it is necessary that such conditions do not imply the stability of the phytosome components. Most of those solvents possess a high toxicity (ICH class 1 or 2) and so the substitution of those for more biocompatible solvents should be taken account in newer formulations as discussed in ICH Q3C technical document[241]. Examples of solvents used for phytosome preparation were present in Annex **1**.

1.4.2.1.2. Combination of factors

The optimal conditions for the synthesis of phytosomes with desired characteristics were generally not found with the adjustment of a single reaction factor but as a combination of factors. The use of different statistic methodologies was seen for the optimization of the phytophospholipid complexation process.

Several examples of quadratic or orthogonal designs were available in literature[233,235,239,242]. The overall tendency was that a higher temperature favours the occurrence of complexation. The drug-to-phospholipid optimized proportion was dependent on the compound characteristics, but the 1:2 molar ratio or similar was the more frequent as result from the optimization.

1.4.2.2. Phytophospholipid complexation methodologies

For the preparation of PPCs the chosen proportions of phospholipids and phytocomponents must be dissolved in suitable medium and react at an optimized

temperature for the adequate time. Then, the complex must be recovered as dry powder or converted into a phytosomal suspension (sub-section **1.5.5**).

1.4.2.2.1. Solvent evaporation

A chosen proportion of natural product and phospholipids were mixed in a reaction vessel containing a suitable solvent system and the reaction is allowed to be carried for 2 to 6h at room temperature or with moderate heating. The solvent is then evaporated leading to the recovery of the dry complex. In this setting, the use of volatile solvents was the advantage of their ease of removal[229]. This is true in the case of solvents with boiling temperature lower than 60ºC, as higher temperatures could impair the stability of the complex and its components. In most frameworks, the solvent evaporation occurs at reduced pressure using temperatures lower than 60ºC, varying the duration of the process from a few hours up to 24h. If the choice of solvent recall in non-volatile solvents, lyophilisation or spray-drying could be valuable alternatives for the solvent removal. For such solvent removal methods, the addition of a carbohydrate (i.e. dextran or mannitol) could be necessary for their cryoprotectant effects on the complex during the lyophilisation process[229,230]. The lyophilisation has also the advantage of dispensing an additional drying step.

1.4.2.2.2. Anti-solvent precipitation

This process has similarities to the solvent evaporation method, being the phyto-components and phospholipids combined in a reaction vessel containing a polar or median polar solvent, being the reaction carried for a predetermined period of time at the selected temperature. The reaction is generally stopped by the addition of an anti-solvent in which the product is generally insoluble, as the aliphatic hydrocarbons (i.e. *n*-hexane), being the PPC recovered after its precipitation (and eventual centrifugation) followed by removal of the solvent[243]. Examples of phytosomes prepared using this methodology were presented at Annex **1**.

In some protocols, the precipitation technique was not very effective for the producing of a complex because the complex is decomposed upon the addition of the anti-solvent[230]. Authors suggested that it could be related very weak interactions during the complex formation and/or to the ability of the anti-solvent to dissolve the phospholipids leaving the crystalline drug precipitated[230].

1.4.2.2.3. Other methods

The use of SCF by the supercritical anti-solvent technique was been used for the preparation of pharmaceutical fine powders. The same principles could be applied for the production of phytosomes, namely by the supercritical fluid solution enhanced dispersion (SEDS). This method has some advantages over traditional PPC preparation techniques, including the controlled particle size and site distribution but also not time consuming and simple[244].

Also, a “mechanical dispersion method” was published[245]. In such, the phospholipid components were dissolved into the minimum amount of an apolar compatible solvent

(i.e. diethyl ether) under sonication. The water dissolved phytocomponent was then added dropwise to the phospholipid solution under sonication for 15 minutes being the phytosome formed[245].

1.4.2.3. From the complex to the vesicle

The PPC presents a disorganized, irregular and amorphous structure that present generally a heterogeneous dispersion. The micellar phytosome structure was only formed when the PPC is added to an aqueous medium leading to the reorganization of the phospholipidic double layer. Some methods for the pharmaceutical preparation of phytosomes from the phytophospholipid complex are here described.

1.4.2.3.1. Film hydration method

Following the formation of a thin film of PPC, using a solvent evaporation method, the process can be continued by the hydration of the PPC using a suitable aqueous medium (purified water, PBS, among others). During the contact between the aqueous and lipidic phases, a gradual swelling of the PPC into the aqueous phase occurs leading to vesiculation of PPC and formation of a phytosome suspension[246,247].

1.4.2.3.2. Nanoprecipitation

This technique used the PPC dissolved in a compatible organic solvent which was extruded dropwise to distilled water with gentle stirring. The organic phase was gradually evaporated by the use of reduced pressure or at room conditions for up to 24h[248,249]. The physical process of nanoprecipitation in the preparation of mitomycin phytosomes from mitomycin-PdC complexes was briefly described by Hou and its collaborators[229]. Initially both mitomycin and PdC were dissolved into an organic solvent and gradually dispersed into the continuous phase leading to interface turbulence and conversion of the system into an O/W suspension. In a second moment, a decrease in the interfacial tension resulted in droplet size reduction and, subsequent, spontaneous emulsification. Then, the organic phase gradually diffuses into the continuous phase with integration of excess water into the nanodroplets. The evaporation of the organic phase contributes to a gradual concentration of the nanodroplets and after the complete removal of the organic phase, phytosomes were precipitated into the aqueous environment (Figure **14**)[229].

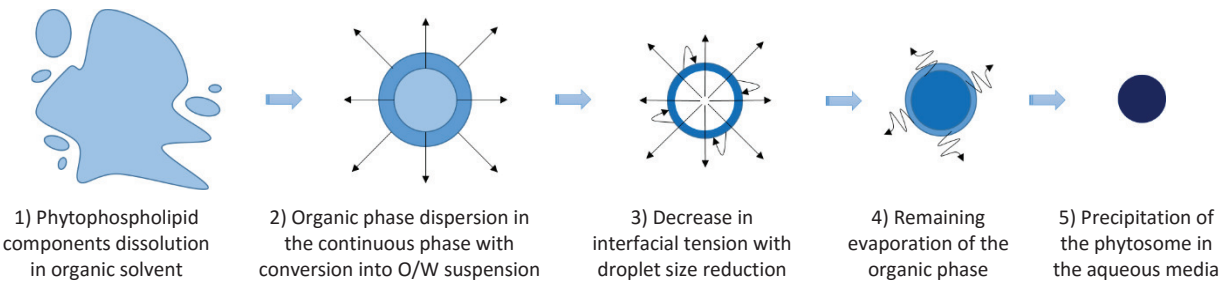


Figure 14. Illustration of the mechanism of phytosome formation by nanoprecipitation technique. Adapted from Hou et al. 2013[229].

1.4.3. Phytosome characterization

Phytosomes are generally characterized according to its morphology, physical properties and chemical composition.

1.4.3.1. Morphology

The shape and morphology of phytosomes are analysed using direct visualization techniques as scanning electron microscopy (SEM)[250] or transmission electron microscopy (TEM) or using topographical characterization, namely by atomic force spectroscopy (AFM)[249]. When directly visualized, PPC were amorphous, fluffy, porous with rough surface and an apparent interaction in the solid state[228]. On the other hand phytosomes presented a liposome-like vesicle, with fairly uniform size, dispersed in the aqueous environment [248].

1.4.3.2. Size and distribution

The particle physical size and its distribution have been analysed mainly by light scattering methodologies as photon correlation spectroscopy (PCS) or dynamic light scattering (DLS) and using computer algorithms to determine the average particle size and the polydispersity index (PI) being the mixture monodisperse when the PI = 0. An alternative methodology of size dispersion is the SPAN value. This measure reflects the width of the size distribution. Smaller values (< 1) are generally obtained when a narrow distribution exists[251].

1.4.3.3. Surface charge

The general charge present at the surface of each phytosome has been evaluated by the measuring the zeta potential (ζP). The knowledge of the surface charge can help to predict the fate of the phytosome particles *in vivo* concerning the ease for aggregation or the attraction to charged tissues. Most phytosomes present a negative or neutral surface charge, depending on the degree of complexation and the availability of negatively charged free phosphate groups from phospholipids[230].

1.4.3.4. Encapsulation efficiency and drug loading

The amount of phytocomponents complexed with phospholipids has been generally evaluated by direct analysis of the dried PPC dissolved into an aggressive media as an organic solvent or extreme pH. The HPLC methodologies were robust, sensible and reproducible state of the art technique for the quantification of drug products into an unknown matrix[227]. Also some authors used simply UV-spectroscopy for the quantification[230]. The amount of natural drug incorporated into a PPC is highly dependent of the drug and matrix physic-chemical characteristics but also from some process variables as starting materials ratio, reaction temperature and reaction time, among others, influence the drug yield in PPC [233,235,239,242].

1.4.3.5. Phytosome stability

Several studies established long-term stability problems when phytosomes were dispersed into an aqueous suspension. The main problems related to this instability were the particle aggregation/leakage, chemical instability (PdC hydrolysis from ester bonds, oxidation) and biological contamination (microorganism growth)[248]. Some strategies that could result in the improvement of the phytosome stability were therefore described.

The addition of an optimized proportion of cholesterol to the vesicular phytosome system was also demonstrated to improve the short-term stability[251]. This effect could be explained by the interaction between cholesterol and PdC (hydrogen bond between the cholesterol hydroxyl and the polar head of PdC) which induces a tighter packing of PdC in the membrane and enhances electrostatic repulsion between phospholipid bilayer. It also enhances the membrane flexibility. Cholesterol also increases the thickness of the phospholipid bilayer[251]. However, the use of cholesterol is limited by its long-term oxidation, that may originate stability problems[252].

Lyophilization has been considered a favourable technique for industrial process in the pharmaceutical field to obtain fine powders. It is easy to manipulate and led to a more stable drug product. The lyophilized phytosome of diosmin maintain their initial physicochemical characteristics at different pH and with the presence of enzymes (simulated gastric fluid). A change in the surface charge was however verified in acidic medium (+24 mV) as compared to a buffered pH 7.4 (-6 mV) or to a higher pH (-27 mV). Such changes in the surface charge could be related to the intrinsic dual charge of PdC (pKa = 0.8). The phosphate negative group is neutralized in acidic pH leading to a positive charge predominance while the opposite occur at higher pH values as alkaline medium should neutralize the choline positive group, leading to the predominance of negative charge[230]. Such occurrence could impair the stability of the nanoparticles as a low ZP could led to particle aggregation and precipitation.

1.4.4. Targeting of phytosomes: coating and functionalization

There is not a standard phytosome for each application. As so, some groups have opted to combine the phytosome technology with other encapsulating agents as polymers, metallic NPs or to functionalizing the phytosome surface in order to obtain improved targeting of bioavailability.

Mitomycin is a water soluble anticancer drug with clinical use limited by aqueous media instability, short elimination half-life and lack of selectivity[253]. The first approaches to overlay such limitations were the encapsulation into a polymeric carrier, dextran[254] or polylactic acid (PLA)[253], or the incorporation in phospholipidic medium: liposome[255] or phytosome[229]. The mitomycin phytosomes were able to reduce the drug degradation and improve its release pattern but did not archive selectivity for the tumour site. The same group opted then to attach to the loaded phytosome a pH-sensitive coating of polyethylene glycol-phosphoethanolamine-polylactic acid (PEG-PE-

PLA) which is stable at physiological pH but originating burst release at endosomal or lysosomal pH. This system was also functionalized with folate (FA) which conferees active targeting to tumour overexpressing the folate receptor[248]. The originated FA-PEG-PE-PLA-phytosome hybrid system exhibited selective tumour accumulation with sustained release and steady-state pharmacokinetics in BALB/c nude mice inoculated with H22 mouse ascitic hepatoma cell line[248]. Those *in vivo* results provide evidences for improved antitumor activity with reduced side effects.

Another example is the hybrid formulation concerning the conjugation of *Calendula officinalis* phytosomes with gold nanoparticles (AuNPs). It was developed for incorporation into wound healing dermal formulations. Those Calendula AuNP-phytosomes had an average particle size of 80±5 nm and showed an *in vitro* protective effect up to 81% in the cytotoxicity induced by H_2O_2 in Vero cells (fibroblasts from African green monkey kidney). Also, in the *in vitro* wound scratch assay[256], those hybrid phytosomes showed an improvement in the gap closure of the cell monolayer of 58.7% (against 42.2% of the equivalent Calendula phytosomes)[257].

The addition of a coating is another option for improvement of the delivery of phytosomal formulations. Polymeric structures as chitosan was used for the improvement of curcumin oral absorption. The chitosan microparticles containing curcumin phytosomes were produced by ionotropic gelation being obtained spherical microspheres with an average particle size of 23.21±6.72 μm[249]. The pharmacokinetic study showed an improvement of the curcumin bioavailability in 1.67 and 1.07-fold when compared with the phytosomes of curcumin and chitosan microparticles of curcumin, respectively. Also the half-life of curcumin in the microencapsulated phytosomes was longer (3.16h) than those of phytosome (1.73 h) and chitosan microparticles (2.34 h) of curcumin upon oral administration in rat model[249].

1.4.5. Applications of the Phytosome strategy

The PPC complexes and their phytosomal equivalents can be formulated in the form of suspension, emulsion, syrup, lotion, gel, cream, pill, capsule, powder, granules, etc. resulting in a product that is better absorbed and produces better result than the conventional herbal extracts.

1.4.5.1. Clinical and nutritional uses

The use as active ingredients in food supplements was the most frequently found application of this technology. Some relevant phytosomes examples of such applications were the silymarin flavonolignans, the curcuminoid polyphenols, the green tea flavan-3-ol catechins and the ginkosenoids from ginko[258] (Figure **15**).

Flavonolignans obtained from milk thistle (*Silymarin marianum*, Compositae) have been used for their ethnomedicinal applications in liver disease for more than 2000 years[258]. The major flavonoligan present in the fruit of milk thistle was silybin (**42**) which was proven to be a liver protectant by its potent antioxidant effect and glutathione conservative effect. Also silybin and related compounds have showed promising results

as chemopreventive or anticancer agents[259,260] due to its inhibition effects on the silent information regulator 1 (SIRT1)[261] and signal transducer and activator of transcription 3 (STAT3)[262]. The phytosome of silybin (Siliphos™, Indena Spa) have showed a huge improvement in the pharmacokinetics of the pure compound. Silybin have a low solubility in water (>0.5 g/L). It suffers an extended phase II metabolism and it is rapidly eliminated by kidney and urine, which results in a low bioavailability and short half-life[263,264]. Siliphos™ showed an significant increase in the oil and water solubility and bioavailability up to 10-fold in rat model[265,266]. This formulation was well tolerated even at high doses[267] and found clinical applications as hepatoprotective[268] and cancer adjuvant[269].

Curcumin polyphenols (**43**-**45**) were obtained from turmeric (*Curcuma longa*, Zingiberaceae) to which conferee the characteristic yellow coloration of the rhizome. Those compounds have potent free radical scavenger, anti-inflammatory and anti-cancer properties that have failed to translate to clinical practice due to their poor bioavailability. Three main curcuminoids were present in standardized preparations

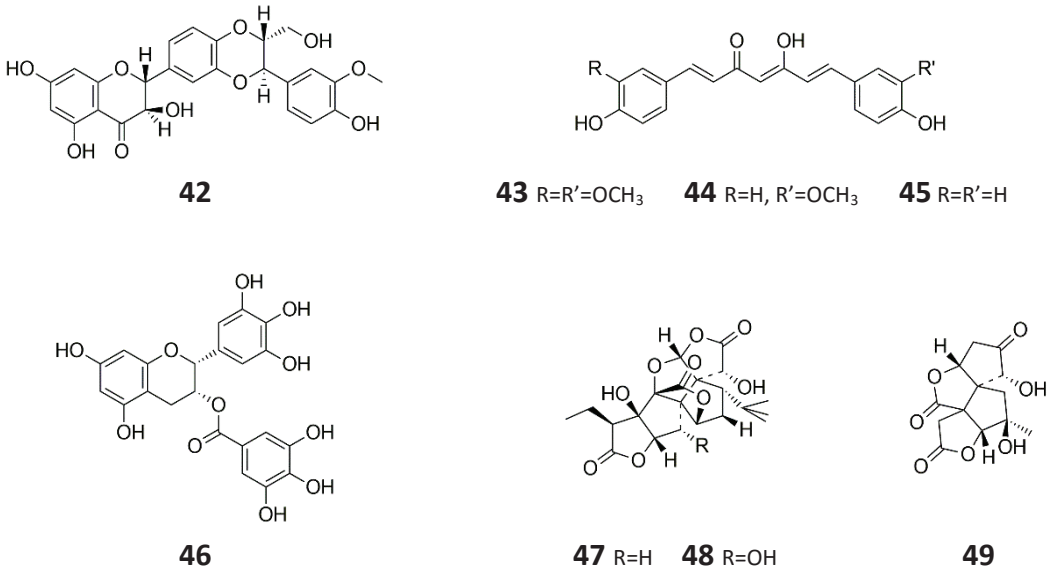


Figure 15. Chemical structure of some natural products included in phytosomal commercially available formulations.

namely curcumin (diferuloylmethane; curcumin I) (**52**), demethoxycurcumin (curcumin II) (**53**) and bisdemethoxycurcumin (curcumin III) (**54**). The phospholipid complexed form of standardized curcumin (Meriva™, Indena Spa) has demonstrated an outstanding improvement in bioavailability. The absorption of curcumin was 18-fold higher and the curcuminoid mixture up to 29-fold higher in comparison to unconjugated curcumin[270]. Two other different studies on curcumin-phospholipid complex demonstrated an improvement of the C_{max} in the range of 1.6[249] to 2.4-fold[227]. This formulation has been used for its protective effects against active radicals. Promising results were obtained in clinical trials in weight management in metabolic syndrome[271],

muscle recovery[272,273] and pain management[274,275]. In addition, its application in cancer chemoprevention, inflammation and neurodegenerative diseases is under study[258].

Green tea catechins and polyphenols obtained from the tea tree (*Camellia sinensis*, Theaceae) have long been used for their antioxidant and anti-inflammatory properties. The major constituents of that complex were epigallocatechin, catechin, epigallocatechin-3-O-gallate (EGCg) (**46**), gallocatechin-3-O-gallate, epigallo-3-O-methylgallate, and epicatechin-3-O-gallate. The main applications of the green tea extracts were in weight lose supplements[276] but can also be included in cancer[277], liver[278] or cardioprotective formulations[279]. A single dose trial demonstrated that Greenselect™ phytosome was able to induce a decrease in the plasmatic oxidative status correlated with the plasmatic levels of ECGg[280]. This formulation conducted also to an improvement of bioavailability of ECGg (C_{max}) in about 2-fold[280]. The weight lose effects were substantially improved by the use of Greenselect® in combination with hypocaloric diet in comparison with the diet only group ($p>0.001$) in a human clinical trial (n = 100)[281]. This lipidic-based formulation was also useful for control of borderline metabolic syndrome as in a 24-week treatment, 68% of the subjects showed a substantial improvement of in weight, lipidic profile and blood pressure while only 20% were able to manage those parameters based on lifestyle changes[282].

Ginkgo (*Ginkgo biloba*, Ginkgoaceae) is one of the anciently used medicinal plants from Chinese traditional medicine[283]. Terpene lactones and flavonoid glycosides were present in extracts of this plant from including ginkgolide A (**47**), B (**48**) and bilobalide (**49**). Although these plant extracts have been used with efficacy for the treatment of cardiovascular[283] and neurologic conditions[283,284], the phytosome form (Ginkgoselect®) originate an improvement of those active components bioavailability[285,286]. Also potent anti-inflammatory[287] and hepatoprotective[285,286] effects were also verified for this phytosome formulation.

With some of the older patents reaching the end of the protection period, it would be expected the appearance of similar pharmaceutical products using the patented formulations.

1.4.5.2. Dermatologic uses

The lipophilic nature of the phytosome let us expect an improvement in the topical absorption of the complex, but as discussed earlier, the transdermal absorption should be negligible, being the local effects the most relevant and also the most appropriate for a cosmetic purpose. Their phospholipidic nature is also adequate for the enhancement of some skin functions as hydration, collagen structure, enzyme balance and restoring the barrier functions of the skin[288]. Standardized extracts from Gingko (*Gingko biloba*), grape seed (*Vitis vinifera*), hawthorn (*Crataegus* spp.), green tea (*Camellia sinensis*), milk thistle (*Silybum marianum*) and ginseng (*Panax ginseng*), among others, have been formulated as phytosomes intended to be used as innovative cosmetic carriers (Annex **2**).

1.5. Hypothesis and Objectives

Cancer and common infectious are two the top-five world death causes, according to World Health Organization. In the past century key advances in the treatment of such diseases were archived, although, few clinically relevant discoveries have been made in more recent years. The spreading of multidrug resistant bacteria and cancer types along with the lack of new antimicrobial and chemotherapeutic agents with a favorable risk-efficacy relationship, highlight the need for the development of new and alternative antimicrobial and anticancer drug classes.

Natural products have been one of the most reliable sources of drugs over the medicine history. Among those, the *Plectranthus* genus have been used for a wide range of ethnomedicinal purposes and is a good raw material since they easily grow in temperate zones. From these genus plants, several diterpenes have been obtained and characterized as antimicrobial or antitumoral agents.

The bioactivity of some natural products was however limited by intrinsic physic-chemical characteristics, namely unfavourable oil/water solubility relationship or susceptibility to rapid degradation in physiologic media, which limit their use as drug products. New drug delivery systems have been on focus in recent years and natural product focused delivery systems as phytosomes promise to overcome such limitations.

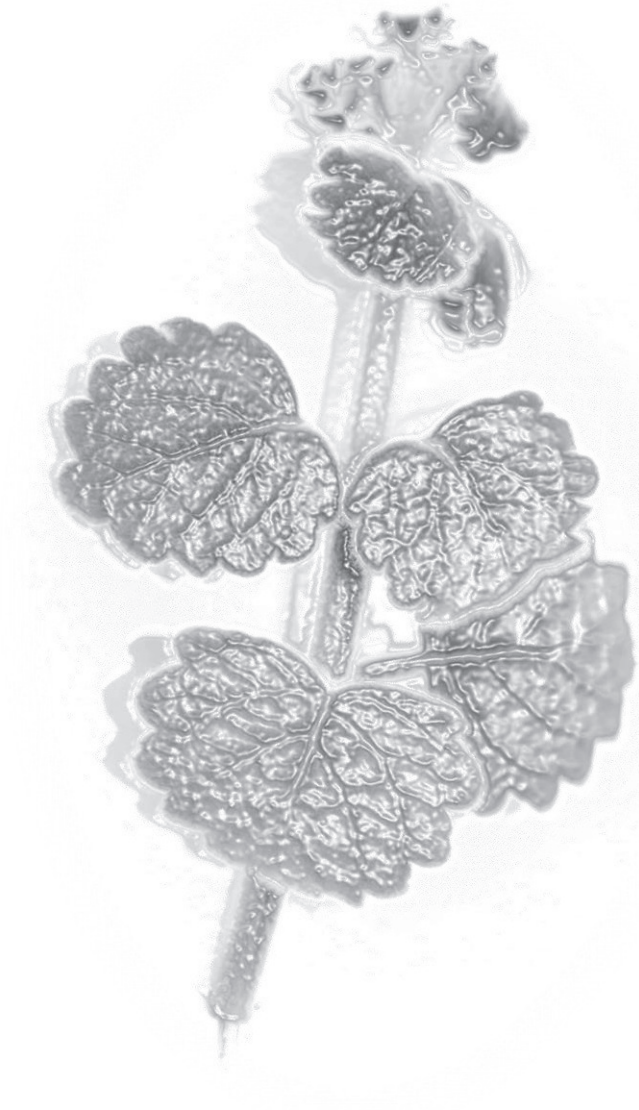
The overall background drives the establishment of some research hypothesis:

- Would the selected *Plectranthus* plants (*P. madagascariensis*, *P. neochilus* and *P. porcatus*) be a source of bioactive compounds?
- Will the extracts and isolated compounds from those plants have potent activity against some microbial pathogens and/or cancer cell lines?
- There will be an advantage of using phytophospholipid complexation (phytosome) to improve the revealed bioactivity?

Following the established thesis hypothesis, within this work some objectives were established:

- **Objective I**: The study of extracts from at least three *Plectranthus* species (*P. madagascariensis*, *P. neochilus* and *P. porcatus*) order to isolate or identify compounds that will be characterized by spectroscopic methods and physicochemical data. Such extracts and compounds would be screened for their antimicrobial and cytotoxic activities;
- **Objective II**: The evaluation of the *in vitro* cytotoxic activities of extracts and their isolated compounds and the establishment of some structure-activity relationships;
- **Objective III**: The more potent antimicrobial components obtained from *Plectranthus* plants will be incorporated into phytosomes in order to improve their bioactivity and/or delivery.

Chapter II



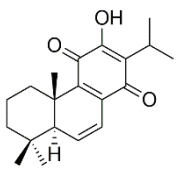
2. Screening of *Plectranthus* spp. for antimicrobial, antioxidant and cytotoxic activities

2.1. Introduction

The first objective of this project was the evaluation of the potential bioactivities, namely antimicrobial and antiproliferative (additionally the antioxidant activity was also measured), of plant extracts from the genus *Plectranthus*. The three species used in this study were selected based on their ethnomedicinal uses and the availability of plant material.

2.3.1. *Plectranthus madagascariensis*

P. madagascariensis (Pers.) Benth is a perennial aromatic herb with procumbent growth resulting in a dense, well-branched shrub up to 1 m high[32] (Figure **2.1**). This plant have been cultivated in Europe for its ornamental and aromatic applications and traditionally used for the treatment of respiratory conditions as cough and asthma, cutaneous wounds and scabies[27]. Previously, *P. madagascariensis* essential oil was characterized as containing high yields of an abietane diterpene, 6,7-dehydroroyleanone (**II.1**)[289]. This diterpenoid was formally studied as a non-toxic weak antimicrobial and potent antioxidant[290]. Kubínová et al. identified rosmarinic acid (**II.2**), 7β,6β-dihydroxyroyleanone (**II.3**), 7β-acetoxy-6β-hydroxyroyleanone (**II.4**) and coleon U-quinone (**II.5**) as the main components of this plant methanolic extract[72]. The acetone extract of *P. madagascariensis* was also studied as an antimicrobial and possesses insect antifeedant activity which was suspected to be related to the presence of coleon U in such extract[83].



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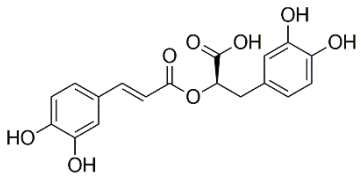

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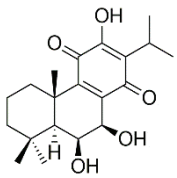

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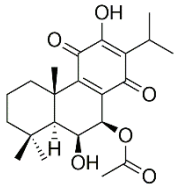

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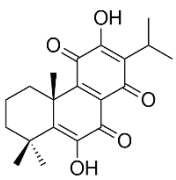
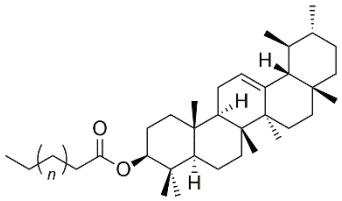


II.5

Figure 2.1. Illustration of the *Plectranthus madagascariensis* (adapted from van Jaarsveld and Thomas, 2006[32]) and some of the known compounds.

2.3.2. *Plectranthus neochilus*

P. neochilus Schltr. is an aromatic herb commonly called "boldo-rasteiro" in Brazil[291,292] (Figure **2.2**). The infusion of this plant has been used in traditional medicine for treating dyspepsia and hepatic insufficiency[291] or for the treatment of chills, cough and a runny or blocked nose[47]. Several studies characterized their essential oil composition and respective biologic activities from different growth locations. Essential oils from plants grown in Portugal[293] and Brazil[294] were similar, however substantially different from plants grown in South Africa[295]. Monoterpenes hydrocarbons were the more prevalent volatile constituents, but sesquiterpenes as β-caryophyllene and its oxidized form, caryophyllene oxide, were also present in high yields[295]. Antimicrobial[293,296], schistosomicidal[294,297],anti-fungal and cytotoxic[294] activities[294,298] were evaluated from *P. neochilus* essential oils. A low toxicity of *P. neochilus* ethanol extract of aerial parts was also described using the brine shrimp lethality assay[146]. The non-volatile composition of this specie remains mostly unclear but some triterpenes and a flavonoid were isolated from the hexane extract of the whole plant, namely a ramified alpha-amyrin (**II.6**), friedelin (**II.7**), a mixture of sitosterol and estigmasterol and the flavonoid cirsimaritin (**II.8**)[299]. Also the diterpene 1,6-di-O-acetyl-9-deoxyforskolin (**II.9**) was obtained from aerial parts of this plant[101].



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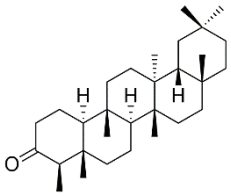

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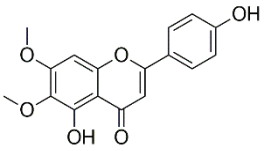

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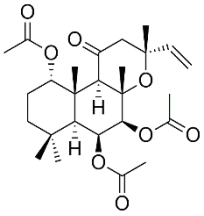


II.9

Figure 2.2. Illustration of the *Plectranthus neochilus* (adapted from Duarte and Lopes, 2007[291]) and main known compounds.

2.3.3. *Plectranthus porcatus*

P. porcatus Van Jaarsv. & P.J.D. Winter has been recently discovered in Leolo Moutains to Sekukuniland and Limpopo Province, South Africa[300]. Those areas present a subtropical climate, with hot summers and dry, sunny winters with light frost. *P. porcatus* is perennial soboliferous multi-stemmed shrub up to 1.5 m in diameter (Figure **2.3**). Its highly aromatic nature could have encourage its collection for medicinal uses[300].

This plant was briefly studied by our group in two occasions. From the acetone extract was obtained a new diterpene with cycloabietane substructure designed [13*S*,15*S*]-6β,7α,12α,19-tetrahydroxy-13β,16-cyclo-8-abietene-11,14-dione (**II.10**)[35]. The microwave aqueous extract was evaluated by HPLC and small yields of polyphenols caffeic (**II.11**) and rosmarinic acid (**II.2**) were detected, which contribute to the antioxidant activity of this extract[25].

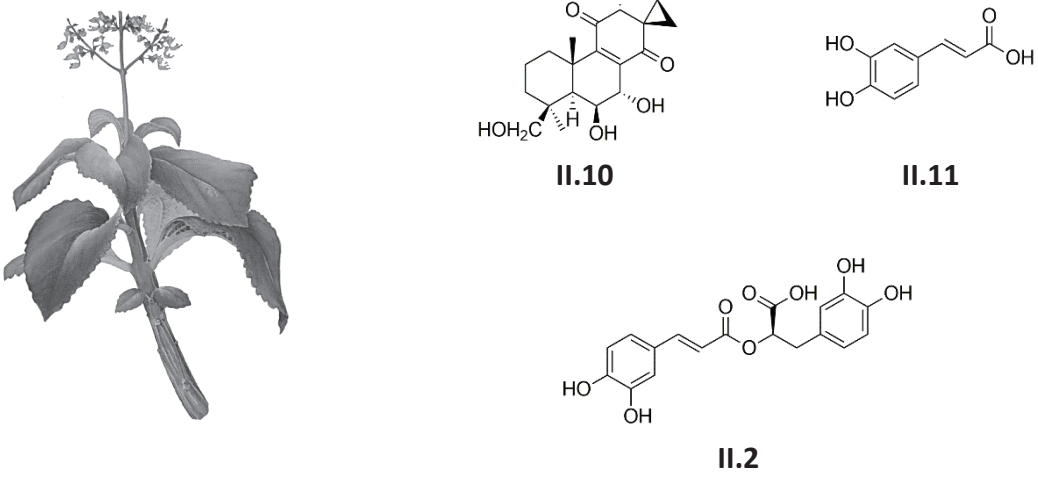


Figure 2.3. Illustration of the *Plectranthus porcatus* (adapted from van Jaarsveld and Thomas, 2006[32]) and main known compounds.

The initial screening was performed with different polarity extracts evaluated of their antimicrobial (bacteria and yeasts), antioxidant (DPPH reduction) and cytotoxic (*in vitro* cancer cell model) activities. Those extracts are also profiled using HPLC-DAD and some of the main compounds were identified by standard co-elution (Figure **2.4**).

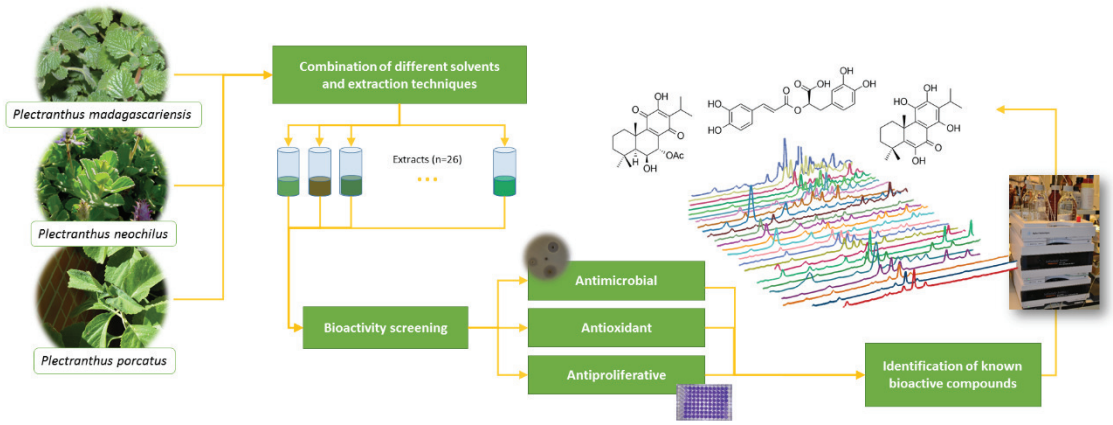


Figure 2.4. General scheme of the workflow described in chapter 2.

In this chapter, the biologic activities screening and initial phytochemical profiling were presented and discussed.

2.2. Experimental

2.2.1. Chemicals and equipment

All the laboratorial work of this chapter has been accomplished at CBIOS facilities (Universidade Lusófona de Humanidades e Tecnologias, Lisbon, Portugal).

2.2.1.1. Chemicals

Extraction and chromatography solvents, namely *n*-hexane, ethyl acetate, methanol and acetone were from analytic grade and purchased from Sigma-Aldrich (Steinheim, Germany). Reverse osmosis water was obtained from a Millipore system (Millipore, MA, USA) system with a resistivity of 18.2 Ω·cm at 25°C. Trichloroacetic acid was obtained from Panreac (Barcelona, Spain). HPLC reagents were from HPLC-grade (VWR Chemicals, Fontenay-sous-Bois, France) and were filtered through a 0.22 µm membrane (Vygon, Ecouen, France) before use. Dimethyl sulfoxide (DMSO) and absolute ethanol was supplied by Merck (Darmstadt, Germany), 2,2-diphenyl-1-picrylhydrazyl (DPPH), tetrazolium chloride and ascorbic acid were supplied by Sigma-Aldrich (Steinheim, Germany). TLC plates (Kieselgel 60 GF254 2mm 1.05554.0001) were purchased from Merck (Darmstadt, Germany) and filter paper nº5 was obtained from Whatman (Maidstone, United Kingdom). Standards of caffeic acid, chlorogenic acid, rosmarinic acid, rutin and naringin were supplied by Sigma-Aldrich (Steinheim, Germany). Authentic standards of coleon U and 7α-acetoxy-6β-hydroxyroyleanone were obtained and fully characterized by Gaspar-Marques[34]. Mueller-Hinton broth was supplied by Sigma-Aldrich (Steinheim, Germany) and and Sabouraud agar was supplied by Biokar Diagnostics (Allonne, France). Fetal bovine serum and penicillin/streptomycin for cell culture were supplied by Sigma-Aldrich (Steinheim, Germany) and Dulbecco's Modified Eagle's medium was supplied by Biowest (Nuaillé, France). Vancomycin, norfloxacin, amphotericin B and doxorubicin were supplied by Sigma-Aldrich (Steinheim, Germany).

2.2.1.2. Equipments

The HPLC-DAD system was composed by an Agilent Technologies 1200 Infinity Series with diode array module (Agilent Technologies, Santa Clara, CA, USA) using a reverse phase (RP-18) HPLC column LiChrospher® 100 (Merck, Darmstadt, Germany). Other equipments used were hotplate magnetic stirrer (MC-8, Bunsen, Madrid, Spain), lyophilizer (Freezone 2.5 L, Labconco, Kansas City, USA), rotary evaporator (IKA RV06-ML 1-B, Staufen, Germany) ultrasonic bath (Bandelin SONOREX RK 510H, Berlin, Germany), ultraviolet 254/366 nm lamp (CAMAG, Muttenz, Swithzerland) and weighting scales (KERN 770, KERN & Sohn GmbH, Balingen, Germany).

2.2.1.3. Plant material

Plectranthus madagascarensis Benth., *P. neochilus* Schltr. and *P. porcatus* Winter & Van Jaarsv were cultivated in Parque Botânico da Tapada da Ajuda (Instituto Superior Agrário, Lisbon, Portugal) from cuttings obtained from the Kirstenbosch National Botanical Garden (Cape Town, South Africa). Voucher specimens were deposited in Herbarium João de Carvalho e Vasconcellos (ISA) with numbers 841/2007 for *P. madagascariensis*, number 570/2008 for *P. neochilus* and 109/2008 for *P. porcatus*. The plant material used in this study was collected between 2007 and 2008, dried at room temperature and stored protected from light and humidity.

2.2.1.4. Microbial strains

The antimicrobial assays were performed in 7 Gram positive bacteria strains: *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis* (ATCC 29212), *Mycobacterium smegmatis* (ATCC 607), *Staphylococcus aureus* (ATCC 25923), methicillin-resistant *S. aureus* (MRSA) (CIP 106760) and *Staphylococcus epidermidis* (ATCC 12228); 3 Gram negative bacteria strains: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 9997) and *Pseudomonas aeruginosa* (ATCC 27853); and 2 yeast strains: *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisiae* (ATCC 9763). Microbial strains were originally obtained from American Type Culture Collection (ATCC) or form “Collection de l'Institut Pasteur" (CIP).

2.2.1.5. Cell lines

The human breast cancer MDA-MB-231 cell line (Cailleau et al., 1974[301]) was obtained from ATCC and maintained at CBIOS at Universidade Lusofóna de Humanidades e Tecnologias (Lisbon, Portugal) facilities.

2.2.1.6. Software editing

Chemical structures were drawn on ChemBioDraw Ultra 12.0.2.1076. ChemStation was used for HPLC-DAD controller and data exportation. Statistical analysis and graphic design were archived using GraphPad Prism 6.01 for Windows 10.

2.2.2. Extract preparation

Dried plant was grinded to small pieces and then pulverized. Plant material was extracted using different combinations of aqueous or organic solvents and extraction techniques. Each crude extract was separated from the remaining plant material by paper filtration (Whatman paper nº1, Sigma-Aldrich, Steinheim, Germany) and the organic solvents were evaporated in a rotary evaporator (IKA RV06-ML 1-B, Staufen, Germany) bellow 40 °C while aqueous extracts were freeze-dried as 1 mL aliquots (Freezone 2.5 L, Labconco, Kansas City, USA). The weight was determined for each extract and were stored at -20 °C until further usage.

2.2.2.1. Decoction (DEC)

Plant material (10 g) is boiled in 100 mL distilled water for 10 min.

2.2.2.2. Infusion (INF)

Plant material (10 g) is added to 100 mL of boiling distilled water and kept in contact for 10 min.

2.2.2.3. Microwave assisted extraction (MW)

Plant material (10 g) is added to 100 mL of distilled water and subject to continuous irradiation (2.45 GHz) for 2 min into a conventional microwave.

2.2.2.4. Maceration (ME)

Plant material (10 g) is added to 200 mL of organic solvent (acetone or methanol) and kept stirring (MC-8, Bunsen, Madrid, Spain) for 24 h.

2.2.2.5. Ultrasound assisted extraction (UAE)

Plant material (10 g) is added to 200 mL of organic solvent (acetone or methanol) and sonicated into an ultrasonic bath (Bandelin SONOREX RK 510H, Berlin, Germany) at 35 kHz for 2 h.

2.2.2.6. Supercritical fluid extraction (SCFE)

The supercritical fluid extraction was performed in an apparatus already described elsewhere[302]. The extraction was performed using 30g of ground plant for 3h at a temperature of 40ºC, pressure 23 MPa and $scCO_2$ flow rate of 0.3 $kg.h^{-1}$.

2.2.2.7. Re-extraction of SCFE remaining plant material (R-SFE)

Remaining plant material resulting from SCFE is recovered, air dried and added to 200 mL of acetone which is kept stirring (MC-8, Bunsen, Madrid, Spain) for 24 h.

2.2.3. Chemical characterization

Extracts were briefly analysed for their phytochemical profile using high performance liquid chromatography (HPLC) and by comparison of the UV spectra and retention time with pure authentic standards[36].

2.2.3.1. HPLC-DAD fingerprinting

Analysis were performed in an Agilent Technologies 1200 Infinity Series system equipped with a LiChrospher® 100, RP-18 (5 mm) column (Merck, Darmstadt, Germany) and ChemStation software**.** Extracts were analysed injecting 20 µL and using a gradient composed of solution A (methanol), solution B (acetonitrile) and solution D (0.3% w/v, trichloroacetic acid in water) as follows: 0 min, 15% A, 5% B and 80% D; 20 min, 80% A, 10% B and 10% D; 25 min, 80% A, 10% B and 10% D; and 28 min, 15% A, 5% B and 80% D. The flow rate was set at 1 mL/min. Detection was carried out between 200 and 600 nm with a diode array detector (DAD). Solvents were filtered and degassed using a 0.22 µm membrane filter previously to the analysis. All analyses were performed in triplicate.

2.2.3.2. Pure standards overlay

Standards in methanol at 1 mg/mL were run injecting 20 µL under the same analytic conditions used for extracts. The UV spectra of each pure compound was retrieved and compared to peaks in the extract with similar retention times. When overlay of UV spectra is verified, a co-elution of extract with pure compound is performed for confirmation.

2.2.4. Microbiology

The antimicrobial activity of each extract was determined in 7 Gram positive bacteria strains: *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis* (ATCC 29212), *Mycobacterium smegmatis* (ATCC 607), *Staphylococcus aureus* (ATCC 25923), methicillin-resistant *S. aureus* (MRSA) (CIP 106760) and *Staphylococcus epidermidis* (ATCC 12228); 3 Gram negative bacteria strains: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 9997) and *Pseudomonas aeruginosa* (ATCC 27853); and 2 yeast strains: *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisiae* (ATCC 9763).

2.2.4.1. Well diffusion assay

For screening of antimicrobial activity, a well diffusion assay was used. Briefly 100 µL of microorganism suspension, concentrated at 0.5 in McFarland scale, were inoculated in a petri dish containing Mueller-Hinton (bacteria) or Sabouraud agar (yeasts). Wells were dug in the agar using a sterile Pasteur pipette. Then 50 µL of sample (at 10 mg/mL), negative control (DMSO) or positive control (vancomycin for Gram positive bacteria; norfloxacin for Gram negative bacteria; amphotericin B for yeast) were added to each well. After incubation at 37ºC for 24 h the growth inhibition zones around the well were measured and the results expressed in millimetres (mm). Assays were performed in triplicate.

2.2.4.2. Minimum inhibitory concentrations

Positive samples from the well diffusion assay screening were subjected to a microplate broth microdilution method[303]. Briefly 100 µL of liquid Mueller-Hinton medium was distributed in each well of a 96-well plate. To the first well of each row was added 100 µL of extract, positive control or negative control solutions at a 1 mg/mL concentration and 1:2 serial dilutions were prepared (1.95–500 µg/mL range). Lastly, 10 µL of bacterial suspension were added to every well and plates were incubated at 37°C for 24h. Bacterial growth was measured with an absorbance microplate reader set to 620 nm. Assays were performed in triplicate.

2.2.4.3. Bioautography

The evaluated extracts were applied on a TLC silica plate (10x4 cm) and developed using a mixture of *n*-hexane:ethyl acetate (8:2; v/v). After elution, the TLC chromatogram was allowed to dry completely and placed on a petri dish. Then, a solution of Mueller-Hilton containing tetrazolium chloride (10 % w/v) and inoculated with *S. aureus* (ATCC 25923) was used to cover the TLC chromatogram. The plate was

incubated overnight at 37°C and the inhibition zones compared to non-incubated TLC chromatogram of the same extract. The assay was performed in quadruplicate.

2.2.5. Radical scavenging activity

The antioxidant activity was screened by the evaluation of DPPH radical scavenging ability[36]. Ten microliters of each plant extract (10 mg/mL) were mixed with 990 µL of DPPH solution (0.002% in ethanol). The resultant solution was incubated for 30 min at room temperature and then, the absorbance was measured at 517 nm against a corresponding blank. The antioxidant activity was calculated as:

$$AA\% = \frac{A_{DPPH} - A_{sample}}{A_{DPPH}}$$

Where *AA%* corresponds to the antioxidant activity in percentage, A_{DPPH} is the absorption verified for the DPPH solution (blank) and A_{sample} is the absorption verified for the sample (extract in DPPH solution). Each assay was carried in triplicate and ascorbic acid was used as positive control.

2.2.6. Cytotoxicity evaluation

The extracts cytotoxicity was assessed in the human breast cancer MDA-MB-231 cell line. Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin. The cultures were maintained at 37ºC, under a humidified atmosphere containing 5% CO_2.

Cell viability was evaluated using the crystal violet staining assay[304]. Briefly, a 96-well microplate was inoculated with approximate 6000 cells per well and incubated for 24 h. The samples were then added to obtain a final concentration of 15 µg/mL. After 48 h the medium was discarded and the cells washed with PBS, fixed with 96% ethanol and stained with crystal violet. The absorbance was measured at 595 nm and the sample cytotoxicity was expressed as the fraction of absorbance comparing with non-treated control cultures. At least two independent experiments were performed, and four replicate cultures were used in each independent experiment. Doxorubicin (5 µM) was used as positive control.

2.3. Results and discussion

2.3.1. Extract preparation

Extracts from *P. madagascariensis*, *P. neochilus* and *P. porcatus* were prepared using combinations of different solvents and extraction methodologies to obtain the preferential extraction of polar (aqueous extracts), median polar (acetone and methanol) and less polar constituents ($scCO_2$) in variable yields (Table **2.1**). The methanol extracts present a higher extraction yields in all studied plants, being maceration extraction able to retrieve generally higher extraction yields (Table **2.1**). This

Table 2.1. Extraction technique, solvents applied, weighted dry residue and calculated yield from studied *Plectranthus* species extracts. n/d – not determined.

Number	Plant	Solvent	Technique	Dry residue (mg)	Yield (mg/g)
E1	*P. madagascariensis*	Water	Infusion	0.23 ± 0.10	2.3
E2			Microwave	0.11 ± 0.04	1.1
E3			Decoction	0.22 ± 0.02	2.2
E4		Acetone	Ultrasound	0.151	1.51
E5			Maceration	0.377	3.77
E6		Methanol	Ultrasound	0.656	6.56
E7			Maceration	1.146	11.46
E8		$scCO_2$	SCFE	0.394	0.01
E9		Acetone	R-SCFE	0.885	0.03
E10	*P. neochilus*	Water	Infusion	0.26 ± 0.04	2.6
E11			Microwave	0.15 ± 0.04	1.5
E12			Decoction	0.22 ± 0.01	2.2
E13		Acetone	Ultrasound	0.180	1.80
E14			Maceration	0.125	1.25
E15		Methanol	Ultrasound	0.702	7.02
E16			Maceration	0.600	6.00
E17		$scCO_2$	SCFE	0.251	0.84
E18		Acetone	R-SCFE	0.417	1.39
E19	*P. porcatus*	Water	Infusion	n/d	n/d
E20			Microwave	n/d	n/d
E21		Acetone	Ultrasound	0.865	8.65
E22			Maceration	0.872	8.72
E23		Methanol	Ultrasound	1.566	15.66
E24			Maceration	2.237	22.37
E25		$scCO_2$	SCFE	0.191	0.64
E26		Acetone	R-SCFE	0.868	2.89

could be related to the ability of ethanoic solvents like methanol to disrupt the cellular wall with higher efficacy than other solvents. The higher extraction yield verified to maceration versus ultrasound extraction should be related to the longer extraction time employed.

2.3.2. Chemical characterization

The phytochemical composition of crude extracts was briefly analysed by HPLC-DAD, in order to identify some of the most prevalent chemicals present in their constitution

(Figure **2.5**). Insights about *P. madagascariensis* constituents have been previously published about its aquaeous[307], acetonic[83] and methanolic[72] extracts.

This analysis confirmed the presence of some known compounds as the polyphenols caffeic acid (**II.11**), chlorogenic acid (**II.12**) and rosmarinic acid (**II.2**) and the abietane diterpenes 7α-acetoxy,6β-hydroxyroyleanone (**II.13**) and coleon U (**II.14**). The flavonoids rutin (**II.15**) and naringenin (**II.16**) were identified for the first time in these plant extracts and was found in both organic and aqueous extracts (Figure **2.6** and Table **2.2**). The abietane diterpenes were more prevalent in organic extracts and should presumably be responsible for the higher antimicrobial and cytotoxic activities verified in those extracts.

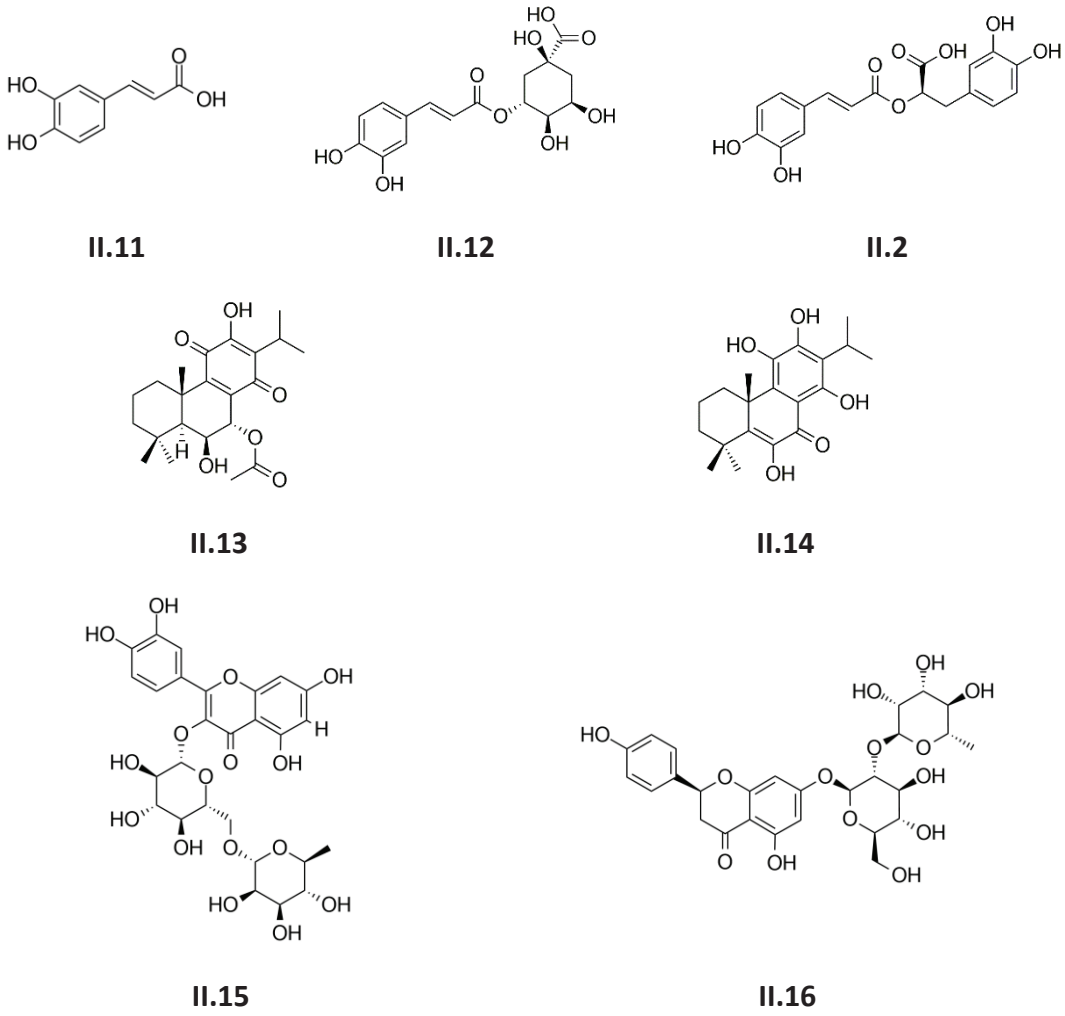


Figure 2.5. Chemical structure of identified phytochemicals from studied *Plectranthus* species extracts: Caffeic acid (II.11), chlorogenic acid (II.12), rosmarinic acid (II.2), 7α-acetoxy,6β-hydroxyroyleanone (II.13), coleon U (II.14) rutin (II.15) and naringenin (II.16).

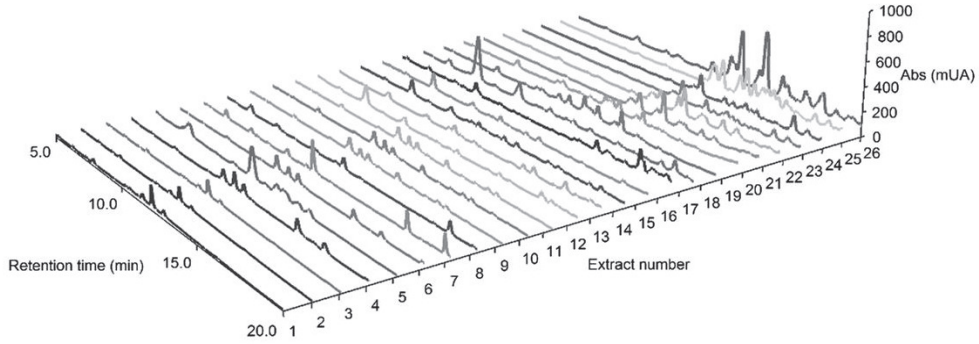


Figure 2.6. HPLC-DAD fingerprinting of each of each extract at 10 mg/mL (5-20 min section; λ=270 nm).

Table 2.2. Detection of analysed compounds in each *Plectranthus* extract and characteristic retention times for the developed HPLC method (within parenthesis, expressed in minutes).

Extract	**II.12** (6.54)	**II.11** (8.32)	**II.16** (10.14)	**II.15** (11.67)	**II.2** (12.43)	**II.13** (14.75)	**II.14** (15.44)
1	+	+	-	+	+	-	-
2	+	+	-	+	+	-	-
3	+	+	-	+	+	-	-
4	+	+	-	+	+	+	-
5	+	+	-	-	+	+	+
6	+	+	-	-	+	-	-
7	+	+	-	-	+	-	-
8	+	+	-	-	+	+	-
9	+	+	-	+	+	+	-
10	+	+	-	+	+	-	-
11	+	+	-	+	+	-	-
12	+	+	-	+	+	-	-
13	+	+	-	+	+	-	-
14	+	+	-	-	+	-	-
15	+	+	-	+	+	-	-
16	+	+	-	-	+	-	-
17	+	-	-	-	-	-	-
18	+	+	-	-	+	-	-
19	-	+	+	+	+	-	-
20	+	+	+	+	+	-	-
21	-	+	+	-	-	-	-
22	+	+	+	-	+	-	-
23	-	-	-	-	-	-	-
24	-	+	+	-	-	-	-
25	+	-	-	-	-	-	-
26	+	+	+	-	+	-	-

Chlorogenic acid (**II.12**), Caffeic acid (**II.11**), Naringin (**II.16**), Rutin (**II.15**), Rosmarinic acid (**II.2**), 7α-acetoxy-6β-hydroxyroyleanone (**II.13**), coleon U (**II.14**); +: detected compound; -: not detected.

To our knowledge there were no data of HPLC-DAD analysis of *P. neochilus*. The extracts from *P. neochilus* were rich in polyphenol compounds, namely chlorogenic acid, caffeic acid and rosmarinic acid but the flavonoid rutin was also detected in each non-acetonic extract. *P. porcatus* possess also the polyphenols chlorogenic acid, caffeic acid and rosmarinic acid in most of its extracts. The flavonoids naringin and rutin were present in aqueous extracts. Many of those compounds demonstrated both antimicrobial[33] and antioxidant[25] activities in previous studies and could be explain the activities verified for some of those plant extracts.

2.3.3. Antimicrobial activity

The initial screening of antimicrobial activity of *Plectranthus* species extracts was performed with the well diffusion assay. This simple procedure was used for the exclusion of inactive extracts from further antimicrobial studies. Extracts of *P. madagascariensis* and *P. neochilus* obtained using acetone and methanol were active in some Gram positive (*Bacillus subtilis* ATCC 6633, *Mycobacterium smegmatis* ATCC 607, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228) and Gram negative (*Klebsiella pneumonia* ATCC 9997 and *Pseudomonas aeruginosa* ATCC 27853) bacteria strains (Table **2.3** and Figure **2.7**). No inhibitory activity was verified for *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and the two yeasts (*Candida albicans* ATCC 10231 and *S. cerevisiae* ATCC 9763) by any of the extracts tested.

The extracts with relevant activity in well assay screening were subjected to the determination of the MIC by a microdilution procedure in the susceptible strains. Extracts from *P. madagascariensis* prepared using ultrasound (**E4**) and maceration (**E5**) in acetone and *P. neochilus* prepared using ultrasound (**E13**) in acetone showed potent activity against both Gram positive and Gram negative bacteria (MIC values ranging between 250-0.48 µg/mL) (Table **2.3**).

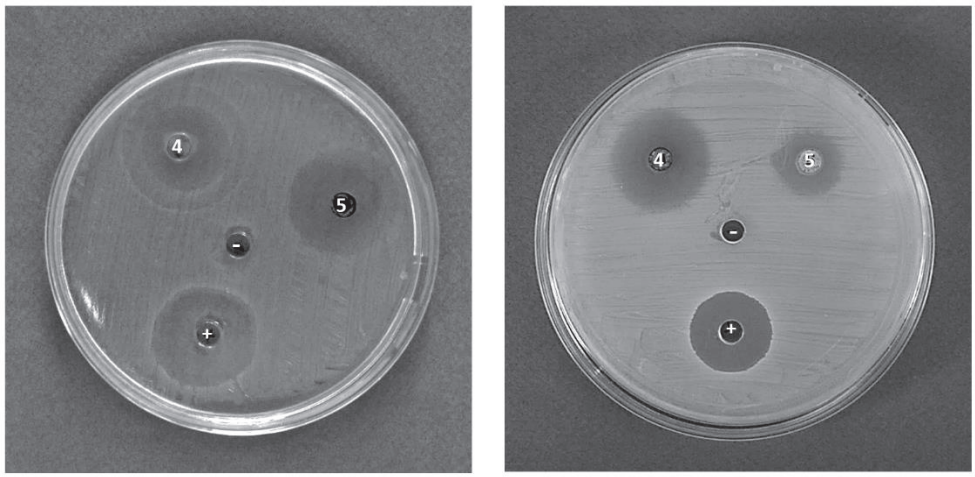


Figure 2.7. Well diffusion assay of extract E4 and E5 against *K. pneumonia* ATCC 9997 (left) and *S. aureus* ATCC 25923 (right). Positive control (+): vancomycin; negative control (-): DMSO.

Table 2.3. Well diffusion assay diameter (mm) of microbial growth inhibition in bioactive extracts. Extracts not presented were inactive against all tested strains. Positive controls: vancomycin (Gram positive bacteria); rifampicin (mycobacteria); norfloxacin (Gram negative bacteria); nystatin (yeasts).

Microbial Strains	Extract number								Control
	E4	E5	E6	E7	E13	E14	E15	E16	
Gram positive strains									
B. subtilis ATCC 6633	24	20	12	11	15	14	11	10	31
E. faecalis ATCC 29212	5	5	5	5	5	5	5	5	27
M. smegmatis ATCC 607	26	26	23	17	20	15	15	20	33
S. aureus *ATCC 25923*	24	20	7	8	8	8	8	8	24
S. epidermidis ATCC 12228	10	25	-	13	15	11	-	5	20
Gram negative strains									
E. coli ATCC 25922	5	5	5	5	5	5	5	5	33
K. pneumonia ATCC 9997	25	22	5	5	5	5	5	5	25
P. aeruginosa ATCC 27853	5	5	5	5	11	11	5	5	35
Yeast strains									
C. albicans ATCC 10231	5	5	5	5	5	5	5	5	17
S. cerevisiae ATCC 9763	5	5	5	5	5	5	5	5	22

A qualitative evaluation of the antimicrobial activity within the extract components was also performed by bioautography. This technique is based on the separation of the extract components by TLC chromatography and evaluate their individual ability to inhibit the growth of a specific microorganism inoculated above the chromatogram. The extracts **E4** and **E13** were selected for this assay as the most active for *P. madagascariensis* and *P. neochilus* respectively. Due to its lack of antimicrobial activity, *P. porcatus* extracts were not included in this study.

Table 2.4. Minimum inhibitory concentrations (µg/mL) determined for the extracts with higher activity in the screening. Positive controls were: vancomycin (Gram positive bacteria); rifampicin (mycobacteria); norfloxacin (Gram negative bacteria).

Microbial strains	Extract number			Control
	E4	E5	E13	
Gram positive				
B. subtilis ATCC 6633	3.91	62.5	125	<0.48
M. smegmatis ATCC 607	31.25	62.5	15.62	<0.48
S. aureus ATCC 25923	3.91	250	250	<7.81
S. aureus CIP 106760	1.95	16.25	31.25	<0.98
S. epidermidis ATCC 12228	7.81	62.5	62.5	<7.81
Gram negative				
K. pneumonia ATCC 9997	<0.48	3.91	0.98	31.25

The results showed clear inhibition zones as yellow areas against a red background (Figure **2.8**). Those areas correspond in both extracts to areas with a lower retention factor (Rf) which considering the solvent system selected (fairly apolar), led as to conclude that the compounds responsible for the anti-staphylococcal activity were, in both extracts, those with more polarity features.

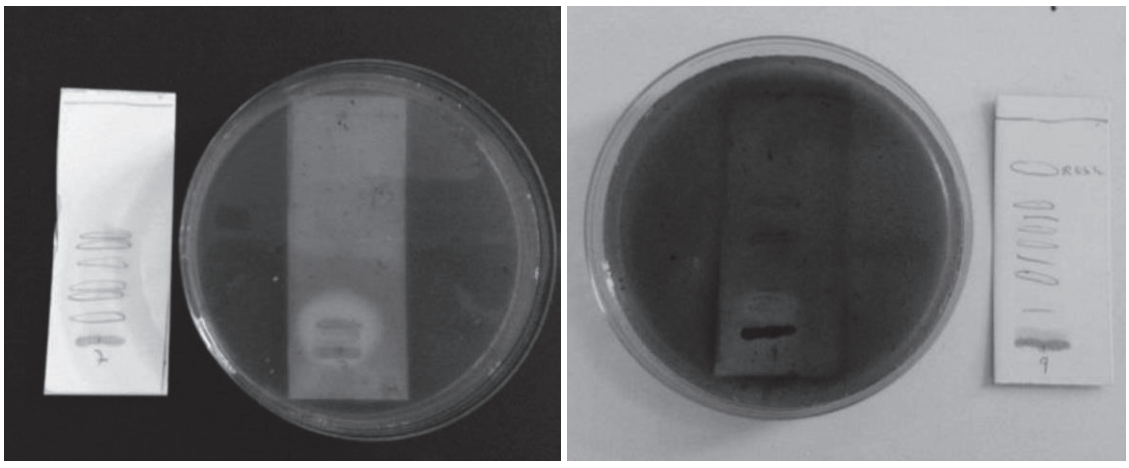


Figure 2.8. Representative digital images of the TLC chromatogram and bioautography of the extracts E4 and E13 from *P. madagascariensis* and *P. neochilus*, respectively.

2.3.4. Antioxidant activity

The antioxidant activity was determined based on *Plectranthus* extracts ability to scavenge the free radical DDPH. Most methanol extracts showed moderate or high antioxidant activity, being the most active, the extracts of *P. madagascariensis* (**E6** - 89.0%; **E7** - 64.8%), *P. neochilus* (**E15** - 64.9%; **E16** - 62.3%) and *P. porcatus* (**E23** - 60.8%;

E24 - 65.9%) (Figure **2.9**). The aqueous extracts of *P. neochilus* also showed relevant antioxidant values (62.5-68.9%) in contrast to aqueous extracts of *P. madagascariensis* (10.2-20.6%) and *P. porcatus* (3.2-17.3%).

The higher antioxidant values verified for methanolic extracts along with the known presence of polyphenols in the plants of the *Plectranthus* genus led us to speculate that those extracts possess higher amounts of such compounds in the extracts obtained using methanol. This is in accordance with previous studies of *Plectranthus* plants that revealed the extraction of high amounts of polyphenols by the use of methanol as solvent[69,72,83].

The antioxidant activity is present in almost every higher plant, due to the plant ability to biosynthetize a wide range of secondary metabolites able to attenuate the oxidative stress damage. However, the extent in which a potent antioxidant activity in vitro translates into a useful modulator of the oxidative status *in vivo* is yet to be determined, being dependent on the compound absorption and its physiologic half-life[305].

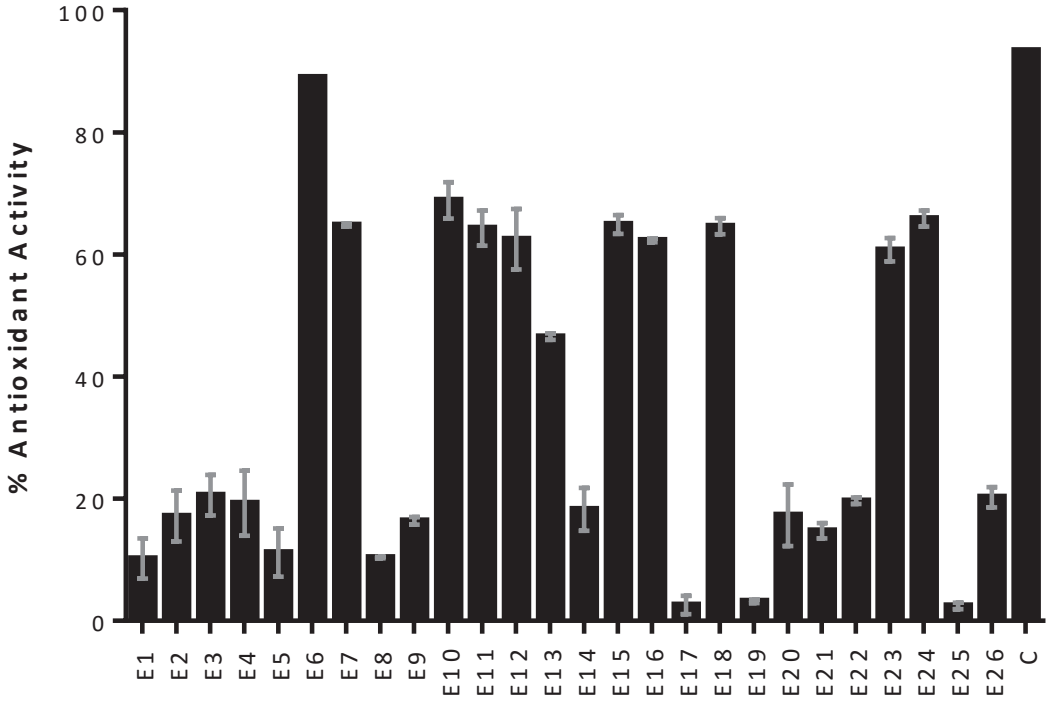


Figure 2.9. Antioxidant activity verified for each extract at 100 ng/mL using DPPH reduction method. Ascorbic acid in the same concentration was used as positive control.

2.3.5. Cytotoxicity screening

The extracts cytotoxicity to cancer cells was evaluated in the MDA-MB-231 breast cancer cells. This cell line was selected due to its availability, ease of manipulation and lack of studies of *Plectranthus* plants extracts in this cell line. When used at 15 µg/mL, all extracts showed low cytotoxicity in this cancer cell line (Figure **2.10**). The most cytotoxic extract was the one obtained by maceration in acetone of *P. madagascariensis* (**E5**), with a reduction in cell viability of 20.13%, followed by the extract obtained by re-extraction in acetone of *P. neochilus* plant material previously extracted by SCFE (11.44% decrease in cell viability). The inhibitory concentration at 50% of the curve (IC_{50}) was determined for the most active extract and a value of 64.52 µg/mL was found (Figure **2.11**). This extract can thus be considered moderately cytotoxic[306] and was selected for further studies (described in Chapter **3**).

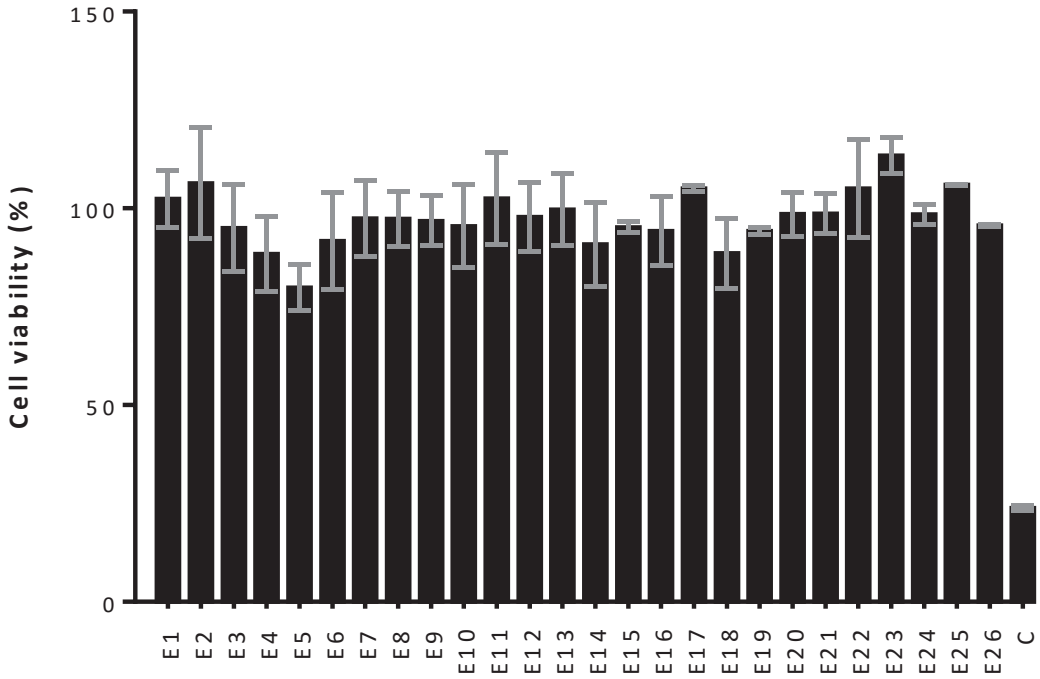


Figure 2.10. Cell viability of MDA-MB-231 cell exposed to 15 µg/mL of each extract for 48 h, assessed by the crystal violet staining assay. Positive control (C) was doxorubicin (5 µM). Results are expressed as mean ± SD from at least two independent experiments.

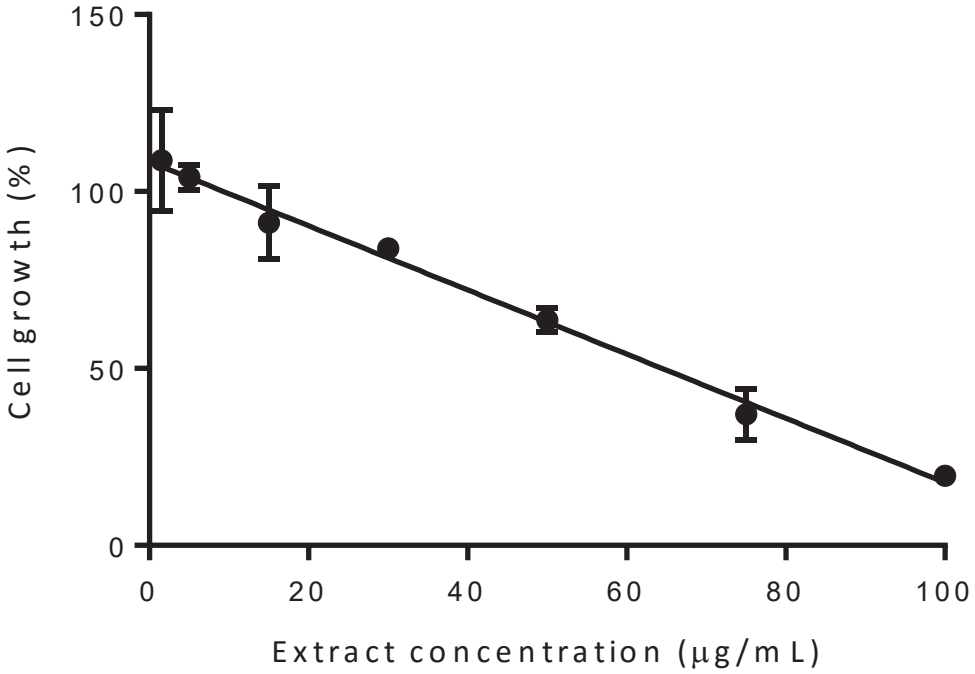


Figure 2.11. Concentration-response profile of *P. madagascariensis* acetonic maceration extract (**E5**) in terms of MDA-MB-231 cells viability (48 h incubation, crystal violet staining assay). Results are expressed as mean ± SD from at least two independent experiments.

2.4. Chapter conclusions

Ethnopharmacological studies on traditionally used medicinal plants led to indications of the presence of bioactive compounds on those plants extracts. In this study the preparation and screening of plant extracts from three *Plectranthus* species led to the identification of some extracts with potent antimicrobial, antioxidant and cytotoxic activities. The acetonic extract from *P. madagascariensis* produced by ultrasound assisted extraction (**E4**) showed an antimicrobial activity comparable to reference antibiotics against *S. aureus* strains (MIC < 3.91 µg/mL), including a MRSA strain (MIC < 0.98 µg/mL), but also against *B. subtilis*, *K. pneumonia* and *S. epidermidis*. This extract possess in its constitution the known abietane diterpenes 7α-acetoxy,6β-hydroxyroyleanone and coleon U which have been isolated from other *Plectranthus* species as antimicrobial compounds[34]. The presence of those compounds could explain the traditional use of this plant for the treatment of respiratory conditions related to infectious agents. The same plant methanolic ultrasound extract presented an antioxidant activity similar to the positive control ascorbic acid. As high yields of polyphenols are also present in this extract, the concomitant radical scavenging activities of both abietane diterpenes and polyphenols could explain this relevant antioxidant activity. The acetonic extract of *P. madagascariensis*, obtained by maceration (**E5**) showed the most relevant cytotoxic effects. This extract was also rich

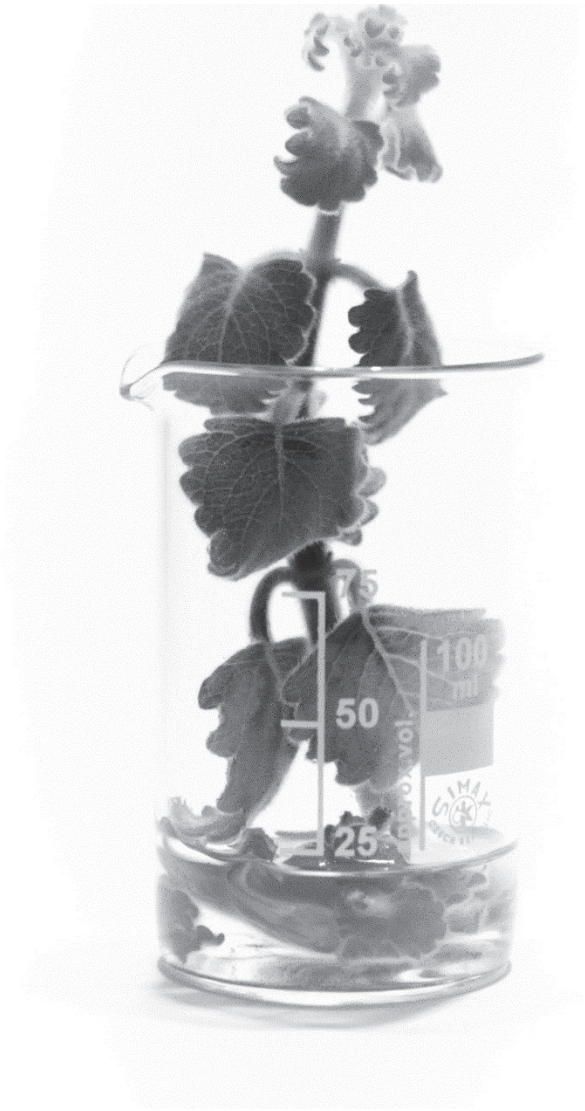
in abietane diterpenes, namely 7α-acetoxy,6β-hydroxyroyleanone and coleon U. Those compounds have been identified as antiproliferative agents against some cancer cell lines[40,82]. Also coleon U was been characterized as a PKCδ activator which could explain, at least partially, its cytotoxic activity[156].

Extracts from *P. neochilus* and *P. porcatus* were also rich in polyphenols and some flavonoids but did not show activities as potent as those from *P. madagascariensis*. However, the interesting antimicrobial activity verified for the acetonic extracts of *P. neochilus* obtained by ultrasound could explain the traditional use for the treatment of infection-related symptoms as cough or chills.

In order to better establish the compounds responsible for individual activities in the plant a bioassay guided fractionation of those plant extracts could help retrieve the most bioactive compounds in order to confirm if the identified compounds were the main responsible for the verified bioactivities in the crude extracts.

Overall, this study helped to characterize the bioactivities and phytochemical composition of three plants traditionally used for medicinal or aromatic purposes.

Chapter III



3. Cytotoxic components from *P. madagascariensis*

3.1. Introduction

The *Plectranthus* genus have been a source of cytotoxic compounds as some forskolin derivatives, coleon U and parvifloron D (detailed in the subsection **1.3.1.**). The presence of such compounds in *Plectranthus* plants, intensified our interest on the screening of this genus in the surge for new compounds with potential anticancer application.

The screening of 26 extracts from three *Plectranthus* plants, described in chapter **2**, supported the identification of organic extracts of *P. madagascariensis* as those with more potent bioactivities. The extract obtained by maceration acetone extraction was the most cytotoxic with an IC_{50} of 64.5 µg/mL in the MDA-MB-231 breast cancer cell line.

In this chapter, the chemical composition of the *P. madagascariensis* extracts was detailed using HPLC-DAD and complementary spectroscopic methodologies being the major compounds identified and quantified. The cytotoxic effects of the pure compounds were evaluated in breast, lung and colon cancer cell lines and some structure-activity relationships were disclosed. Those results were used to establish potential connections between the chemical composition of the plant extracts and their therapeutic uses in skin cancer[27].

3.2. Experimental

3.2.1. Chemicals and equipment

Most of the laboratorial work described in this chapter was been done at the CBIOS facilities (Universidade Lusófona de Humanidades e Tecnologias). Some procedures were performed in collaborating institutions, namely Faculdade de Farmácia da Universidade de Lisboa (RMN studies), Faculdade de Farmácia da Universidade do Porto (cytotoxicity assays) and Institute for Biological Research “Sinisa Stankovic” at University of Belgrade, Serbia (cytotoxicity assays).

3.2.1.1. Chemicals

Extraction and chromatography solvents, namely *n*-hexane, ethyl acetate, methanol and acetone were from analytic grade and purchased from Sigma-Aldrich (Steinheim, Germany). Reverse osmosis water was obtained from a Millipore system (Millipore, MA, USA) system with a resistivity of 18.2 Ω·cm at 25°C. Trichloroacetic acid was obtained from Panreac (Barcelona, Spain). HPLC reagents were from HPLC-grade (VWR Chemicals, Fontenay-sous-Bois, France) and were filtered through a 0.22 µm membrane (Vygon, Ecouen, France) before use. Dimethyl sulfoxide (DMSO) and absolute ethanol was supplied by Merck (Darmstadt, Germany). TLC plates (Kieselgel 60 GF254 2mm 1.05554.0001) were purchased from Merck (Darmstadt, Germany) and filter paper nº5 was obtained from Whatman (Maidstone, United Kingdom). Standard of rosmarinic acid was supplied by Sigma-Aldrich (Steinheim, Germany). Authentic standards of coleon U,

7α,6β-dihydroxyroyleanone and 7α-acetoxy-6β-hydroxyroyleanone were obtained and fully characterized by Gaspar-Marques[34]. Fetal bovine serum and penicillin/streptomycin for cell culture were supplied by Sigma-Aldrich (Steinheim, Germany) and Dulbecco's Modified Eagle's medium was supplied by Biowest (Nuaillé, France). Doxorubicin was supplied by Sigma-Aldrich (Steinheim, Germany).

3.2.1.2. Equipments

The HPLC-DAD system was composed by an Agilent Technologies 1200 Infinity Series with diode array module (Agilent Technologies, Santa Clara, CA, USA) using a reverse phase (RP-18) HPLC column LiChrospher® 100 (Merck, Darmstadt, Germany). Other equipments used were hotplate magnetic stirrer (MC-8, Bunsen, Madrid, Spain), lyophilizer (Freezone 2.5 L, Labconco, Kansas City, USA), rotary evaporator (IKA RV06-ML 1-B, Staufen, Germany) ultrasonic bath (Bandelin SONOREX RK 510H, Berlin, Germany), ultraviolet 254/366 nm lamp (CAMAG, Muttenz, Swithzerland) and weighting scales (KERN 770, KERN & Sohn GmbH, Balingen, Germany). Magnetic resonance experiments were performed on a INOVA-400 (Varian, Palo Alto, CA, USA).

3.2.1.3. Plant material

Plectranthus madagascarensis Benth., *P. neochilus* Schltr. and *P. porcatus* Winter & Van Jaarsv were cultivated in Parque Botânico da Tapada da Ajuda (Instituto Superior Agrário, Lisbon, Portugal) from cuttings obtained from the Kirstenbosch National Botanical Garden (Cape Town, South Africa). Voucher specimens were deposited in Herbarium João de Carvalho e Vasconcellos (ISA) with numbers 841/2007 for *P. madagascariensis*, number 570/2008 for *P. neochilus* and 109/2008 for *P. porcatus*. The plant material used in this study was collected between 2007 and 2008, dried at room temperature and stored protected from light and humidity.

3.2.1.4. Cell lines

The human breast cancer MDA-MB-231 cell line (Cailleau et al., 1974[301]) was obtained from ATCC and maintained at CBIOS at Universidade Lusofóna de Humanidades e Tecnologias (Lisbon, Portugal) facilities.

Human estrogen-dependent breast carcinoma (MCF-7), colorectal carcinoma (HCT116) and non-small cell lung carcinoma (NCI-H460) cells were obtained from ATCC and cultivated at Faculdade de Farmácia da Universidade do Porto.

MRC-5 normal human embryonal bronchial epithelial cells and NCI-H460 non-small cell lung carcinoma cell line were maintained at Institute for Biological Research "Sinisa Stankovic" at University of Belgrade (Belgrade, Serbia) and obtained from American Type Culture Collection (ATCC), Rockville, MD. From the original NCI-H460 was obtained the multidrug resistant non-small cell lung carcinoma cell line with P-glycoprotein overexpression by continuous treatment with stepwise increasing concentrations of doxorubicin (5-100 nM) for a period of 3 months in a procedure optimized by Pesic et al. 2006[308].

3.2.1.5. Software editing

Chemical structures were drawn on ChemBioDraw Ultra 12.0.2.1076. ChemStation was used for HPLC-DAD controller and data exportation. MestreNova was used for the RMN analysis and editing. Statistical analysis and graphic design were archived using GraphPad Prism 6.01 for Windows 10.

3.2.2. *P. madagascariensis* extraction

The *P. madagascariensis* extraction with acetone or methanol by UAE or ME, by SCFE with $scCO_2$ and R-SCFE with acetone, was performed in accordance to previously described in sub-section **2.2.1.**

3.2.3. HPLC-DAD profiling and identification of their major compounds

The extract profiling was performed using an Agilent Technologies 1200 Infinity Series LC System (Santa Clara, CA, USA) coupled to a diode array detector (DAD) and processed using the ChemStation Software. From each dried extract, a sample was prepared in methanol (methanolic extracts) or acetone (other extracts) at a concentration of 20 mg/mL. A 20 µL sample was injected to a LiChrospher® 100 RP-18 5 µm (4.0 x 250 mm) column (Merck, Darmstadt, Germany) and eluted in a gradient of methanol (A), acetonitrile (B) and 0.3% (w/v) trichloroacetic acid in ultrapure water (C) as follows: 0 min, 15% A, 5% B and 80% C; 20 min, 70% A, 30% B and 0% C; 25 min, 70% A, 30% B and 0% C; and 28 min, 15% A, 5% B and 80% C. The flow rate was established at 1 mL/min at room temperature. Solvents were filtered and degassed using a 0.22 µm membrane filter previously to the analysis. All analyses were performed in triplicate.

The major peaks from each extract sample were identified by comparing the retention time and UV-vis spectra overlay with commercial standards (rosmarinic acid, Sigma-Aldrich, Steinheim, Germany) or authentic standards previously obtained from *Plectranthus* spp., namely 7α,6β-dihydroxyroyleanone, 7α-acetoxy-6β-hydroxyroyleanone and coleon U (Gaspar-Marques et al. 2006)[34].

The calibration curves were constructed as a linear regression of the analyte concentration (mM) *versus* the average peak area. The limit of detection (LOD) and limit of quantification (LOQ) were determined to evaluate the sensitivity of the analysis corresponding to the concentrations of analyte that resulted in signal-to-noise ratios of 3 (LOD) and 10 (LOQ) following the guidelines from ICH Q2(R1) on validation of analytical procedures[309].

The LOD and LOQ were calculated as:

$$LOD = \frac{3.3\sigma}{S} \qquad LOQ = \frac{10\sigma}{S}$$

Where σ correspond to the standard deviation of the response and *S* correspond to the slope of the calibration curve. The slope S is estimated from the calibration curve of the analyte.

3.2.4. Isolation of 7α-formyloxy-6β-hydroxyroyleanone (III.3)

An ultrasound acetonic extract of *P. madagascariensis* was obtained by sonicating 100 g of plant material into 2000 mL of acetone for 2 h. This extract was treated with activated charcoal and immediately applied to a silica gel (Merck 60 gel 463934, Germany) chromatography column (30 g). Using a gradient of hexane and ethyl acetate, 10 fractions were obtained and grouped by their similarity in TLC (silica gel 60 F254 plate 0.2 mm thicknesses, Merck, Germany; using as eluent system hexane:ethyl acetate, 7:3). Compound **III.3** was purified from the fraction eluted with hexane:ethyl acetate (95:5) by repeated elution (eluent system: hexane/ethyl acetate, 75:25 v/v) using a preparative chromatography (Merck 60 F254 20x20 cm 1.05715) followed by crystallization (methanol).

3.2.5. Spectroscopic structure elucidation (III.3)

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian INOVA-400 spectrometer equipped with a 5 mm inverse detection z-gradient probe. The ^{1}H and ^{13}C NMR spectra (respectively at 400 and 100 MHz) were measured at room temperature (22-23 °C) using $CDCl_3$ as solvent. The ^{1}H and ^{13}C NMR chemical shifts are reported with respect either to the residual $CHCl_3$ signal (δ7.25 and δ77.00, respectively). One-dimensional ^{1}H and ^{13}C NMR spectra were acquired under standard conditions. The pulse programs of the COSY, HSQC and HMBC experiments were taken from the Varian software library. Homonuclear two-dimensional spectra (COSY) and inverse proton-detected heteronuclear two-dimensional spectra (HSQC) were acquired in the phase-sensitive mode and HMBC spectra were acquired in the absolute value mode.

3.2.6. Cytotoxicity assays

The cytotoxicity of the extracts and pure compounds was assessed using human breast cancer MDA-MB-231 cell line. The cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin. The cell viability was evaluated using the crystal violet staining assay[304,310]. Briefly, cells were inoculated on a 96-well microplate and incubated for 24 h. Cells were exposed to the testing solutions for 48 h, and afterwards the medium was discarded and the cells were washed with phosphate saline buffer, fixed with 96% ethanol and stained with crystal violet. The absorbance was read at 595 nm and cell viability was calculated as a fraction of the absorbance presented by non-treated control cultures. At least two independent experiments were performed, and four replicate cultures were used in each independent experiment.

The cytotoxicity of pure compounds (**III.1**-**III.5**) on HCT116, MCF-7 and H460 cells lines was assessed after 48 h treatment using the sulphorhodamine B assay by the procedure

supervised by Saraiva[311]. Briefly, cells were cultured in RPMI-1640 with ultraglutamine medium from Lonza, supplemented with 10% fetal bovine serum from Gibco and maintained in a humidified incubator at 37 C with 5% CO2 in air. For the evaluation of cytotoxic effects of tested compounds, cells were plated in 96-well plates at a final density of 5.0 x 10^3 cells/well and incubated for 24 h. Then, cells were exposed to serial dilutions of each compound (from 1.85 to 150 µM), being the effect of the compounds analysed after 48h of incubation, using the sulforhodamine B (SRB) assay. Briefly, following fixation with 10% trichloroacetic acid from Scharlau, plates were stained with 0.4% SRB and washed with 1% acetic acid. The bound dye was then solubilized with 10 mM Tris Base and the absorbance was measured at 510 nm in a microplate reader (Biotek Instruments Inc., Synergy MX, USA).

The cytotoxicity on NCI-H460 cells and its multidrug resistant selected strain, NCI-H460/R[308], along with the normal cell line MCR-5 was evaluated in the concentration range of 2.5-50 µM by (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) reduction assay by the procedure described by Pesic et al., 2013[308,312]. Briefly, the cells were incubated with compounds at 8000 cells/cm^2 for NCI-H460, 16,000 cells/cm^2 for NCI- H460/R in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 4.5 g/L glucose, 10,000 U/mL penicillin, 10 mg/mL streptomycin, 25 mg/mL amphotericin B solution at 37ºC in a humidified 5% CO_2 atmosphere for 72 h. Afterwards, 100 ml of MTT solution (1 mg/mL) was added to each well and plates were incubated at 37ºC for 4 h. Formazan product was dissolved in 200 ml of DMSO and the absorbance of obtained dye was measured at 540 nm using an automatic microplate reader (LKB 5060–006 Micro Plate Reader, Vienna, Austria).

3.3. Results and discussion

3.3.1. *P. madagascariensis* extraction

The extraction yield was evaluated for all prepared extracts (mg of extract/g of dried plant) considering the combination of several extraction processes and solvents (Table **3.1**). The extracts with higher extractive yield were those obtained using methanol as solvent (**E6**, **E7**). This can be explained by the ability of ethanolic solvents to disrupt biologic membranes, favouring the diffusion of secondary metabolites to the bulk extract. Moreover, considering the relative polarity of the extraction solvents (dielectric constant, ε), it could be verified a trend for the more polar solvents to archive higher extraction yields ($\varepsilon_{methanol} > \varepsilon_{acetone} >> \varepsilon_{scCO_2}$). The SCFE method using $scCO_2$ (**E8**) was the least efficient extraction technique with the lower yield. Due to this low extraction efficiency, a re-extraction of the remaining plant material (R-SCFE) was performed in order to extract the remaining non-extracted secondary metabolites. The reminiscent plant material of the SCFE was macerated in acetone for 3 weeks, resulting in a higher extraction yield when compared to SFE (Table **3.1**).

Table 3.1. Total extraction yields of selected extracts and substance quantification in those extracts.

Extract	Solvent	Technique	Extraction yield (mg/g)	Substance yield in extract (mg/g)			
				III.1	III.2	III.3	III.4	III.5
E4	Acetone	UAE	1.51	29.85	4.62	1.64	1.04	15.52
E5	Acetone	ME	1.45	17.49	3.19	6.74	1.21	5.77
E6	Methanol	UAE	6.56	4.60	4.20	0.81	0.77	t
E7	Methanol	ME	11.96	26.44	1.05	0.24	t	t
E8	$scCO_2$	SCFE	1.31	17.79	4.98	0.20	0.84	n/d
E9	Acetone	R-SCFE	2.95	50.52	0.33	0.17	n/d	n/d

ME – Maceration extraction; UAE – Ultrasound assisted extraction; SCFE – Supercritical fluid extraction; R-SCFE – Re-extraction of SCFE remaining plant material. t – traces; n/d – not detected.

3.3.2. HPLC-DAD extract profiling and identification of major compounds

Previous phytochemistry studies on the *Plectranthus* genus revealed the presence of polyphenols and diterpenes as the most frequent secondary metabolites (as reviewed previously in section **1.2.2**). These compounds have characteristic absortion parterns in the UV spectra zone due to the presence of conjugated carbonyl groups (270 nm), aromatic rings (280 nm) and phenolic groups (330 nm). For this reason measurements at 270, 280 and 330 but also the reference 254 nm were selected to monitoring the high peak resolution of extracts profile chromatograms (Figure **3.1**).

The peak eluted at 10.47 min was presented in all extracts and exhibited a UV spectrum with a characteristic maximum absorption (λ_{max}) at 330 nm and at 265 nm. This could be associated with a phenolic compound with aromatic rings[36,307]. After co-elution with rosmarinic acid (**III.1**) an overlay of both UV spectra and retention time was verified being this peak positively identified as rosmarinic acid (Figure **3.2**). This polyphenol has been found in numerous *Plectranthus* species and was previously identified in P. *madagascariensis*[72,307].

The peaks obtained at the average retention times of 17.80, 19.40 and 19.80 min possess the typical royleanone-type abietane UV spectra with λ_{max} at 272 nm and a secondary broad λ_{max} between 300 and 500 nm[56]. This is in accordance to other phytochemical studies of *Plectranthus* species[55]. The co-elution of extracts with authentic samples of 7α,6β-dihydroxyroyleanone (**III.2**) and 7α-acetoxy-6β-hydroxyroyleanone (**III.4**) demonstrated the overlay of the UV spectra at 17.80 and 19.80 min (Figure **3.2**). This allowed the identification of the peak at 17.80 and 19.80 min as corresponding to compound **III.2** and **III.4**, respectively (Figure **3.3**).

A major peak eluted at 21.08 min presented a UV spectrum with λ_{max} at 259, 283, 331 and 383 nm, which supported the presence of a conjugated carbonyl with an aromatic ring (Figure **3.2**). These spectral characteristics were comparable to those verified for coleon U[83], a diterpene often found in *Plectranthus* species. The co-elution of the extracts with authentic sample of coleon U demonstrated an overlay of the UV spectra (Figure **3.2**). This led to the attribution of the 21.13 peak to coleon U (**III.5**) (Figure **3.3**).

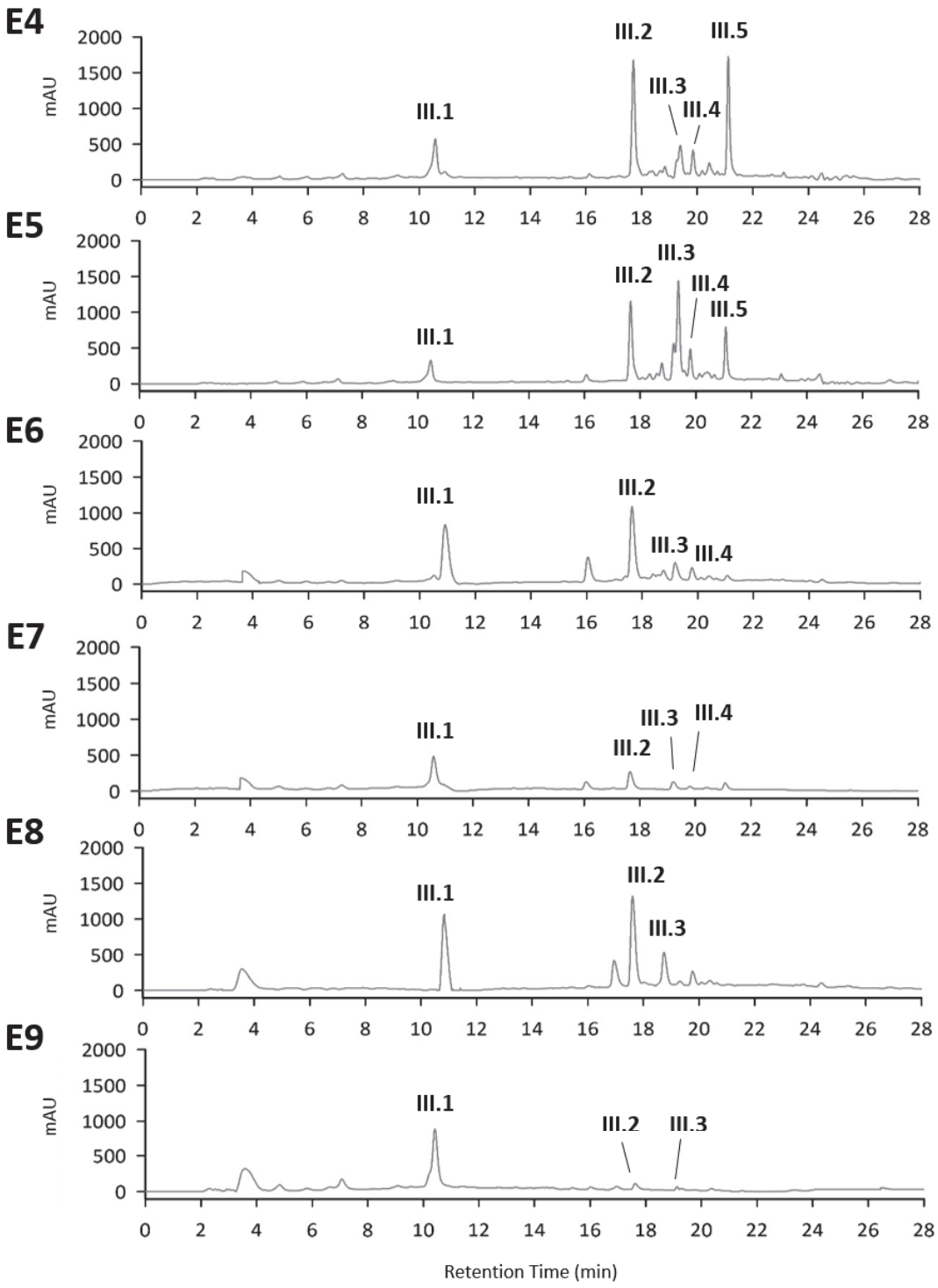


Figure 3.1. HPLC representative chromatogram from *P. madagascariensis* extracts E4-E9 (270 nm).

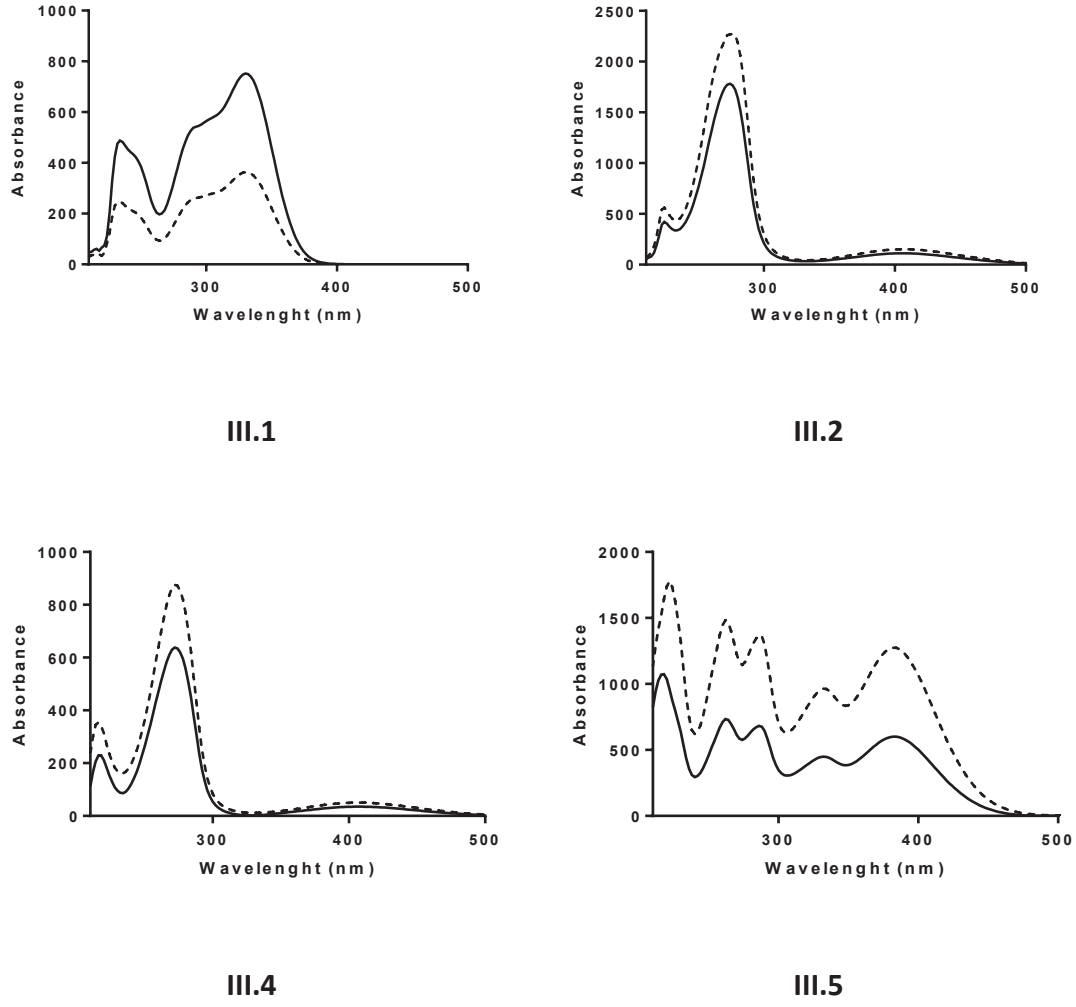


Figure 3.2. Overlay of sample extracts and a standard solution of rosmarinic acid (III.1), 6α,7β-dihydroxyroyleanone (III.2), 7α-acetoxy-6β-hydroxyroyleanone (III.4) and coleon U (III.5). Full line corresponds to 1 mg/mL of each standard and discontinued line to the corresponding component in the sample extracts.

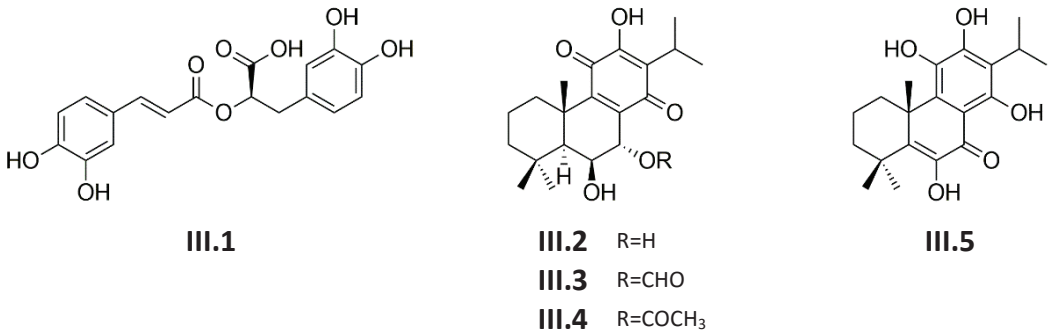


Figure 3.3. Chemical structure of the compounds III.1-III.5 quantified from *P. madagascariensis* organic solvent extracts: rosmarinic acid (III.1); 7α,6β-dihydroxyroyleanone (III.2); 7α-formyloxy-6β-hydroxyroyleanone (III.3); 7α-acetoxy-6β-hydroxyroyleanone (III.4); coleon U (III.5).

The latter compound was reported to have some intrinsic instability, being easily converted to the oxidized form coleon U-quinone (**III.6**)[56,83]. This degradation product was also detected in some *P. madagascariensis* extracts as a trace at the retention time of 19.19 min. The periodic analysis of an authentic sample of **III.5** demonstrated the gradual increase of the peak at retention time 19.19 min with respective reduction of the peak at 21.08 min, which represents a gradual conversion of the compound **III.5** into the oxidized form **III.6**. (Figure **3.4**).

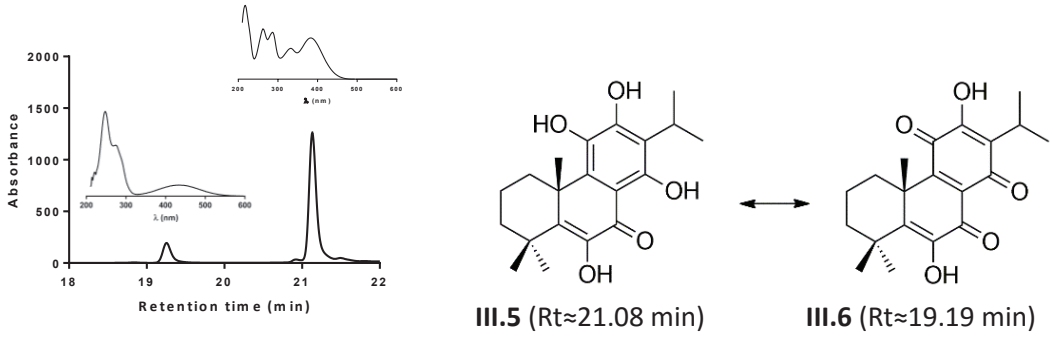


Figure 3.4. Proposed mechanism of coleon U (III.5) degradation on coleon U-quinone (III.6) and evidence of decomposition over time, followed by HPLC. Rt = retention time.

Kubínová et al. was previously studied the methanolic extract of *P. madagascariensis* which resulted in the identification of rosmarinic acid, 7β,6β-dihydroxyroyleanone, 7β-acetoxy-6β-hydroxyroyleanone and coleon U-quinone as the major components of that extract[72]. However, the full spectroscopic elucidation of those compounds was not accomplished by those authors.

3.3.3. Isolation of 7α-formyloxy-6β-hydroxyroyleanone (III.3)

The ultrasound acetone extract of *P. madagascariensis* yielded 480 mg (0.48% (w/w)). The extract was subjected to active charcoal to eliminate the high content in chlorophylls plant pigments and the procedure was monitored by TLC.

Compound **III.3** was purified from the fraction eluted with hexane:ethyl acetate (95:5) by repeated elution in a preparative chromatography yielding 6.1 mg (1.27% (w/w)) of yellow needles after crystallization. The purified compound **III.3** showed a retention time and UV-spectra in HPLC-DAD that overlay the peak detected in extracts at 19.40 min.

3.3.4. Spectroscopic characterization of III.3

The ^{1}H NMR spectra of **III.3** was very similar to that of **III.4**[37]. The main differences were the absence of the signal at δ 2.02 (s, 3H) corresponding to the methyl protons of

the acetoxyl group at C7 in **III.4** and the presence of a distinct doublet at δ 8.05 (*J* = 1.2 Hz) which might correspond to an acidic proton from a secondary formyloxyl group[31] (Table **3.2**) . This observation suggested the substitution of the 7α-acetoxyl group of **III.4** by a 7α-formyloxyl in **III.3,** which was confirmed by the literature[31] and extensive NMR studies (^{1}H and ^{13}C NMR, COSY, HMBC and HMQC) (Table **3.2**). The compound **III.3** was identified as 7α-formyloxy-6β-hydroxyroyleanone, which was previously isolated from *P. hadiensis*[80] and *P. myrianthus*[56]. This was, to our knowledge, the first isolation of this compound from *P. madagascariensis* and the first fully spectroscopic characterization of this compound (^{1}H and ^{13}C NMR).

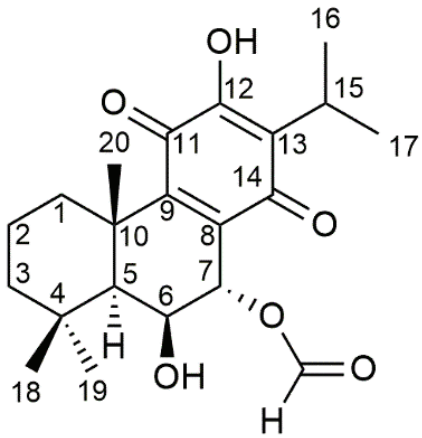


Figure 3.5. Structure of 7α-formyloxy-6β-hydroxyroyleanone (III.3).

Table 3.2. NMR data of compound III.3 in $CDCl_3$ (400 MHz).

C	^{13}C (ppm)	^{1}H (*m*, J) (ppm)	COSY (^{1}H x ^{1}H)	HMBC (^{1}H x ^{13}C)
1	38.49	1.19 (*s*) 2.64 (*d,* 12.9)	2.64 (1β) 1.19 (1α)	
2	19.09	1.50 (*m*)* 1.85 (*d*, 4.5)	1.19 (1α)	
3	42.45	1.20 (*m*)* 1.50 (*m*)*		
4	33.83			
5	49.80	1.38 (*s*)		21.71 (10)
6α	67.25	4.37 (*m*)	5.80 (7Hβ)	
7β	68.42	5.80 (*m*)	4.37 (6Hα)	
8	136.45			
9	150.38			
10	38.79			
11	183.34			
13	124.35			
15	24.34	3.17 (*qi*, 14.1; 7.1)	1.21 (16Me); 1.24 (17Me)	124.35 (13)
16	33.85	1.24 (*d*, 7.1)	3.17 (15H)	
17	20.01	1.21 (*d*, 7.1)	3.17 (15H)	124.35 (13)
18	33.57	0.96 (*s*)		24.06 (19); 42.38 (3); 49.80 (5)
19	23.97	1.25 (*s*)		33.57 (18); 42.38 (3); 49.80 (5)
20	21.71	1.62 (*s*)		38.49 (1); 49.8 (5)
6-OH	33.83	2.31 (*t*, 7.5)		
12-OH	151.13	7.20 (*m*, 3.1)		
CHO	159.64	8.04 (*d*, 1.2)		

3.3.5. HPLC-DAD quantification of major compounds

The quantification of the major compounds in *P. madagascariensis* extracts was based on the validated calibration curves for the main polyphenol rosmarinic acid (**III.1**) and the main abietane diterpenes 7α,6β-dihydroxyroyleanone (**III.2**), 7α-formyloxy-6β-hydroxyroyleanone (**III.3**), 7α-acetoxy-6β-hydroxyroyleanone (**III.4**) and coleon U (**III.5**) (Table **3.3**). Linear responses and high sensitivity were obtained for all compounds.

Table 3.3. Linear regression analysis parameters for the proposed HPLC-DAD method

Analyte	λ (nm)[a]	RT (min)[b]	C_{range} (mM)	Regression equation	R^2	LOD	LOQ
III.1	330	10.47 ± 0.065	0.1 – 1.5	y = 9733.1x - 378.8	0.999	0.001	0.003
III.2	280	17.80 ± 0.022	0.02 – 0.5	y = 85490,4x - 155,2	0.998	0.001	0.002
III.3	280	19.40 ± 0.014	0.04 – 0.26	y = 40748x - 245.93	0.999	0.001	0.004
III.4	280	19.80 ± 0.020	0.02 – 0.27	y = 65383x – 204	0.997	0.003	0.009
III.5	330	21.13 ± 0.004	0.02 – 0.12	y = 82084x + 818.47	0.987	n/d	n/d

[a] Wavelength used for the calibration curve; [b] Retention time of the compound as average of 12 samples ± standard deviation; n/d – not determined.

Rosmarinic acid (**III.1**) was the secondary metabolite present in higher yields in the extracts A-D and F with yields ranging from 4.60 to 50.52 mg/g (Table **3.1**). This finding is in agreement with the described abundance of this polyphenol in other *Plectranthus* species[36]. High yields of royleanone-type diterpenes were found in all extracts, but particulary in the acetonic extracts (Table **3.1**). Coleon U (**III.5**) was the diterpene quantified in higher yields (5.77-15.52 mg/g), followed by 7α-formyloxy-6β-hydroxyroyleanone (**III.3**) (6.74-0.17 mg/g), 7α,6β-dihydroxyroyleanone (**III.2**) (0.33-4.98 mg/g) and lastly 7α-acetoxy-6β-hydroxyroyleanone (**III.4**) (0.11-1.21 mg/g).

The cytotoxic effects of the *P. madagascariensis* extracts have been previouly evaluated in the MDA-MB-231 cancer cells and those results presented in chapter **2**. The quantification of the major compounds adds new information that could help explain the differences in the cytotoxicity of those extracts. The higher content in diterpenoids seems to be related to the higher cytotoxic effect as verified in by the extract **E5** which had the higher combinatory yield of coleon U and royleanone-type abietane diterpenes of all the extracts prepared (Table **3.1**). The royleanone-type compounds are known to have cytotoxic activities namely against breast cancer cell lines[39,40]. Aditionally, coleon U (**III.5**) was described in literature as a potent cytotoxic, active against breast, leukemia and melanoma cancer cell lines[39,40,82,155]. Those *P. madagascariensis* components would contribute for the cytotoxicity verified in this plant extracts. However, the extract **E4**, with the highest yields of compounds **III.4** and **III.5** do not correspond to the extract with the highest cytotoxicity. This can be explained by synergistic effects between the extract elements. The most cvytotoxic extract (**E5**) present a lower yield of rosmarinic acid

compared to other extracts. This compound is known to be a potent antioxidant[58], and some studies related the cytotoxic effect of abietane diterpenes possessing quinone methide moieties to the induction of radicalar reactions[112,113]. Those factors could let us speculate a potential antagonistic effect between antioxidant polyphenols and quinone methide diterpenes.

3.3.6. Cytotoxicity of the pure compounds (III.1-III.5)

The cytotoxicity of compounds **III.1**-**III.5** was tested in breast cancer (MDA-MB-231 and MCF-7), colon cancer (HCT116), non-small cell lung cancer (NCI-H460) and normal lung bronchial (MCR-5) cell lines (Table **3.4**). Those results showed growth inhibition effects in most cancer cell lines by all the abietane diterpenes. It is especially relevant the high selectivity of the royleanones **III.2** and **III.4** based on the comparison of GI_{50} of a cancer (NCI-H460) and normal lung cell line (MCR-5). Also, these two royleanones showed similar growth inhibition of the NCI-H460 cancer cell line and of its multidrug resistant variant (NCI-H460/R) which overexpress the multidrug resistance protein 1 (MDR1) (also known as P-glycoprotein)[308]. This is a strong indication that compounds **III.2** and **III.4** were not substrates for such efflux pumps.

The growth of MDA-MB-231 cancer cells was not particularly affected by the *P. madagascariensis* abietane diterpenes. This cell line is a highly metastatic triple negative breast cancer cell line, not displaying estrogenic receptors (ER), progesterone receptors (PR) or human epidermal growth factor receptor 2 (HER2), and thus, being clinically difficult to target[313]. It was known that the ER negative cells have a higher expression of PKC classic isoforms when compared to ER positive cell lines[314,315]. The upregulation of the classic isoform PKCα promotes the invasiveness and metastasis formation[315–317] along with increased drug resistance[318] in breast cancers. On the other hand, the PKCδ activation supports both prosurvival[319] and proapoptotic functions in breast cancer cells[320,321]. Also, some abietane diterpenes (coleon U[156] and a benzyloxy derivative of 7α-acetoxy,6β-hydroxyroyleanone[322]) have demonstrated to exert proapoptotic effects by the specific activation of the PKCδ. In this cell line, where its verified an overexpression of classic PKC isoforms, in detriment to new PKC isoforms, the preferential mechanism of apoptosis induction by coleon U (and eventually of other abietanes) by activation of PKCδ should be less effective, which justifies a lower growth inhibition of this cell line by such compounds.

There have been demonstrated some relationship between the PKC overexpression and the drug resistance by MDR1. Following the treatment with the PKC activator TPA, a cellular increase of the MDR1 expression was verified, which was suppressed by the use of a PKC inhibitor (staurosporine)[323]. Also, the use of a PKC inhibitor, bryostatin 1, potentiate the cytotoxic effects of anticancer drugs transported by efflux pumps as vincristine, by the reduction of MDR1 expression[324]. Those findings could be especially relevant in the case of abietane diterpenes not transported by MDR1, in which the PKC activation and the secondary MDR1 overexpression shouldn't increase the cell resistance to those compounds.

Table 3.4. Growth inhibitory effects of the *P. madagascariensis* major compounds in different cell lines.

	Cell line / GI_{50} (μM)						
	MDA-MB-231	MCF-7	HCT116	NCI-H460	NCI-H460/R	MCR-5	SI[#]
III.1	> 100	nt	nt	> 100	> 100	> 100	-
III.2	> 100	26.0 ± 0.6	≥ 50	25 ± 2	25 ± 2	91 ± 13	4.3
III.3	> 100	7.9 ± 0.8	7.9 ± 1.2	14.9 ± 2.9	nt	nt	-
III.4	> 100	6.4 ± 0.4*	-	2.7 ± 0.4	3.1 ± 0.4	8.6 ± 0.4	3.2
III.5	46.9	5.5 ± 0.8*	-	3.0 ± 0.2*	nt	nt	-

[#]Selectivity index. SI= GI_{50} (MCR-5)/GI_{50} (NCI-H460); nt – not tested; *Previously published results from our group[39].

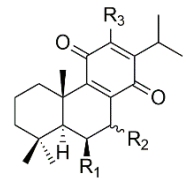
3.4. Structure activity relationships

The conjugation of the new data on abietane diterpene cytotoxicity (Table **3.4**) with other studies on the cytotoxicity of this compound classes (Table **3.5**) allowed the prospection of some structure-activity relationships (SAR). The compounds **III.2**-**III.4** present as main difference the polarity of the group 7α (Figure **3.3**). In this SAR, a clear tendency could be verified for the increasing cytotoxicity with the higher lipophilicity of the 7α substituent. The same tendency was verified between horminone (Figure **3.6**, R_1=H, R_2=OH and R_3=OH) and its more cytotoxic 7α-acetoxy derivate (Figure **3.6**, R_1=H, R_2=OAc and R_3=OH)[325,326].

The bibliographic exhaustive surge of reported cytotoxicity of royleanone-type compounds, bearing a *p*-benzoquinone moiety in the C-ring (Figure **3.6**), additional tendencies could be explored (Table **3.5**). Burmistrova et al. (2013) studied the cytotoxic effects from a series of derivatives of the 7α-acetoxy-6β-hydroxyroyleanone (**III.4**)[40]. The overall compounds affect the cell proliferation with an intensity apparently cell-type dependent. When such compounds were displayed by their log P value, a strong tendency for higher cytotoxic effects was verified for log P values between 2 and 5.5. As the "Lipinski rule of 5", establish that the log P for an oral bioavailable compound should be under 5, the useful compounds must be considered in the 2-5 range of log P[327]. The only exception for this trend was royleanone (Figure **3.6**, R_1=H, R_2=H and R_3=OH) that showed only slight cytotoxic effects in some cell lines[40,325,328]. This could indicate that the presence of a lipophilic substituent was needed at position 6 and/or 7 for the cytotoxic effects to take place. However, the royleanone 6,7dehidro derivative has showed some potent cell-type-specific cytotoxic effects[329–331]. This way, the presence of an electron donating group at position 6 or 7 seems necessary for the cytotoxicity.

Table 3.5. Cytotoxicity of several royleanone derivatives, expressed as GI_{50} (μM) in different cell lines.

Compound features / Cell line (GI_{50} in μM) [reference]				Breast	CNS	Colon	Gastric	Leukaemia/Lymphoma					Melanoma		Papil.	Pancreas	Renal	Lung	Normal
R_1	R_2	R_3	logP	MCF-7 [39,40]	SF-268	HCT116	AGS [328]	HL-60 [329,330]	P-388 [331]	U937 [40]	Molt-3 [40]	MEL-1 [40]	UACC-62 [39]	MV-3 [325]	KB [331]	PaCa-2 [325]	TK-10 [39]	H460	MCR-5 [328]
OH	OH	OH	0.85	+	-	-							-				-	-	+
OH	OCHO	OH	0.97	++	++	++													
OH	$OCOCH_3$	OH	1.08	++				+		+	+	+	+++				++	+++	++
$OCOCH_3$	$OCOCH_3$	OH	1.31	+				+		+	++	+							
$OCOCH_3$	$OCOCH_3$	$OCOCH_3$	1.54	++				++		++	+++	++							
H	=O	OH	1.57											-		-			
H	βOH	OH	1.74				-												+
H	OH	OH	1.74				+		+++					+	+	+			+
$OCOCH_2CH_3$	$OCOCH_3$	OH	1.96	+				+		++	++	++							
H	$OCOCH_3$	OH	1.97											++		+++			
OH	OCH_3	OH	2.10						+++						+				
OH	$OCOCH_3$	$OCOBzNO_2(p)$	2.30	+				++		+++	++	++							
$OCO(CH2)_2CH_3$	$OCOCH_3$	OH	2.38	++				++		+++	+++	+++							
$H\Delta^{6,7}$	$H\Delta^{6,7}$	OH	2.51				-	+++/+	+++						++				+/-
H	H	OH	2.83				-	-		-	+	-		-		-			+
$OCOCH_2CH_3$	$OCOCH_3$	$OCOCH_2CH_3$	2.85	+				+++		+++	+	+							
OH	$OCOCH_3$	$OCOBzCH_3(p)$	3.69	+++				+++		+++	+++	++							
OH	$OCOCH_3$	OCOBzCl(*p*)	3.77	+++				+++		+++	+++	+++							
$OCOBzNO_2(p)$	$OCOCH_3$	OH	4.31	++				+++		+++	+++	++							
OCOBz	$OCOCH_3$	OCOBz	5.33	+				+++		+++	++	+++							
OCOBzCl(*p*)	$OCOCH_3$	OCOBzCl(*p*)	6.45	-				++		++	-	-							
OH	O-FA	OH	>7	-	-								-				-	-	



Otherwise stated all R_2 conformation was β; logP values estimated using ChemBioDraw; –: not active (GI_{50}>30); +: low cytotoxic (10<GI_{50}≤30); ++: cytotoxic (5<GI_{50}≤10); +++: highly cytotoxic (GI_{50}≤ Papil. – papilloma; Bz – benzene; FA – fatty acid.

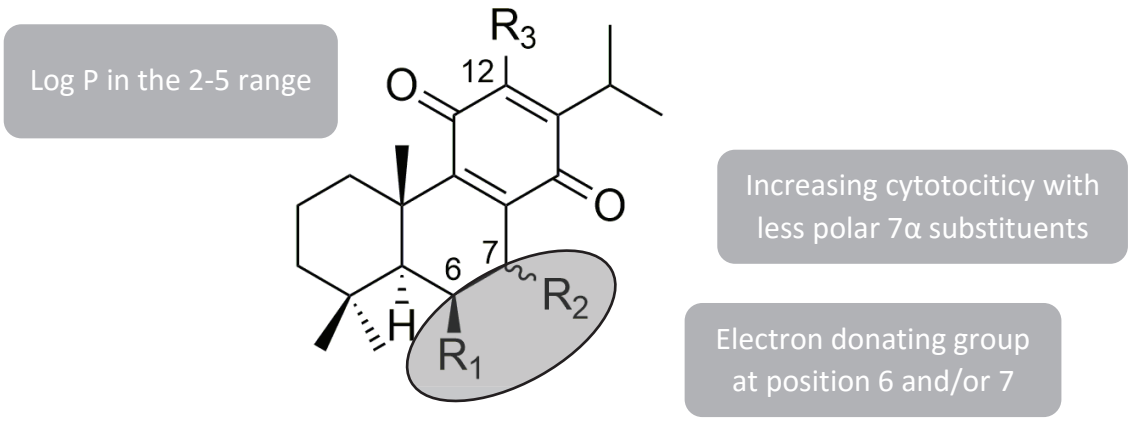


Figure 3.6. Structure and proposed SAR of 6,7,12-substituted royleanone-type abietane diterpenes based on data compilated at Table **3.5**.

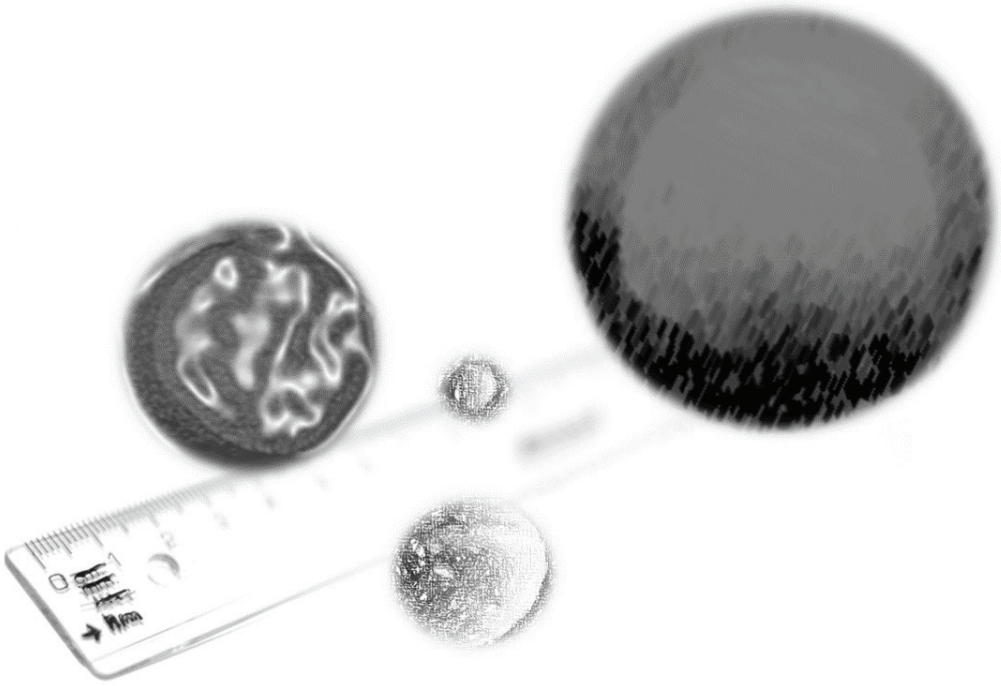
3.7. Chapter conclusions

This chapter study presented the preparation of several *P. madagascariensis* extracts, which have been screened for their cytotoxic activity. The main compounds in these extracts were identified by HPLC-DAD and the compound **III.3** was isolated and spectroscopically characterized. The acetonic maceration extract (**E5**) showed high yields of abietane diterpenes, presenting also the highest cytotoxic effect in MDA-MB-231 breast cancer cells (IC_{50} of 63.9 µg/mL). The observed cytotoxic activity may be justified by the presence of known cytotoxic diterpenes, namely 7α,6β-dihydroxyroyleanone (**III.2**, 3.19 mg/g), 7α-formyloxy-6β-hydroxyroyleanone (**III.3**, 6.74 mg/g), 7α-acetoxy-6β-hydroxyroyleanone (**III.4**, 1.21 mg/g) and coleon U (**III.5**, 5.77 mg/g). To the best of our knowledge this is the first report of the presence of compounds **III.2**-**III.5** in *P. madagascariensis*. The complete ^{13}C RMN data for compound **III.3** is also presented for the first time.

The compounds **III.1**-**III.5** were tested for their individual cytotoxic effects in a battery of cell lines and all abietane diterpenes originated the growth inhibition of some cancer lines. The royleanones **III.2** and **III.4** displayed a high selectivity toward cancer cells and were not target of efflux proteins overexpressed in the NCI-H460/R multidrug resistant cell line. An initial SAR of the cytotoxicity of 6,7 and/or 12-substituted royleanone abietanes was established with a clear trend for log P values between 2 and 5 corresponding to compounds with improved cytotoxic effect. The whether those structural alterations correspond to an improved fitting on the target or the cytotoxic activity was instigated by favorable log P for the membrane crossing is still to be answered.

Overall, compounds **III.2**-**III.5** may be considered a very promising compound for further studies in order to fully understand the potential of the abietane diterpenoids as chemopreventive, chemoadjuvancts or chemotherapeutic agents.

Chapter IV



4. Antibacterial phytosomal formulations

4.1. Introduction

The antibiotic resistance problem had been recently highlighted by key organizations including the World Health Organization[119] and the World Economic Forum[332] as a major concern for the humanity in the following years. Bacteria can acquire resistance to antibiotics by gene mutations, horizontal gene transfer or they could be intrinsically resistant to some antibiotic classes[333]. The most relevant predisposal factor of non-intrinsic resistance is considered to be the worldwide misuse of antibiotics, including medical overprescribing practices, patient self-medication and use as veterinary growing stimulants[334,335]. Such issues could originate a life threating situation in the case of hospital associated infections (i.e. septicaemia, pneumonia), but can also complicate the treatment of apparently simple situations as a superficial wound. In recent years, the arise of some microorganisms with resistance to the traditional topical treatments, namely mupirocin[336] and fusidic acid[337], have shown to be a public health problem, namely in the management of both acute or chronic skin and soft tissue infection[338].

The skin has protection and barrier functions, that could be compromised by the occurrence of physical trauma or wounding. The following skin repair processes intend the complete reestablishment of the damaged tissue integrity. However, this processes could be impaired if microbial infection occurs. It is often recommended by dermatology guidelines to use topical antibiotic agents for the prophylaxis of wound bacterial infection[339,340]. Also, in the wound environment often occur the formation of biofilms which enhance the bacterial tolerance to topical antibiotics[341]. Such reasons highlight the need for new therapeutic alternatives for topical antibacterial treatment.

The screening performed by Weckesser and collaborators (2009)[342], suggested that natural extracts from plants as *Gentiana lutea*, *Harpagophytum procumbens*, *Boswellia serrata*, *Usnea barbata*, *Rosmarinus officinalis* and *Salvia officinalis* could be effective in controlling common skin pathogenic agents. Following such results, Elston et al.[338] suggested that such natural compounds could be alternative options for topical skin antimicrobial treatments. Also the concomitant use of natural products, as essential oils, with traditional antibiotics was suggested to be an effective strategy in reverting the bacterial resistance[343].

The investigation presented in chapter **2**. showed that the ethnomedicinal use of *P. madagascariensis* as wound disinfectant could be justified by the presence of several antibacterial oxygenated abietane diterpenes and some polyphenols. One of the extracts (**E4**) obtained in that study, showed outstanding activity against some of the typical skin bacteria, as *S. epidermidis* and *S. aureus,* including a methicillin-resistant strain. Considering those results along with the need of new antimicrobial agents and the acceptance that natural products could be a reliable alternative to traditional antibiotics, it is proposed the development of an optimized antibacterial system for possible topical application. Based on some reports[344], we state that an ideal antibacterial topical formulation should be (i) active against the expected skin

pathogens and including MDR strains to prevent the emergence of superinfections; (ii) non-toxic for keratinocytes at the concentration of activity; (iii) the formulation elements should preferentially have synergic effects and different modes of action; (iv) should not contain any antimicrobial that would be used parentally; and (v) should not occur systemic toxicity caused by the absorption of the antibacterial formulation through the intact skin or wound.

The use of nanotechnology has been also suggested as an additional resource for the improvement of antibacterial activityp[345,346]. Their favourable effects occur through mainly three mechanisms: by incorporating different components with synergic or concomitant antibacterial effects[347,348]; by overcoming existing mechanisms of resistance favouring increased uptake[348] and disrupting the biofilm formation[347]; and by drug targeting assuring higher concentrations of the drug to the infection site[348].

In order to test the feasibility of *Plectranthus* extracts encapsulation in polymeric matrixes, a preliminary study focused on the production of alginate beads containing an antioxidant and anti-*S. epidermidis* microwave aqueous extract of *P. madagascariensis*, which was selected from a screening of 5 *Plectranthus* plants (annex **10**)[307]. In this study, the obtained particles were spherical, homogenous in colour and particle size and showed very high encapsulation efficiency of the main extract component, rosmarinic acid. The long-term stability studies also demonstrated the viability of this system to the improvement of the stability of this extract and, consequently, of its biological activities. These properties may be beneficial for the healing.

This initial approach in the encapsulation of *P. madagascariensis* extracts, led as to test the phytosome technology as strategy for the improvement of antibacterial activity of *Plectranthus* components. Phytosomes were mainly known for their uses in oral formulations due to its improvement of bioavailability. Although, considering the phytosome high phospholipid content, it could exert a nourishing effect in the skin, and also being highly biocompatible with the skin layers could increase the penetration of bioactive compounds to inner layers of the skin and thus intensifying the therapeutic outcome[349].

Some excellent reference texts have focused on the basis of transdermal penetration of drugs and particulate materials[350,351]. The free drug percutaneous penetration potential trough skin was mainly characterized based on the maximum flux (J_{max})[352] being this parameter related in many mathematic models to the log of octanol solubility (log S_o), molecular weight, octanol water partition coefficient (o/w coefficient) and melting point[352,353].

Micro- and nanoparticle could penetrate the stratum corneum by three main mechanisms: particles smaller than 5-7 nm through lipidic intercellular route; particles smaller than 36 nm through aqueous pores; and larger particles (up to 21 µm) through transfollicular route but only offering a negligible contribution (less than 0.1% of total skin surface) to the overall permeation[354]. As most phytosome possess an average particle size higher than 50 nm it is unlikely that a high degree of transdermal permeation occurs through the epidermis layers for those particles, being the main

mechanism of transdermal penetration the intrafollicular route. The eventual permeation of phytosomes would also be affected by factors as superficial charge, pKa, o/w partition coefficient, particle shape and deformability, composition and stability of the particle could influence the permeation ability of such nanoparticles[354]. Those factors also have influence in the physical stability and aggregation upon contact to skin medium maintaining this way the maximum potential for permeation. Another factor was the interactions with skin components which were influenced by the particle charge, the formation of dipole, hydrophobic and/or hydrogen-bond interactions[351]. Those factors stated that phytosome could not be appropriated for transdermal delivery, although, Das and collaborators demonstrated that their rutin phytosomes with an average size of 1.2 µm presented a 24h-permeation of the stratum corneum about 60% higher when compared to the lipophilic free drug[355]. Being due to the higher permeation or due to a still unidentified mechanism, there was an evident improvement of topical activity of some natural products as boswellic acid (anti-inflammatory)[247] and curcumin (antioxidant)[246] with advantage over their equivalent niosome and liposome formulations[246,247]. The functionalization of phytosomal systems seems a valid strategy for the improvement of the intended biologic activity. This is highlighted by the *Calendula* extract phytosomes associated to gold nanoparticles (AuNP) with antioxidant and wound healing effects superior to the equivalent phytosome formulation[257].

Chitosan is a semi-natural polysaccharide being the most important derivative of chitin (poly-β-(1→4)-N-acetyl-d-glucosamine) from which is obtained by alkaline or enzymatic deacetylation[356]. Chitosan is weak base, becoming soluble in acidic media by the protonation of -NH_2 group on the carbon 2 of the d-glucosamine polysaccharide (Figure **4.1**). The combination of its aqueous solubility with the natural cationic character finds it many applications in the production of viscous solutions, gels, films and fibres. Chitosan matrixes have shown interesting antimicrobial properties against a wide range of bacteria[357,358]. Such biologic activity was currently accepted to be more bacteriostatic rather than bactericide[359] and resulting from the independent or simultaneous contribution of three mechanisms: 1) interaction between the positively charged chitosan molecules and the negatively charged bacterial membrane, involving a cross-linkage between the chitosan protonated groups and the anions on the bacterial surface and changes in membrane permeability with possible cell rupture[359,360]; 2) inhibition of the mRNA and protein synthesis via the penetration of chitosan into the nuclei of the

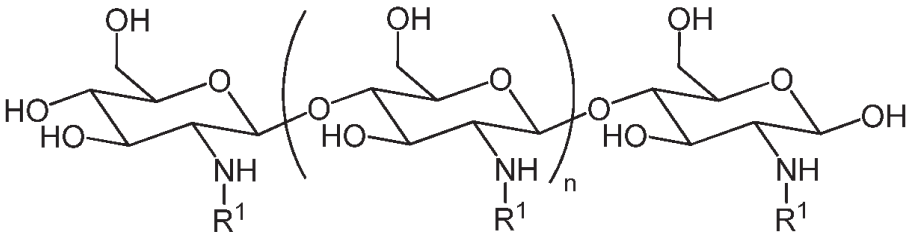


Figure 4.1. Structural representation of the chitosan polymer. Depending on the deacetylation degree, R could represent be H (amine) or $COCH_3$ (amide). A more deacetylated sample is richer in H substituents.

microorganisms[359]; 3) the formation of an external barrier, chelating metals and led to the suppression of essential nutrients to microbial growth[361]. Such versatile antimicrobial mode of action led some groups to use those properties for the production of nanocomposite antibacterial films, wound coating materials[362] and antibacterial nanoparticles[363].

Chitosan nanoparticles have been obtained mainly by the spontaneous formation of complexes between chitosan and polyanions as the tripolyphosphate (TPP) or dextran. The better established method for those nanoparticle preparation is internal gelation. Is such method, a chitosan solution in acetic acid is prepared and extruded dropwise into an aqueous solution of magnetically stirred TPP.

The insights through which the cross-linking occurs were still badly understand with few mechanistic approaches made so far. Various authors suggest a simplified model in which the anionic groups of TPP form stable interactions with chitosan amine groups[364–366]. A recent study demonstrated that could exist simultaneously three types of primary ionic cross-linking interactions between TPP and chitosan oligomers designed H-links, M-links and T-links. However, due to the highest interaction energies and the availability of free spatial area for their occurrence, H-links were proposed as the most frequent cross-linking types[367].

The aim of this chapter is to prepare phytosomes and optimize the production method. The selection of both formulation and process parameters will be done according physic-chemical and biologic characterizations.

4.2. Experimental

4.2.1. Chemicals and equipment

Most of the laboratorial work has been performed at the CBIOS facilities (Universidade Lusófona de Humanidades e Tecnologias). Some procedures were performed in collaborating institutions, namely Faculdade de Farmácia da Universidade de Coimbra (animal experiments), Faculdade de Ciências da Universidade de Lisboa (AFM, DSC, DRIFTS and SEM studies) and Institute for Biological Research "Sinisa Stankovic" at University of Belgrade, Serbia (cytotoxicity assays).

4.2.1.1. Chemicals

Phosphatidylcholine (48% purity from soybean), cholesterol, acetic acid, sodium laureth sulfate, carboxymethyl cellulose, sodium tripolyphosphate (technical grade 85%) and chitosan (low molecular weight) were purchased from Sigma-Aldrich (Steinheim, Germany). Extraction and reaction solvents, namely dichloromethane, ethanol and acetone were from analytic grade and purchased from Sigma-Aldrich (Steinheim, Germany). Reverse osmosis water was obtained from a Millipore system (Millipore, MA, USA) system with a resistivity of 18.2 Ω·cm at 25°C. Trichloroacetic acid was obtained from Panreac (Barcelona, Spain). HPLC reagents were from HPLC-grade (VWR Chemicals, Fontenay-sous-Bois, France) and were filtered through a 0.22 µm membrane (Vygon, Ecouen, France) before use. Dimethyl sulfoxide (DMSO) was

supplied by Merck (Darmstadt, Germany) was supplied by Sigma-Aldrich (Steinheim, Germany). Filter paper nº5 was obtained from Whatman (Maidstone, United Kingdom). An authentic standard of 7α,6β-dihydroxyroyleanone was obtained and fully characterized by Gaspar-Marques[34]. Mueller-Hinton broth was supplied from Sigma-Aldrich (Steinheim, Germany). Vancomycin was supplied by Sigma-Aldrich (Steinheim, Germany). Polydimethylsiloxane (PDMS) membranes with a thickness of 75 µm were kindly donated by Dow Corning Europe S.A (Seneffe, Belgium).

4.2.1.2. Equipments

The HPLC-DAD system was composed by an Agilent Technologies 1200 Infinity Series with diode array module (Agilent Technologies, Santa Clara, CA, USA) using a reverse phase (RP-18) HPLC column LiChrospher® 100 (Merck, Darmstadt, Germany). Other equipments used were hotplate magnetic stirrer (RT15 power, IKA, Staufen, Germany), centrifuge (Z36 HK HERMLE Labortechnik, Wasserburg, Germany), lyophilizer (Freezone 2.5 L, Labconco, Kansas City, USA), particle size and zeta potential analyser (Delsa Nano C, Coulter, CA, USA), rotary evaporator (IKA RV06-ML 1-B, Staufen, Germany) ultrasonic bath (Bandelin SONOREX RK 510H, Berlin, Germany) and weighting scales (KERN 770, KERN & Sohn GmbH, Balingen, Germany). The atomic force microscopy (AFM) was performed in a Multimode Nanoscope IIIa (Digital Instruments, Veeco, Cambridgeshire, UK) and the scaning electron microscopy (SEM) was performed in a 5200LV (JEOL Ltd., Tokyo, Japan). Infrared spectroscopy experiments were performed in a Nicolet 6700 (Thermo Fisher Scientific, Waltham, MA, USA) while DSC experiments were performed in a DSC 7 with TAC 7/3 controller (PerkinElmer, Waltham, MA, U.S.A.) with weightings performed in an ultra-microbalance (XP2U, Mettler-Toledo, Greifensee 8606 Switzerland).

4.2.1.3. Plant material

Plectranthus madagascarensis Benth. was cultivated in Parque Botânico da Tapada da Ajuda (Instituto Superior Agrário, Lisbon, Portugal) from cuttings obtained from the Kirstenbosch National Botanical Garden (Cape Town, South Africa). A voucher specimen was deposited in Herbarium João de Carvalho e Vasconcellos (ISA) with the number 841/2007. The plant material used in this study was collected between 2007 and 2008, dried at room temperature and stored protected from light and humidity.

4.2.1.4. Microbial strains

The antimicrobial assays were performed in 3 Gram positive bacteria strains: *Staphylococcus aureus* (ATCC 25923), methicillin-resistant *S. aureus* (MRSA) (CIP 106760) and *Staphylococcus epidermidis* (ATCC 12228). Microbial strains were originally obtained from American Type Culture Collection (ATCC) or form “Collection de l'Institut Pasteur" (CIP).

4.2.1.5. Cell lines

HaCaT is an immortalised non-tumorigenic human keratinocyte cell line derived from normal human trunk skin and able to stratify (Boukamp et al., 1988)[368]. This line was

maintained at Institute for Biological Research "Sinisa Stankovic" at University of Belgrade (Serbia) and obtained from CLS (Cell Lines Service), Eppelheim, Germany.

4.2.1.6. Software editing

Chemical structures were drawn on ChemBioDraw Ultra 12.0.2.1076. ChemStation was used for HPLC-DAD controller and data exportation. Statistical analysis and graphic design were archived using GraphPad Prism 6.01 for Windows 10.

4.2.2. *Plectranthus madagascariensis* extract

The bioactive *P. madagascariensis* extract was prepared as previously reported in sub-section **2.2.2**. Briefly, **E4** was prepared by USAE carried using 10 g of dried and pulverized plant, immersed in 100 mL of acetone, into an ultrasonic bath (Bandelin SONOREX RK 510H, Germany) with a working frequency of 35 kHz and 400 W of power and sonicated for 1 hour. The bulk extract was separated from the remaining plant material by filtration through paper nº5 (Whatman, Maidstone, United Kingdom) and evaporated under reduced pressure in a rotary evaporator (IKA RV06-ML 1-B, Staufen, Germany) until drying.

The extract was characterized by HPLC-DAD and main compounds identified and quantified using the methodology described at subsection **3.2.3**.

4.2.3. Optimization of phytosome preparation

Different solvents (acetone, dichloromethane and ethanol), reaction time (60, 120 and 240 min) and cholesterol concentration in the formulation (0, 2.5 and 5 molar %) were tested in order to obtain the smallest and more uniform phytosomes. Briefly, molar equivalent concentrations of PdC and extract were solubilized in 20 mL of solvent and kept magnetic stirring (RT15 power, IKA, Staufen, Germany) at 50ºC during the reaction time. To obtain the phytosomes, 40 mL of reverse osmosis water was then added and the organic solvent was partially eliminated in the rotary evaporator (IKA RV06-ML 1-B, Staufen, Germany) and the pellet was recovered by centrifugation at 23,540 G for 10 min (Z 36 HK HERMLE Labortechnik, Germany).

4.2.4. Preparation of loaded-phytosomes (PS)

Based on previous section results, PdC (424 mg), extract (200 mg) and cholesterol (11.6 mg) were dissolved into 40 mL of acetone and the mixture was magnetic stirred at 55ºC for 2 h (HTS 1003, LMS, Tokyo, Japan). The solution was then added to 80 mL of 2% (w/v) acetic acid being the organic fraction evaporated under reduced pressure (IKA RV06-ML 1-B, Staufen, Germany) for about 2h at 50ºC until the volume became stable.

4.2.5. Chitosan microencapsulation of PS (ChiPS)

The prepared phytosome suspension was mixed with 400 mL of a 1% chitosan (w/v) solution in 2% acetic acid (v/v). The resultant solution was then added dropwise to 600 mL of the counter-ion sodium tripolyphosphate solution (0.3% w/v) under vigorous magnetic stirring (500 rpm) (HTS 1003, LMS, Tokyo, Japan). The resultant suspension

was centrifuged at 33,320 x *g* for 5 minutes to recover the particles in the pellet. The pellet was washed several times with distilled water and freeze-dried using a temperature of −50°C and a vacuum of 0.020 mbar (Freezone 2.5 L, Labconco, Kansas City, USA).

4.2.6. Determination of encapsulation efficiency and drug loading

Encapsulation efficiency of the extract in the phytosomes was determined by evaluation of the content of the major peak in the extract (7α,6β-dihydroxyroyleanone). For this compound, a calibration curve was established using authentic samples. Three independent batches of Chi-Ps were freeze-dried using a temperature of −50°C and a vacuum of 0.020 mbar (Freezone 2.5 L, Labconco, Kansas City, USA) and precisely weighted for the quantification of the ChiPS recovery yield after production.

The encapsulation efficiency and drug loading were determined by dissolving 10 mg of freeze-dried particles in 1 mL methanol overnight[249]. The mixture was then centrifuged at 23,540 x *g* and the supernatant filtered through a 0.46 µm filter previously to the analysis. The amount of 6β,7α-dihydroxyroyleanone (Roy) detected by HPLC-DAD was used to estimate the encapsulation efficiency (EE) (**I**) and drug loading (DL) (**II**) using the following equations:

$$(\boldsymbol{I})\ EE\ (\%) = \frac{Roy_{encapsulated}}{Roy_{total}} \times 100$$

$$(\boldsymbol{II})\ DL\ (\%) = \frac{Roy_{encapsulated}}{ChiPS} \times 100$$

Were $Roy_{encapsulated}$ corresponds to the weight of Roy detected after the disruption of the particles, Roy_{total} is the total weight of Roy used for particle production and ChiPS is the weight of particles. Tests were performed in triplicate being results expressed as the means ± S.D.

4.2.7. Physic and morphological characterization

Loaded phytosomes and loaded ChiPS were diluted with distilled water and analysed in by DLS in a Delsa Nano C (Coulter, CA, USA) to obtain the average particle size, polydispersity index (PI) and Zeta potential (ζP). Morphology was assessed by SEM[369] (5200LV SEM, JEOL Ltd., Tokyo, Japan) or AFM[370] (Multimode Nanoscope IIIa, Digital Instruments, Veeco, Cambridgeshire, UK) using previously optimized methods.

4.2.8. Physic-chemical analysis

The differential scanning calorimetry (DSC) measurements were carried out on a PerkinElmer DSC 7 with TAC 7/3 instrumental controller (PerkinElmer, Waltham, MA, USA). The samples with 2.5-4.9 mg mass were sealed in air, inside aluminium sample pans. Each crucible was transferred to the apparatus and heated at a rate of 10ºC/min

in the range 25-250ºC. Nitrogen (Air Liquide N45, Lisbon, Portugal) at a flow rate of 0.5 cm^3/s was used as the purging gas. The temperature and heat flow scales of the instrument were calibrated at the same heating rates with indium (mass fraction: 0.99999; T_{fus} = 429.75K, $\Delta_{fus}h_0$ = 28.45 J/g). All weightings were performed with a precision of ±0.1 µg on a XP2U ultra-microbalance (Mettler-Toledo, Greifensee 8606 Switzerland).

Table 4.1. Samples evaluated for interaction between its components by DSC and DRIFTS.

Sample	DSC	DRIFTS
A	Cholesterol	Cholesterol
B	Extract **E4**	Extract **E4**
C	PdC	PdC
D	Mixture of Cholesterol, **E4** and PdC	Mixture of Cholesterol, **E4** and PdC
E	Loaded-phytosomes	Loaded-phytosomes
F	-	TPP
G	-	Chitosan
H	-	Mixture of TPP/Chitosan
I	-	Mixture of TPP/Chitosan/PS
J	-	Loaded-ChiPS

The diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) experiments were performed in the range 400-4000 cm^{-1}, with a resolution of 2 cm^{-1} and 528 scans on a Nicolet 6700 spectrometer with Omnic software (Thermo Fisher Scientific, Waltham, MA, USA), being the samples dispersed in pulverized KBr and analysed directly.

4.2.9. *In vitro* release studies

The release pattern of extract components from ChiPS was studied *in vitro* using a membrane-free model. Briefly, 10 mg of PM-PS-Chi were added to 100 mL of PBS pH 5.5 and pH 7.4 and kept stirring at 300 rpm at 37ºC. One aliquot of 1 mL of the mixture was collected at fixed time-points, centrifuged at 23,540 x *g* for 5 min (and the supernatant stored at -20ºC until analysis. The same volume of collected medium was used to solubilize the pellet and added to the medium. Extract components released from the nanoparticles were quantified by HPLC. All results were made in triplicate and expressed as the mean of the repetitions with SD.

4.2.10. *In vitro* skin permeation studies

The permeation studies were performed using an adapted Franz cell model. The Franz cells were kept immersed in thermostated water (32ºC) and receptor chambers were filled with 5 mL of PBS pH 7.4 and stirred continuously with mini-magnetic stir bars.

Polydimethylsiloxane (PDMS) membranes with a thickness of 75 µm were used in the Franz cell interface (0.98 cm^2) and the formulations (extract **E4** and loaded ChiPS) were applied at donor chambers (average chamber volume of 3.9 mL). Collections were made at specific time intervals (2, 4, 6, 8, 10 and 24h) by withdraw 200 µL of sample followed by reposition of the same volume of fresh PBS pH 7.4 at each time point. Components released from the extract or ChiPS were quantified by HPLC. Experiments were made in quadruplicate and expressed as the means ± SD.

4.2.11. Biological activities

4.2.11.1. Antibacterial activities

The antimicrobial activities of Roy, extract **E4**, phytosome and ChiPS (Table **4.2**) were evaluated qualitatively by well diffusion assay and quantitatively by broth microdilution method as described in sub-section **2.2.2**.

Table 4.2. Samples evaluated for its antibacterial effects in well diffusion and broth microdilution assays.

Sample	Well diffusion assay	Broth microdilution		
	S. epidermidis	*S. aureus*	MRSA	*S. epidermidis*
A	Roy	Roy	Roy	Roy
B	E4	E4	E4	E4
C	-	Loaded PS	Loaded PS	Loaded PS
D	Loaded ChiPS	Loaded ChiPS	Loaded ChiPS	Loaded ChiPS
E	Unloaded Chi	-	-	-
Control	Vancomycin	Vancomycin	Vancomycin	Vancomycin

4.2.11.2. Cytotoxicity in human keratinocyte cell line

The cytotoxicity profile of the extracts was characterized in the human keratinocyte cell line, HaCaT using the MTT test by the procedure described by Pesic et al., 2013[308,312]. Briefly, the cells were incubated with compounds at 32,000 cells/cm^2 in DMEM supplemented with 10% FBS, 4 g/L glucose, L-glutamine (2 mM) and 5000 U/ml penicilin, 5 mg/mL streptomycin solution at 37ºC in a humidified 5% CO_2 atmosphere for 72h. Afterwards, 100 ml of MTT solution (1 mg/mL) was added to each well and plates were incubated at 37ºC for 4 h. Formazan product was dissolved in 200 mL of DMSO and the absorbance of obtained dye was measured at 540 nm using an automatic microplate reader (LKB 5060–006 Micro Plate Reader, Vienna, Austria).

4.2.11.3. Acute and Sub-chronic mice irritation study

Male hairless Sho® SCID mice (Charles River Laboratories, Massachusetts, USA) with 12 weeks old and 20-30 g were housed under normal conditions according to established animal care guidelines as follows: temperature of 20±2ºC, humidity of 60-90% RH and a 12h light/dark cycle and provided with ad-labium access to a commercial

mice-diet and drinking water. This study was conducted in accordance to the internationally accepted principles for laboratory animal use and healthcare as found in Directive 2010/63/EU. The project was approved by the Portuguese Veterinary General Division (DGAV).

ChiPS and extract **E4** were incorporated into 5% carboxymethyl cellulose (CMC) hydrophilic gel, with very high viscosity, as vehicle. Eleven mice were randomly divided into 4 groups. Sodium laureth sulfate (SLES) solution at 5% (w/v) was used as positive control[371].

The animals were treated as follows: Group I (*n* = 4): CMC gel with 5% (w/w) ChiPS; Group II (*n* = 3): CMC gel containing 1% (w/w) extract **E4**; Group III (*n* = 2): CMC gel; Group IV (*n* = 2): SLES 5% (w/v).

To each group 0.1 mL of the test or control formulation was applied with a gloved finger in an area of approximate 10 cm^2 on the back of each animal, housed in individual cages, daily for 15 days. Each animal was monitored during the first hours after application in order to guarantee that the formulation remain in the target area enough time to be absorbed. Mice were observed daily. Reactions, defined as erythema and/or edema, were observed at 24, 48 and 72h after the formulation application. Photos were taken at time of observation and the reaction degree was evaluated according to the scoring system for skin reactions (Table **4.3**).

The Score of Primary Irritation (SPI) was calculated[372] for test and control in each mice as following:

$$SPI = \frac{\sum Erythema\ and\ edema\ grade\ at\ 24, 48\ and\ 72h}{Number\ of\ observations}$$

The Primary Irritation Index (PII) was calculated as follows according to table 4.4:

$$PII = \frac{\sum SPI_{test} - \sum SPI_{base}}{Number\ of\ animals}$$

From the midback region of each mice skin samples from each animal were collected at day 16 of consecutive application and fixed in 10% buffered formalin for further toxicological assessment of the skin samples.

Table 4.3. Classification system for skin reaction, adapted from [372,373].

Erythema	**Reaction Score**
No erythema	0
Very slight erythema	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to eschar formation	4
Edema	**Reaction Score**
No edema	0
Very slight edema	1
Well defined edema (edges of the area well defined by define raising)	2
Moderate edema (raising approximately 1mm)	3
Severe edema (raised > 1 mm and extended beyond the area of exposure)	4
Total possible score for irritation	8

Table 4.4. Response categories of irritation, adapted from from[372,373].

Erythema	Primary Irritation Index (PII)
Negligible	0-0.4
Slight irritation	0.5-1.9
Moderate irritation	2-4.9
Severe irritation	5-8

4.2.12. Statistical analysis

All results are expressed as means ± standard deviation. t-student analysis was applied to demonstrate statistical differences in all tested parameters. All analyses were performed using a software program (GraphPad Prism 5®, GraphPad Software, San Diego, CA) with a statistical significance level of 0.05.

4.3. Results and discussion

4.3.1. *Plectranthus madagascariensis* antibacterial extracts

The selected extract was analysed by the previously described HPLC-DAD method (subsection **3.2.2**). The **E4** was obtained as a yellowish gum with a yield of 0.22 % ($w_{extract}/w_{dry\ plant}$). This extract profiling in HPLC-DAD showed that polyphenols and diterpenes were the main components of this extract (Figure **4.2**). The main compound yield was also quantified, namely 7α,6β-dihydroxyroyleanone (Roy) as 4.59±0.02% ($w_{compound}/w_{extract}$).

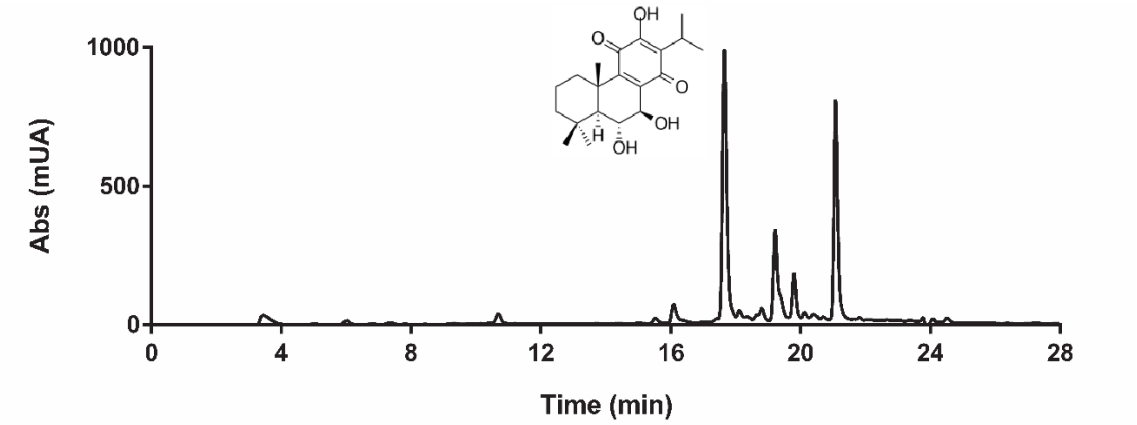


Figure 4.2. Representative chromatogram of the P. madagascariensis extract **E4** (270 nm) and structure of the main component: 7α,6β-dihydroxyroyleanone.

4.3.2. Optimization of phytosome preparation methodology

The results from the optimization process were presented in the tables **4.5** to **4.7**. Under the process conditions studied, the smallest and most uniform particles were produced using acetone, a reaction time of two hours and adding 2.5% of cholesterol.

Original methodologies for the preparation of PPC state the use of an aprotic solvent in order to avoid solvent interferences in the complexation process. However, in the recent years, some protic ethanolic solvents as ethanol or methanol have been used for the preparation of PPCs (as reviewed in Annex **1**). In this preliminary study, two polar aprotic solvents (acetone and dichloromethane) and a polar protic solvent (methanol) were tested. The overall smaller particles were obtained by the use of acetone (Table **4.5**). This solvent was also the solvent used in the plant extraction methodology and for such reason should be most efficient in re-solubilizing the extract. This improved solubility of the components would allow more favourable conditions for the complexation process to occur, leading to more compact and uniform particles. Also, previous studies have demonstrated superior efficiency of acetone over ethanoic solvents for the preparation of drug-phospholipid complexes[239]. Acetone is also considered less toxic than dichloromethane in pharmaceutical formulations (ICH class 2 vs ICH class 3).

Table 4.5. Influence of solvent choice in phytosome physical properties. Results are present as the means ± S.D. of three independent batches.

Solvent	Acetone	Dichloromethane	Ethanol
Particle size (nm)	107.2 ± 16.55	142.2 ± 23.3	175.7 ± 91.65
Polydispersity index	0.419 ± 0.06	0.511 ± 0.12	0.359 ± 0.001

Smaller and more uniform particles were obtained with an intermediate reaction time of 2h (Table **4.6**). This is in accordance with many PPCs preparation examples[235,239,240,246,374,375] (Annex **1**). This time should be adequate for the total complexation of the PPC components while not enough for the occurrence of further aggregation with longer reaction times, as occur at time 4h. The substantially higher particles and heterogenic distribution obtained at 1h reaction time should be originated by the incomplete solubilisation of some components with the formation of precipitate aggregates along with the phytosomes.

Table 4.6. Influence of reaction time in phytosome physical properties. Results are present as the means ± S.D. of at three independent batches.

Reaction time (h)	1	2	4
Particle size (nm)	469.85 ± 201.95	107.2 ± 16.55	182.1 ± 64.35
Polydispersity index	0.772 ± 0.04	0.410 ± 0.06	0.458 ± 0.01

The inclusion of cholesterol at molar percentages of 2.5 or 5% resulted in non-substantial change of phytosome size. No statistically significant differences ($p > 0.05$, t-student test) were found between the particle size in the formulation with a cholesterol molar percentage of 2.5 or the formulation with cholesterol at 5%. However, the mean size of the particles obtained with acetone and reaction time of two hours (Table **4.6**) was smaller than the obtained with the inclusion of 2.5% of cholesterol (Table **4.7**). Moreover, the use of the 2.5% cholesterol proportion resulted in a two-fold reduction in PI which would improve the formulation stability. In other studies, the inclusion of cholesterol in phytosomes has been justified as stability enchantment functions on such nanosystems[257,376]. For such reasons, the phytosomal formulation obtained with reaction solvent acetone, reaction time of 2h and the inclusion of 2.5% of cholesterol was selected as the optimized formulation.

Table 4.7. Influence of cholesterol addition in phytosome physical properties. Results are present as the means ± S.D. of three independent batches.

Cholesterol (molar %)	0	2.5	5
Particle size (nm)	332.2 ± 159.7	191.3 ± 75.3*	203.3 ± 55.8*
Polydispersity index	0.247 ± 0.03	0.243 ± 0.18	0.263 ± 0.15

*p > 0.05, t-student test

4.3.3. Preparation and characterization of optimized formulations

The optimized suspension of loaded phytosomes had a mean size of 191.3 ± 75.3 nm while the chitosan coated loaded phytosomes (ChiPS) presented a mean size of to 1082 ± 363 nm (Table **4.8**). Both formulations demonstrated similar polydispersity indexes of 0.243±0.18 and 0.22 ± 0.10 respectively.

The particle size and its distribution were factors influencing the fate and stability of nanoparticle formulations[377]. The determined mean size for each formulation is adequate for topical application by minimizing the unintended transdermal penetration (intracellular pathways or via aqueous pores) that could occur in particles smaller than 36 nm. Still, some transdermal absorption could occur through the transfollicular route[373], but its contribution to the overall process is very low as follicles correspond to less than 0.1% of total skin surface[354].

Table 4.8. Size distribution of loaded phytosomes and chitosan coated phytosomes (ChiPS). Data is present as mean ± standard deviation with n = 3.

Formulation	Particle Size (nm)	Polydispersity index (Pi)
Phytosomes	191.3±75.3	0.243±0.18
ChiPS	1082 ± 363	0.22 ± 0.10

The optimized suspension of loaded phytosomes presented an approximately neutral surface charge with ζP of +0.07±0.15 mV while the ChiPS presented a positive charged surface with ζP of +20.59 ± 12.02 mV (Table **4.9**).

The neutral character of the suspended uncoated phytosomes could represent the occurrence of a complete reaction. The phospholipids containing a choline group behave as Bronsted acids which possess tendency to become deprotonated. The PdC intrinsic pKa estimated as 0.8 confirms this character[378]. On the other hand, the hydroxyl group of many natural products, namely phenolic hydroxyls present in the major components of this extract, can act as electron acceptors which makes the complexation with PdC possible. The occurrence of complexation would have led to the charge

distribution within the formed complex being an overall neutral charge expected, as verified in the literature[230].

Chitosan is a biodegradable, biocompatible, cationic polymer which have been used for the production of polymeric nanoparticles for variable applications. It was expected that the microencapsulation step would led to a positive charge of the particles. It was verified with the increasing from neutral to +20.59 ± 12.02 mV. Also the coating with chitosan by ionotropic gelation, using TPP as the cross-linking agent, led to an enlargement of the particle size to 1082 ± 363 nm with a lowering of the polydispersity index. Similar methodologies have produced chitosan microspheres with higher dimension, namely the curcumin phytosomes coated by chitosan, that presented a mean particle size of 23.21±6.72 μm[249]. Particles presenting a higher size generally tend to present a worst stability, with more frequent formation of interparticle aggregates[379].

It was demonstrated that negatively charged particles have a more favourable penetration in the inner skin layers that those that were positively charged[380]. Possessing a positive charge, the ChiPS have less predisposal for transdermal penetration. The human skin presents a negative surface charge at neutral pH, mainly due to the presence of negatively charged phospholipids and carbohydrates[381–384]. This would originate an attraction between the negative charged skin surface and the positive charged ChiPS particles. Taken together with the bioadhesivity properties of chitosan based materials, this indicate that the ChiPS present a relevant affinity for the surface of the skin.

Table 4.9. Zeta potential of phytosomes, and chitosan coated phytosomes (ChiPS). Data is present as mean ± standard deviation with n = 3.

Formulation	Zeta potential (ζP) (mV)
Phytosomes (loaded)	+0.07±0.15
ChiPS (loaded)	+20.59 ± 12.02

SEM and AFM imaging showed that the phytosomes present an amorphous spherical shape with somewhat heterogeneous size distribution (Figure **4.3** and **4.4**). The topographical analysis of the uncoated phytosomes showed a spherical shape of the nanoparticle vesicles with size ranging mostly from 30 to 200 nm. Those observations were in accordance with the values registered by DLS.

The chitosan coated phytosomes by AFM imaging (Figure **4.4 A**) presented a spherical shape by with a rough texture and higher size when compared to uncoated phytosomes. The spherical shape is favourable for the delivery of such particles as this would promote the higher surface area possible[385]. The SEM imaging of ChiPS (Figure **4.4 B**) resulted in the observation of an aggregate of smaller phytosomes. This could be related to the sample preparation as it seems that the water loss in the drying process favours the aggregation of the water rich ChiPS. In AFM phase contrast visualization (Figure **4.5**), it

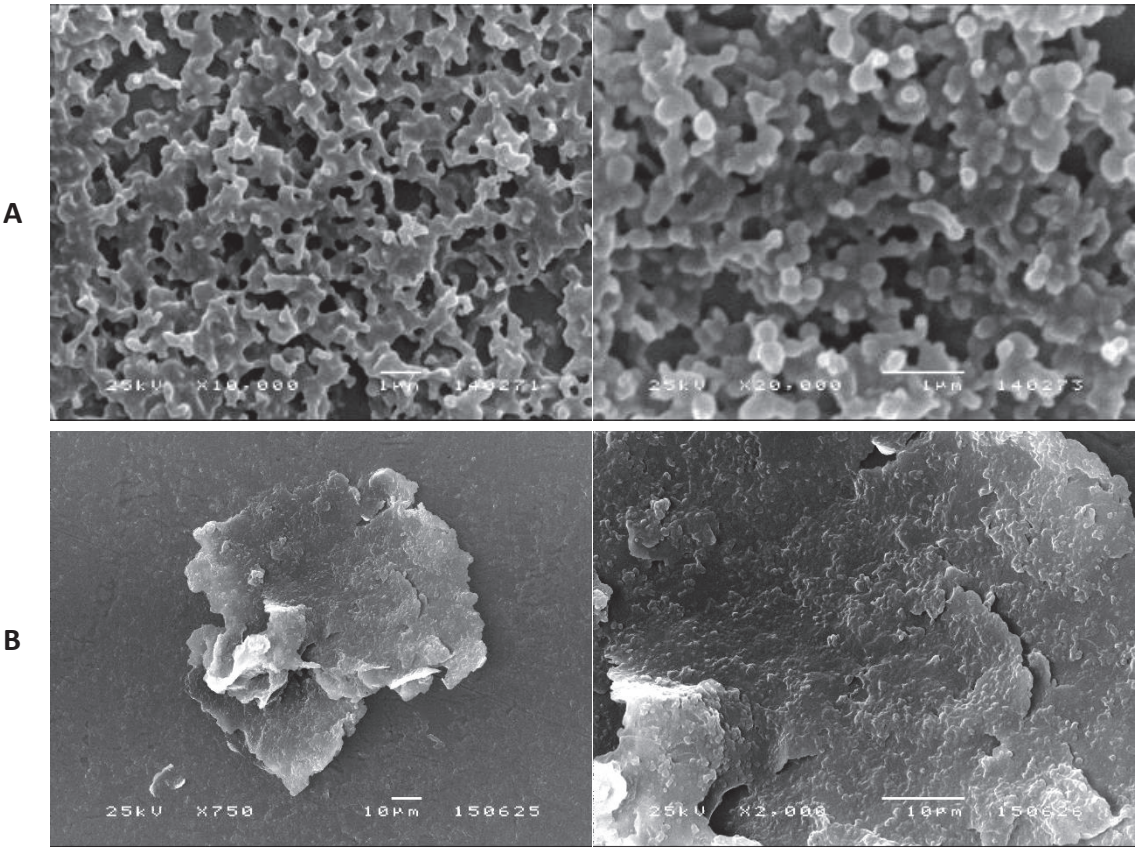


Figure 4.3. SEM images of uncoated phytosomes (A) and coated phytosomes (B). A, left: magnification = 10,000x, scale bar = 1 µm; A, right: magnification = 20,000x, scale bar = 1 µm; B, left: magnification = 750x, scale bar = 10 µm; B, right: magnification = 2,000x, scale bar = 10 µm.

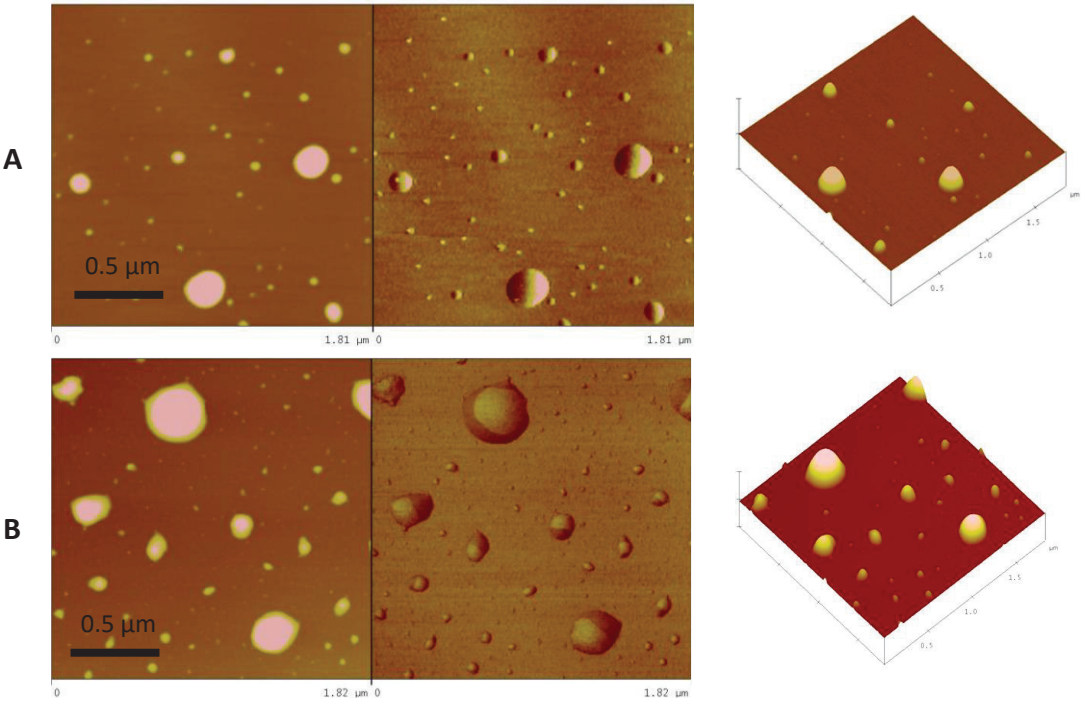


Figure 4.4. Topographical, phase contrast and 3D AFM images of the uncoated phytosomes (**A**) and of the chitosan coated phytosomes (**B**). Scale bars represents 0.5 µm.

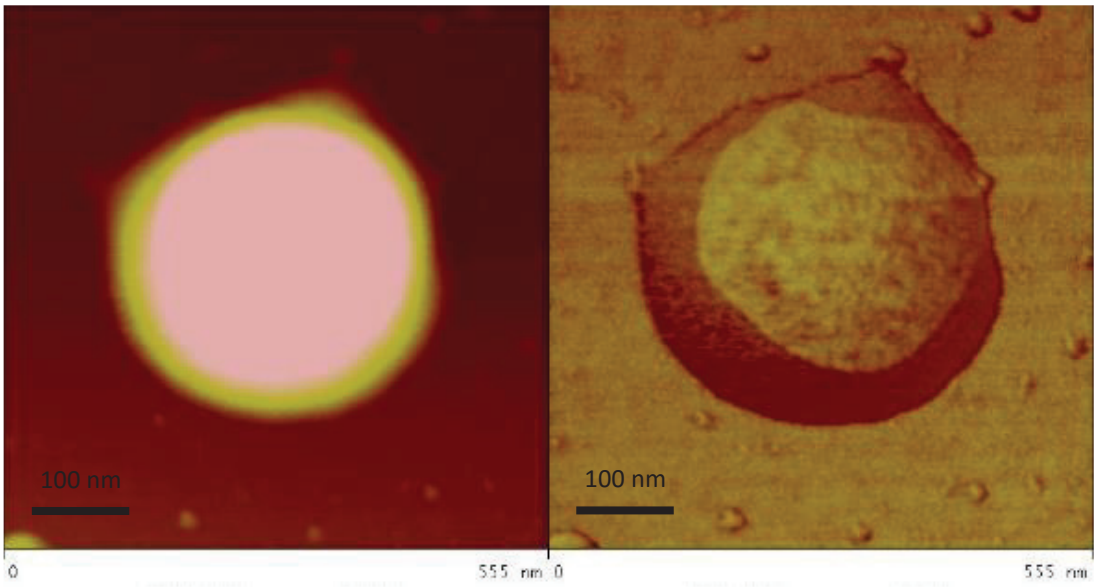


Figure 4.5. Topographical and phase contrast of a single chitosan coated phytosome nanoparticle. Scale bar represents 100 nm.

was clear the presence of a rough coating involving a softer material. The inner, softer material is similar to the verified for the uncoated particles, and thus this image could represent a phytophospholipidic core involved by the TPP cross linked chitosan coating. Additional AFM images were presented at annex **9**.

The formation of the complex between the PdC, cholesterol and the antibacterial extract **E4** was confirmed by DSC (Figure **4.6**) and DRIFTS (Figure **4.7** and **4.8**).

Thermograms for cholesterol (A), extract **E4** (B), PdC (C), physical mixture (1.2:20:42.4 w/w) of cholesterol, extract and PdC (D) and loaded phytosomes (E) were obtained as presented in Figure **4.6**. The cholesterol thermogram (Figure **4.6 A**) possess a single sharp peak with onset at 147.77ºC and maximum at 150.17ºC which should occur due to the pure compound melting (reported melting point of 147-149ºC). As in the case of the *P. madagascariensis* extract **E4** (Figure **4.6 B**), a single mild endothermic peak was verified starting at 34.33ºC and with maximum at 58.33ºC. The phase diagram of PdC (figure **4.6 C**) presented two resolved peaks. The first peak at 133.83ºC was mild and might be related to the movement of phospholipidic polar head during the heating process[224–227]. The second peak was sharp with onset at 169.30ºC and maximum at 180.33ºC. This highly endothermic (ΔH = 28.25 J/g) peak should occur due to the phase transition from gel to liquid crystalline state during which occur the non-polar tail melting[226,227].

When the physical mixture of cholesterol, extract and PdC (Figure **4.6 D**) is analysed, an apparent summation between those elements was verified. The endothermic peak from **E4** was maintained and also peaks with similar thermal behaviour to those of PdC were verified but presenting a lower onset and peak value. This implies the occurrence of minimal interactions between those elements. However, the analysis of the

thermogram corresponding to phytosomes (Figure **4.6 E**) showed the disappearance of the endothermal peaks corresponding to the extract **E4** or cholesterol, which occur due to the presumable formation of interactions between those molecules and the phospholipids. A broad and mild peak was present at 135ºC which could correspond to the PdC polar head movement due to the heating process as its onset (125.06ºC) was similar but lower to the verified for the first peak of PdC (126.23ºC). The disappearance of the sharp second peak of phospholipids (Figure **4.6 C**) and the decrease of the transition temperature from 180.33 to 170.67ºC after the formation of phytosomes (Figure **4.6 E**) was been suggested to be related to the establishment of strong interactions between the polar head of the phospholipids and natural products and the consequent formation of a new entity[224–227]. Those interactions allow the free turning of the hydrocarbon chains of the PdC and favours its enwrapping of the polar head containing the natural product[224–227].

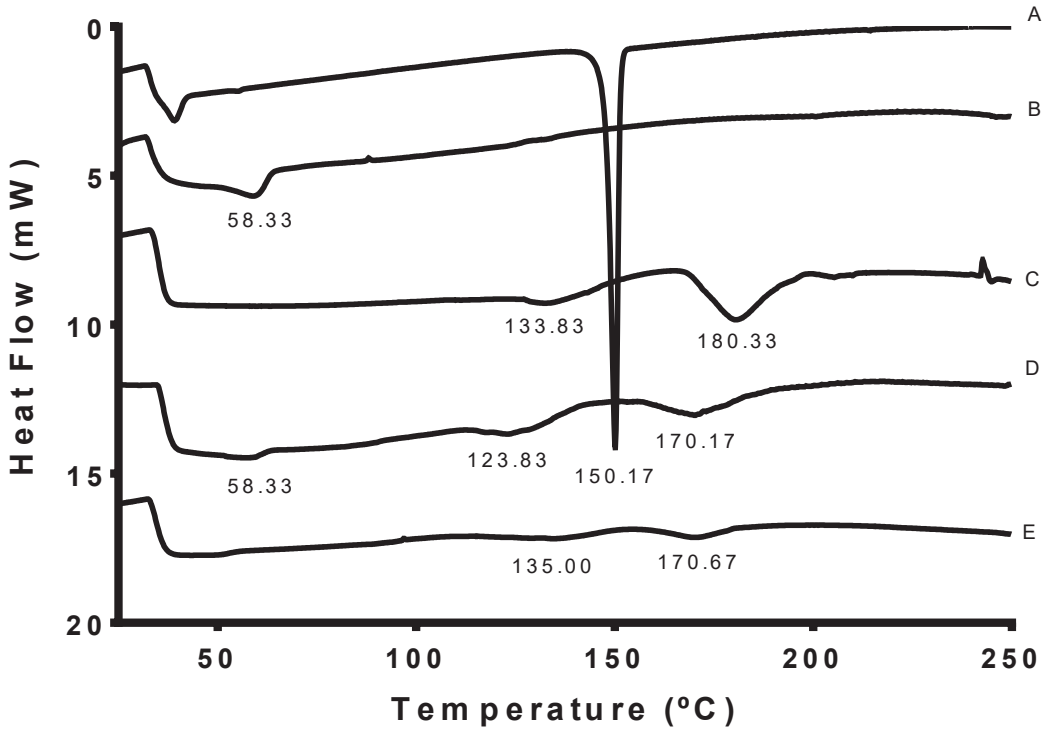


Figure 4.6. DSC thermogram of (A) cholesterol, (B) extract E4, (C) PdC, (D) physical mixture of cholesterol, extract and PdC and (E) phytosomes. The isolated values represent the peak temperature (ºC). Individualized spectra were presented at annex 9.

The infrared spectroscopy analysis of the coated and uncoated phytosomes, its components and their mixtures was performed in order to confirm the complexation of phytosomes (Figure **4.7**) and the chitosan/TPP coating (Figure **4.8**). While the cholesterol IR spectra was concordant with literature references, for the extract **E4**, no standard reference was available. From the HPLC-DAD characterization of this extract presented

in chapter **3**, it is known that compounds bearing ketone, phenol and hydroxyl groups were present, and thus, those signals were found between 3500 and 3200 cm^{-1} (medium broad peak, hydrogen bonded O-H stretching vibrations), 2918 and 2848 cm^{-1} (C-H stretching in aromatics); 1707 cm^{-1} (C=O stretching), 1585 cm^{-1} (C=C stretching), 1456 cm^{-1} (CH_3-CH_2 bending), 1165 cm^{-1} (C-O stretching).

The characteristics peaks of PdC were present in the DRIFTS spectra as a broad peak between 3500 and 3200 cm^{-1} (hydroxyl and/or amine stretching), 2926 and 2856 cm^{-1} (C-H stretching at fatty acid residues), 1741 cm^{-1} (carbonyl stretching of the fatty acid ester), 1456 cm^{-1} (CH_3-CH_2 bending), 1228 cm^{-1} (P=O stretching), 1059 cm^{-1} (P-O-C stretching) and 970 cm^{-1} (N-$(CH_3)_3$ stretching)[228,229,249].

The analysis of phytosome IR spectra presented a shift and broadening of the signal corresponding to the hydroxyl O-H stretching (3500-3200 cm^{-1}), to the aliphatic phosphate (P=O stretching) and to the choline quaternary ammonium (N-$(CH_3)_3$ stretching) groups of the phospholipids. No relevant alterations were found in the bands of the aliphatic carbon chains of the fatty acids (2924, 2850 and 1460 cm^{-1}). These observations suggest that some weak physical interactions between free hydroxyl of the natural components and the polar groups of phospholipids took place during complex formation with dispersion of the extract components into the phospholipid matrix[228,229,249].

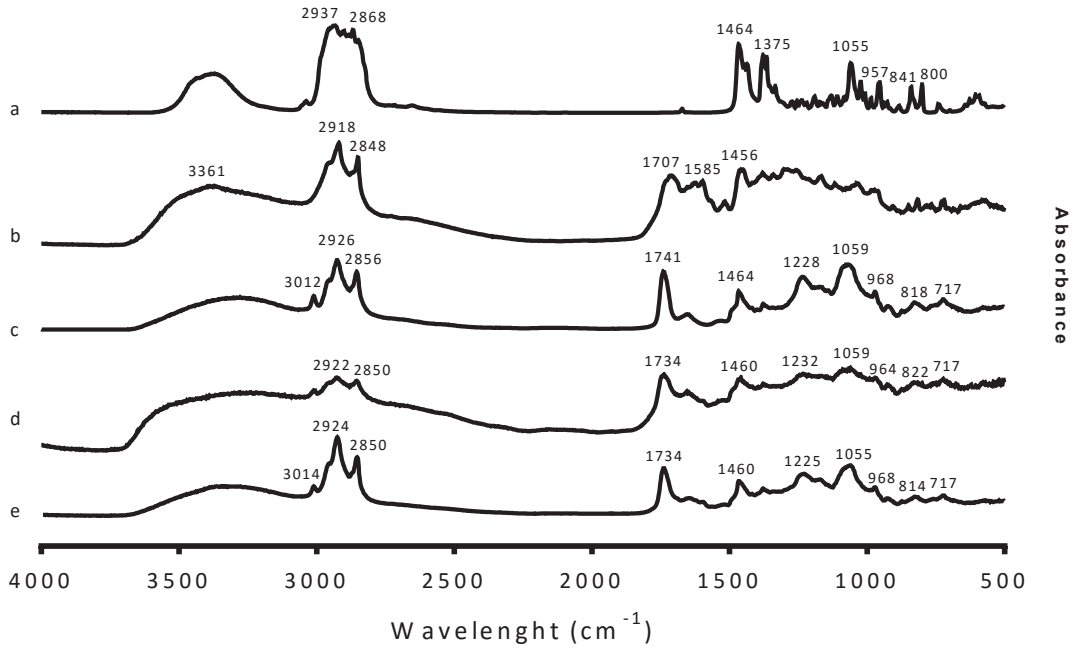


Figure 4.7. DRIFTS spectra of (a) cholesterol, (b) extract, (c) PdC, (d) physical mixture of cholesterol/extract/PdC and (e) phytosome. Individualized spectra available at annex **9**.

The evaluation of chitosan/TPP coating by DRIFTS was based on the reference peaks of chitosan at 2865 cm^{-1} corresponding to C-H stretching and those at 1646, 1569 and

1371 cm^{-1} corresponding to different vibrational modes of the N-H amide[386]. At the physical mixture of phytosomes with chitosan and TPP, the reference signals for chitosan appear diluted in the phospholipidic base, however, when analysing ChiPS, the chitosan reference signals remain identifiable at 1633, 1531 and 1371 cm^{-1} with some shift which could be due to a conformational change due to the crosslinking with TPP. Also, a substantial change in the signals for the phytosome were noted, with disappearance of the major peaks at 3014 and 1055 cm^{-1} along with a clear diminishing of the peaks at 2924, 2850, 1734 and 1460 cm^{-1}. This should represent the occurrence of coating by chitosan at most of the surface of the phytosomes, which is coherent with the observations of AFM visualization and the zeta potential shift for a positive value.

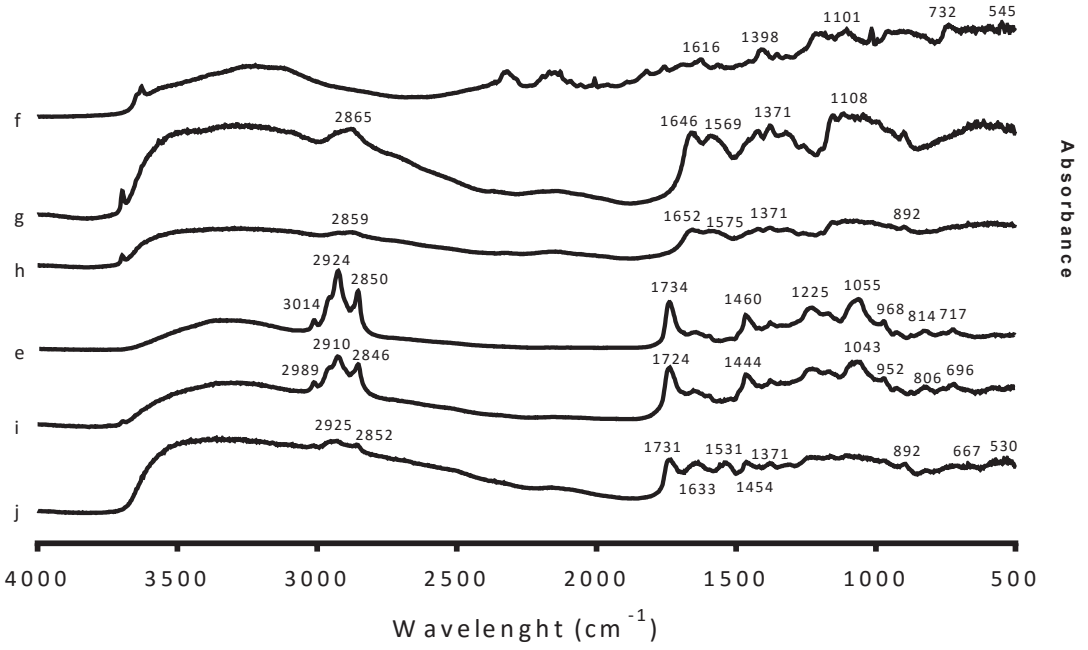


Figure 4.8. DRIFTS spectra of (f) TPP, (g) chitosan, (h) physical mixture of TPP/Chi, (e) loaded phytosomes, (i) physical mixture of TPP/Chi/PS and (j) loaded Chi coated phytosomes. Individualized spectra available at annex 9.

4.3.4. Determination of encapsulation efficiency (EE%) and drug loading (DL)

The EE% of the *P. madagascariensis* extract in the nanoparticle system was estimated based on the major antibacterial diterpenic compound (7α,6β-dihydroxyroyleanone, Roy) present in the extract. In order to calculate the content of Roy in the ChiPS a standard curve was established with high linearity (R^2 = 0.9999) in the range of 0.005 to 1 mg/mL (y = 32946x - 139.43) with adequate sensitivity (LOD: 0.002; LOQ: 0.004). The EE% in the ChiPS system was of 57.7±0.1%. In other studies, when phytosomes were combined with a polymer coating[249] or conjugated with a metallic core[257], similar or lower encapsulation efficiencies were verified.

The DL was of 0.670±0.001% for Roy. The low drug loading for the reference compound in the extract was expected as the extract contains only 4.6±0.02% (w/w) of Roy. However, if we based on the amount of extract that contains 0.67 mg of Roy, we estimate an extract DL of 14.56% which is similar or superior to the drug loading of phytocomponents in similar phytosome formulations[249].

4.3.5. *In vitro* release studies

The evaluation of Roy release from the lyophilized ChiPS in PBS at different physiologic pH was used to establish the release profile of those nanoparticles (Figure **4.9**). The particle mass to volume proportion selected was adequate in order to assure the maintenance of the sink conditions. An initial burst release was verified with approximately 70% of the Roy being released from the ChiPS after 1h. A second phase of slower release was verified, reaching maximum release at 10h (100.8% at pH 7.4 and 82.9% at pH 5.5). Those media represent the physiologic pH verified at the surface of the skin (5.5)[387] and at blood vessels (7.4)[388].

It was known that quinone-methide compounds, as the case of Roy, possess an improved reactivity in acidic media, due to the protonation of the carboxyl oxygen with possible further esterification or nucleophilic substitution of this group[389]. This improved reactivity could explain the incomplete release curve verified at pH 5.5, in which some Roy degradation could occur at this more acidic pH.

The skin pathogens *S. aureus* and *S. epidermidis* possesses a doubling time, in a reconstructed epidermis model, of about 55 and 230 min, respectively[390]. According to this study, after 1h, about three fourths of the total Roy have already been released (70.18-75.86% of total Roy), the release curve of Roy from phytosomes should be adequate for the control of those bacterial strains multiplication.

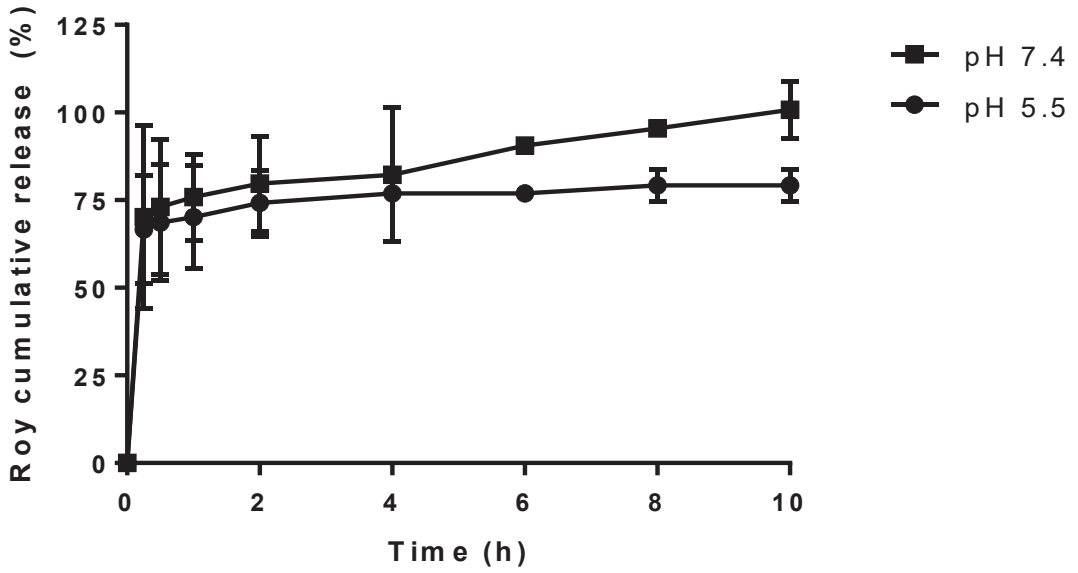


Figure 4.9. *Cumulative release of Roy from Chi-PS at phosphate buffer saline adjusted to pH 5.5 or pH 7.4 (T = 37ºC).*

4.3.6. *In vitro* permeation studies

The diffusion patterns of permeation of Roy through PDMS in a Franz cell model showed a lower flux value for the Roy permeation from ChiPS (0.52±0.17 μg/cm^2/h) in comparison to this compound permeation from the non-encapsulated extract E4 (1.90±0.08 μg/cm^2/h) (Figure **4.10** and **4.11**). This observation is favourable for the formulation as a high permeability of the antibacterial compounds was unintended. This result could be explained by a conjugation of factors. At first, in the extract, their components were solubilized in the PBS which favourers the direct contact to the membrane while in ChiPS the Roy was incorporated into the polymeric-phytosome formulation and it is required that Roy is released from this matrix for the membrane permeation to occur. This effect was only responsible by a partial contribution on the lower flux as the release studies demonstrated a rapid initial release up to 75% in the first hour. A second factor could be related to the saturation of PDMS membrane by substances adsorbed at its surface that could therefore limit the crossing of Roy. The main substance that could be retained is chitosan due to its cationic properties which could led to adsorption at the negative charged PDMS membrane[391]. This strengthens our speculation that only residual permeation of ChiPS would occur through transdermal pathways other than the follicular pores.

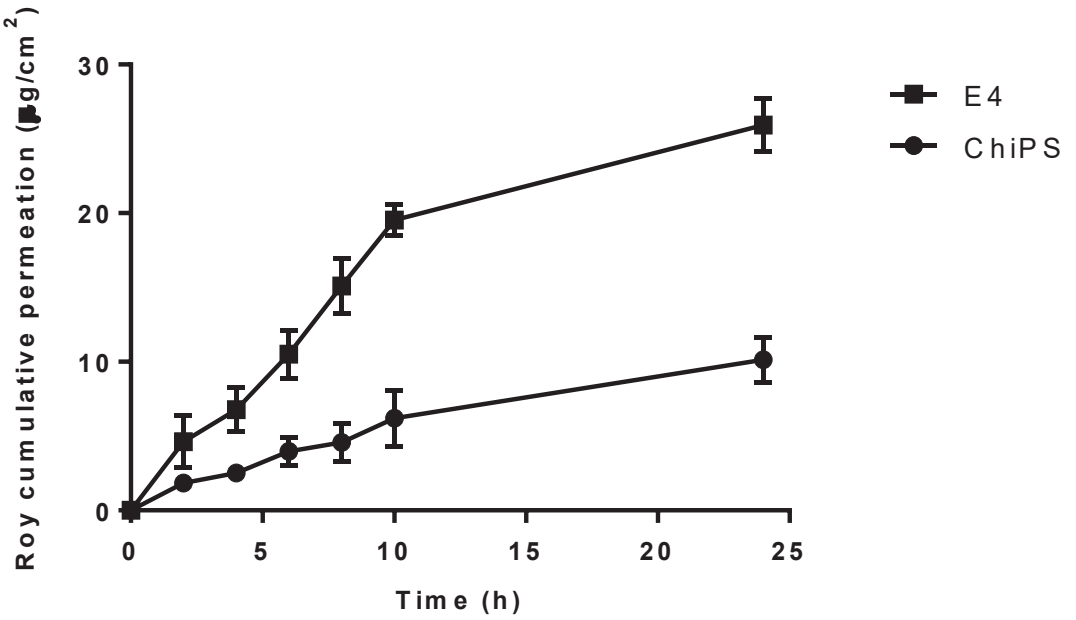


Figure 4.10. Permeation of Roy from the *P. madagascariensis* extract **E4** or loaded ChiPS in Franz cell permeation model using PDMS membrane.

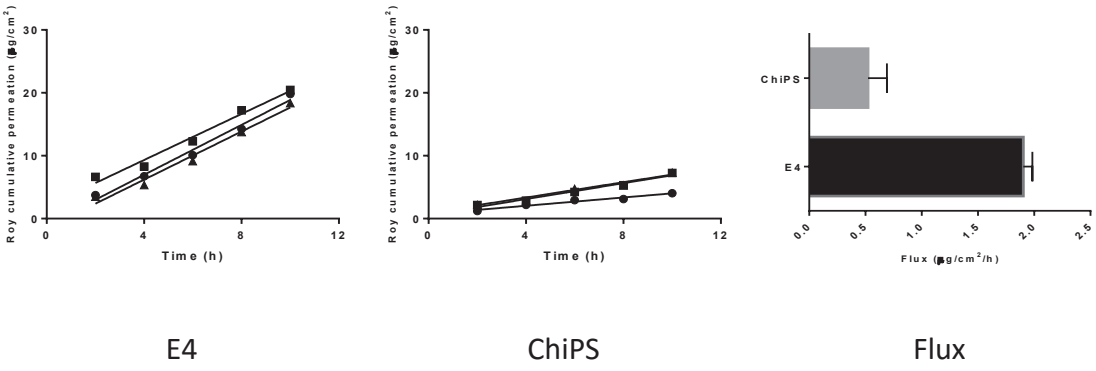


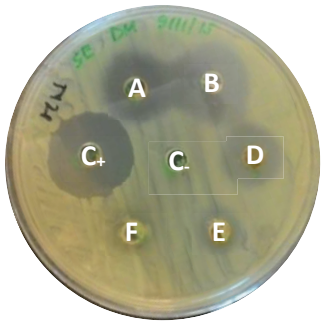
Figure 4.11. Permeation of Roy from the *P. madagascariensis* extract **E4** or ChiPS in Franz cell permeation model using PDMS membrane.

4.3.7. Biological activities

4.3.7.1. Antibacterial activities

The activity of the selected extract from *P. madagascariensis* (**E4**), the main diterpene (6β,7α-dihydroxyroyleanone, Roy) and the phytosomes incorporating the extract or the blank sample. A preliminary qualitative evaluation by well diffusion assay in *S. epidermidis* (Table **4.8**) demonstrated an apparent maintenance of the anti-staphylococcal activity with similar inhibition halo verified between the extract **E4**, the main compound Roy and the ChiPS. Also, chitosan/TPP mixture presented a slight inhibition which could indicate potential bacterial growth inhibition effects at higher concentrations.

Table 4.10. Inhibition diameter against *S. epidermidis* ATCC 12228.

Analyte		Inhibition (mm)	Plate Image
A	Roy	19	
B	*P. madagascariensis* **E4**	21	
D	ChiPS	17	
E	Chi/TPP mixture	8	
F	PBS	5	
C$_-$	DMSO	5	
C$_+$	Vancomycin	22	

Samples **A-D** were tested at 1 mg/mL. **A** and **B** were diluted in DMSO; **C** and **D** were diluted in PBS. Roy - 7α,6β-dihydroxyroyleanone; **C**$_+$ **=** PBS – phosphate saline buffer pH 7.4; **C**$_-$: DMSO - Dimethyl sulfoxide.

The quantitative evaluation of the antibacterial activity of the extract **E4**, Roy, loaded phytosome and the correspondent chitosan coated loaded phytosomes was performed by the determination of the MIC value for two bacterial pathogens of the human skin: *S. aureus* (including a MRSA strain) and *S. epidermidis*. The extract **E4** showed potent antibacterial activity with MIC values in the range of 1.95-7.81 μg/mL. The isolated compound Roy showed inhibitory effects lower than those of the extract (MIC 15.6-62.5 μg/mL), which suggests the existence of synergic effects between this compound and other antibacterial abietane diterpenes.

The antibacterial activity of the loaded phytosomes (MIC 3.91-15.6 μg/mL) and of the chitosan coated loaded phytosomes (MIC 0.98-31.3 μg/mL) was proportional or superior to those of the reference antibiotic vancomycin (MIC 0.98-7.81 μg/mL). Those values represent a clear increment in the antibacterial activity of the phytosome formulations over the extract, which become evident by the normalized MIC values as function of the Roy content in each sample (Table **4.9**).

Table 4.11. Antibacterial activity of *P. madagascariensis* components including phytosomal forms.

Analyte	Microorganism strain / MIC (μg/mL)		
	S. aureus ATCC 25923	*S. aureus* (MRSA) CIP 106760	*S. epidermidis* ATCC 12228
Roy	62.5	15.6	-
E4	3.91 (0.18)	1.95 (0.09)	7.81 (0.36)
Phytosome	3.91 (0.05)	3.91 (0.05)	15.6 (0.21)
ChiPS	0.98 (0.02)	15.6 (0.25)	31.3 (0.50)
Control (Vancomycin)	0.98	7.81	7.81

Roy - 7α,6β-dihydroxyroyleanone; E4 – *P. madagascariensis* extract 4; ChiPS – chitosan coated phytosomes of E4.

Values under brackets represent MIC values normalized for the Roy content: ( ) = MIC x $[Roy]_{sample}$

It was also verified that the ChiPS showed pronounced selectivity for *S. aureus* over *S. epidermidis*. This was a favourable observation due to the commensal character of *S. epidermidis* which unfrequently present a pathogenic behaviour and contrasts with *S. aureus* which is the most common skin pathogen[339,392].

4.3.7.2. Cytotoxicity in human keratinocyte cell line

The evaluation of *in vitro* cytotoxicity in HaCaT human skin keratinocyte cell line showed low or none cytotoxicity. At the MIC concentration for *S. aureus* strains the ChiPS formulation showed less than 10% reduction in cell viability (Figure **4.12**). Also, it seems that the encapsulation process originates a ChiPS formulation with lower cytotoxicity (IC_{50} = 85.87 μg/mL) over the original extract **E4** from *P. madagascariensis* (IC_{50} = 56.77 μg/mL). Similar results have been observed for *Calendula* phytosomes with the phytosome formulation presenting lower cytotoxicity than the plant extract[257]. Also, all formulation components of ChiPS were environmental friendly, biocompatible and approved for skin use[393–395]. This means that such components should not be responsible for any toxicological effects. The residual toxicity verified for higher concentrations of ChiPS could be explained by toxicological specificities of the nanosystems. A recent review on nanotoxicological effects of lipid carriers pointed that occlusive effects possess relevant influence in the cytotoxicity profiles of nanosystems at higher concentrations[396]. Such effect was verified even in nanoparticles formulations composed only by biocompatible ingredients as in the betamethasone lipidic nanoparticles developed by Silva et al. (2015)[397]. The use of *in vivo* models could help to exclude such effects.

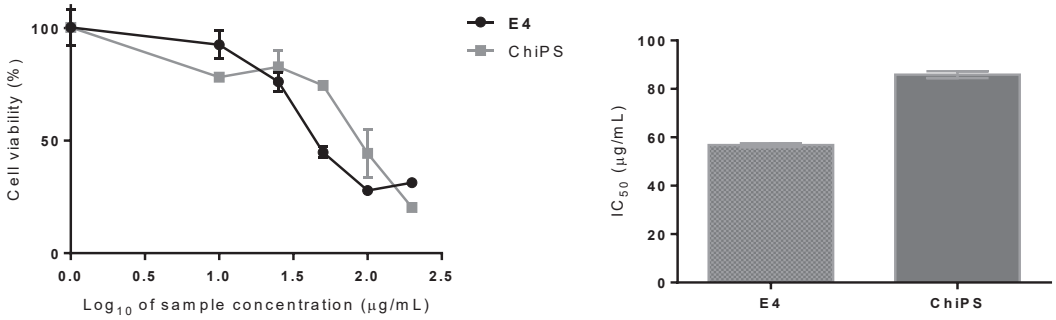


Figure 4.12. Growth inhibitory effects of the *P. madagascariensis* extract E4 and its correspondent ChiPS.

4.3.7.3. Acute and sub-chronic mice irritation study

This *in vivo* study was intended to discriminate the presence of any potential irritation effect due to the application of the extract **E4** or the correspondent chitosan coated loaded phytosomes. The overall result showed negligible irritation or edema in each treatment group with PII < 0.5 (Figure **4.12** and Table **4.9**). As expected, the positive control group had a slight irritation based on a PII score of 0.67.

Table 4.12. Irritation and edema score of each mice by group.

Mice	Group	Erythema + Edema			SPI	PII
		24h	48h	72h		
1	**A** (CMS gel + ChiPS)	0 + 0	0 + 0	0 + 0	0	0.25
2		0 + 0	0 + 0	0 + 0	0	
3		0 + 0	1 + 0	2 + 0	1	
4		0 + 0	0 + 0	0 + 0	0	
5	**B** (CMS gel + E4)	0 + 0	0 + 0	0 + 0	0	0
6		0 + 0	0 + 0	0 + 0	0	
7		0 + 0	0 + 0	0 + 0	0	
8	**C** (CMS gel)	0 + 0	0 + 0	0 + 0	0	0
9		0 + 0	0 + 0	0 + 0	0	
10	**D** (SLES 5%)	0 + 0	0 + 0	4 + 0	1.33	0.67
11		0 + 0	0 + 0	0 + 0	0	

4.4. Chapter conclusions

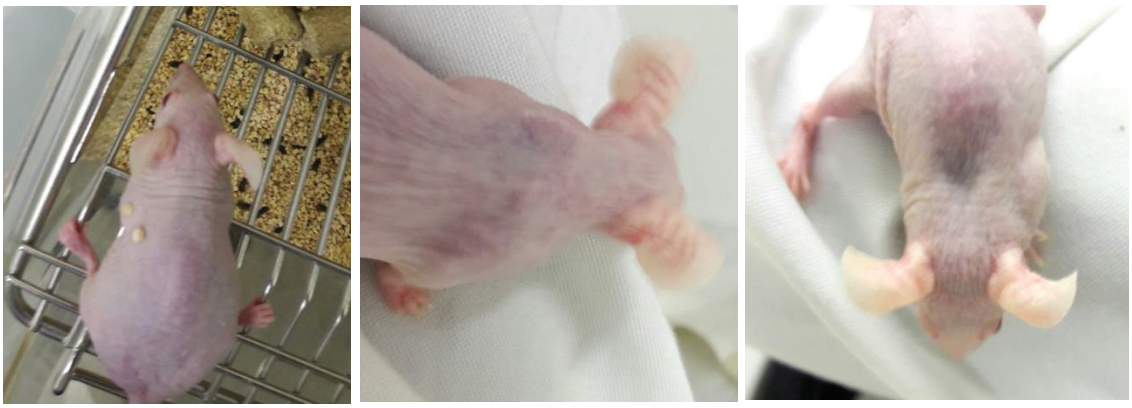
The present study was focused on the development of an antibacterial formulation based on the phytosome of a potent antibacterial extract from *P. madagascariensis* (**E4**). This plant had been used in traditional African medicine for the treatment of cutaneous

wounds. As the extract possesses high yields of abietane diterpenes as 6β,7α-dihydroxyroyleanone (Roy) those should be responsible for its antibacterial activity.

The use of phytosome strategy have been successful for both *per oz* as topical delivery of natural compounds. Also, chitosan has been on focus because of the combination of the properties of a water soluble, cationic, bioadhesive, biodegradable polymer of natural origin with some intrinsic antibacterial activity. The combination of the phytosome strategy with the encapsulation by chitosan surged as a strategy for the improvement of the antibacterial activity of the extract **E4**.

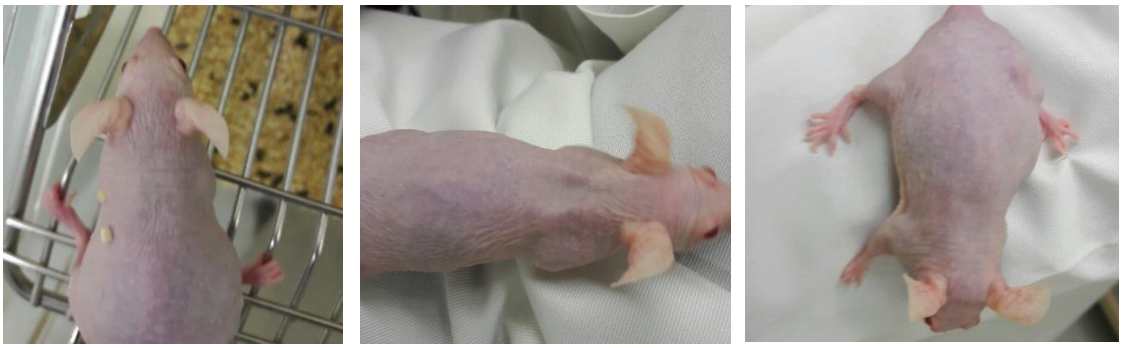
The phytosomes were produced by the conjugation of PdC, cholesterol and the extract into acetone by the effect of stirring and heat, and recovered by solvent evaporation with dispersion in acetic acid solution and complexation confirmed by DLS and DRIFTS. Those nanovesicles were spherical, amorphous, with an average size of 191.3±75.3 nm (DLS), a low size dispersion and neutral charge. The positive charge is important for the skin adherence of the formulation. The spherical shape was maintained but phase contrast in AFM showed the presence of a substance (presumably chitosan) involving the phospholipidic particles. This was confirmed by the IR

i)



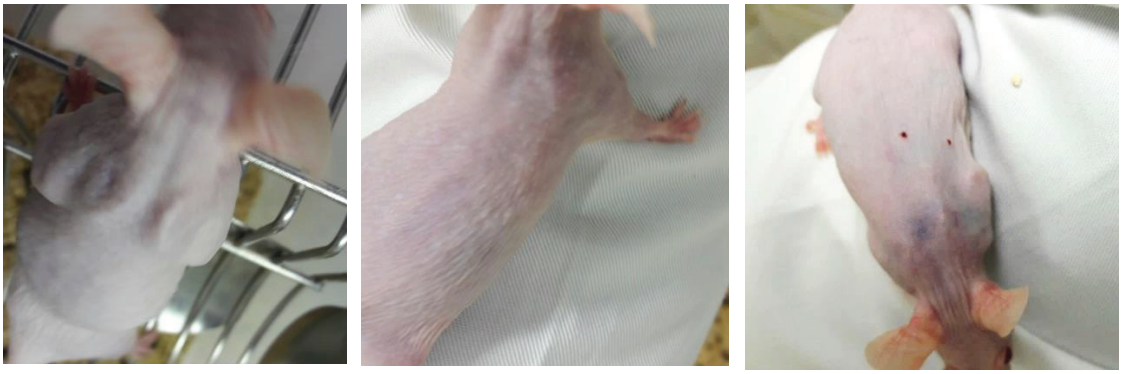
24h **48h** **72h**

ii)



24h **48h** **72h**

iii)



24h **48h** **72h**

Figure 4.13. Photography of three animals at 24, 48 and 72h of sample application: i) Only a slight erythema was observed after 48h of application in this subject, but a well-defined erythema was visible after 72h; ii) No visible alterations from 24-72h; iii) No erythema or edema visible up to 72h when an eschar was formed at the zone of application.

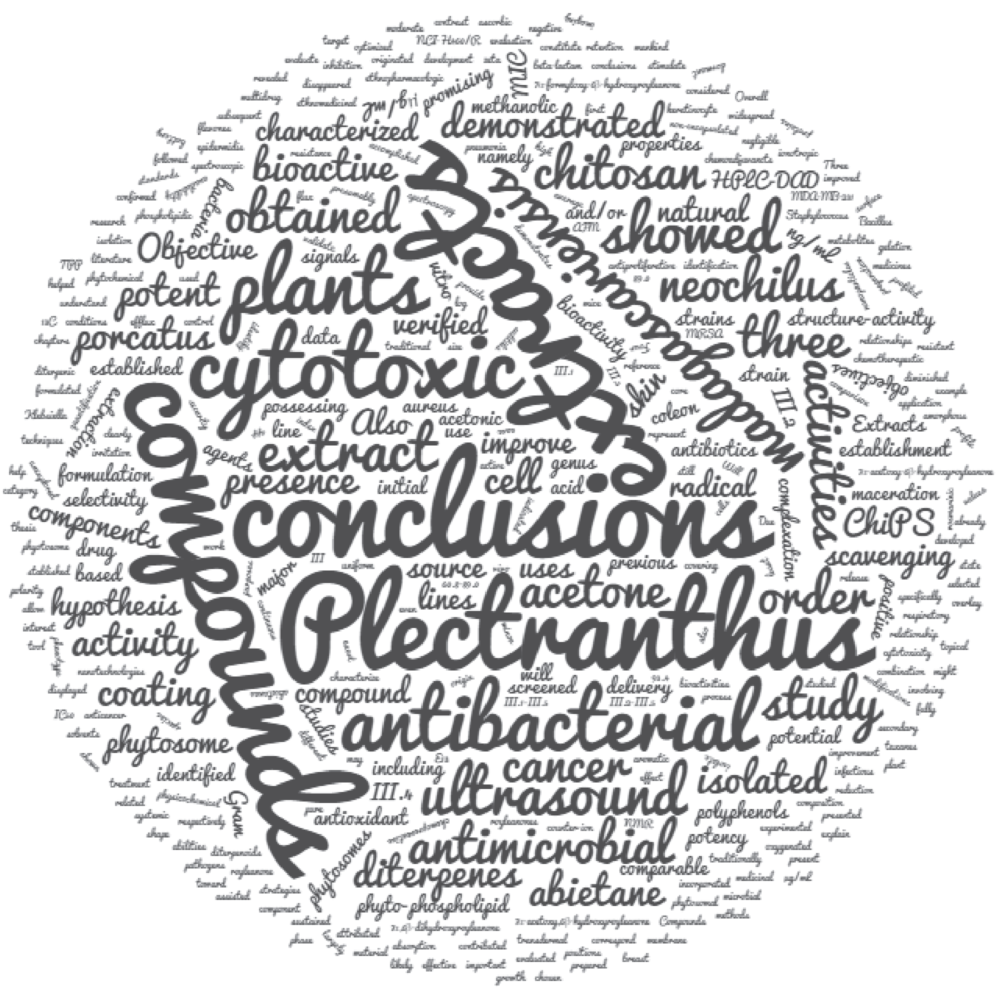
spectroscopy of the ChiPS in which, the signals for the phytosome were clearly diminished or even disappeared but the chitosan signals were still present, which might represent the presence of a coating of chitosan covering the phospholipidic core. Those nanoparticles showed a sustained release profile with a reduction of the membrane flux in comparison to the non-encapsulated extract. This could be important for the retention of the active compounds at the skin surface were they should exert its antibacterial effects, with minor transdermal systemic absorption.

The antibacterial effects were enhanced by this strategy, being the anti-staphylococcal activity superior even to the reference antibiotic, vancomycin, and active against MRSA. Also, negligible *in vitro* keratinocyte cytotoxicity and *in vivo* mice skin irritation were verified, which implies the security of this formulation topical application.

This promising formulation could be used as an innovative ideal skin antibacterial as it is active against skin pathogens, including MDR strains; non-toxic for keratinocytes at MIC; different antibacterial mechanisms of its elements (chitosan and extract diterpenes), including synergic effects between some of them (extract diterpenes).

Future work directions should focus on the long term stability of those particles along with its activity against other skin pathogens, including other resistant strains, and biofilm forming bacteria.

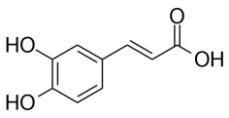
Chapter V



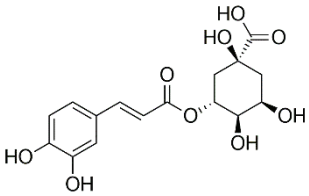
conclusions
Plectranthus
compounds
cytotoxic
antibacterial
extracts
madagascariensis
plants
ultrasound
obtained
showed
chitosan
demonstrated
neochilus
three
activities
extract
antimicrobial
acetone
study
order
isolated
cancer
abietane
diterpenes
coating
activity
hypothesis
phytosome
porcatus
potent
Objective
bioactive
characterized
components
presence
scavenging
ChiPS
compound
phyto-phospholipid
antioxidant
identified
verified
natural
structure-activity
strains
establishment
maceration
radical
antibiotics
polyphenols
potency
comparable
source
uses
improve
cell
delivery
screened
potential

5. Conclusions

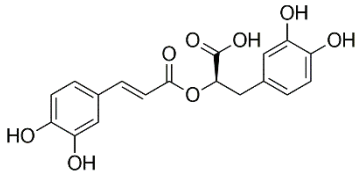
In this study, three plants of the *Plectranthus* genus (*P. madagascariensis*, *P. neochilus* and *P. porcatus*) were selected, based on their ethnopharmacological uses (respiratory conditions as cough and asthma, cutaneous wounds and scabies for *P. madagascariensis*; dyspepsia, hepatic insufficiency, chills, cough and a runny or blocked nose for *P. neochilus*; and aromatic purposes for *P. porcatus*). From those plants, 26 extracts (**E1**-**E26**) were obtained by the combination of extraction techniques (infusion, decoction, microwave, ultrasound, maceration and supercritical fluid extraction) with different polarity solvents (water, acetone, methanol and $scCO_2$). All the prepared extracts were briefly profiled by HPLC-DAD and the majority of the components were identified as polyphenols (caffeic acid (**IV.1**), chlorogenic acid (**IV.2**) and rosmarinic acid (**IV.3**)), diterpenes (7α-acetoxy,6β-hydroxyroyleanone (**IV.4**) and coleon U (**IV.5**)) and flavones (rutin (**IV.6**) and naringenin (**IV.7**)).



IV.1



IV.2



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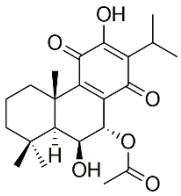

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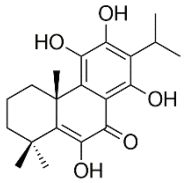

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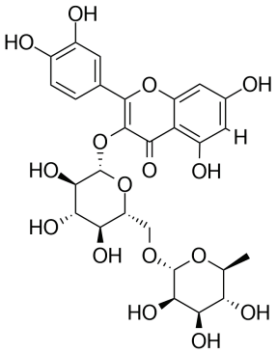
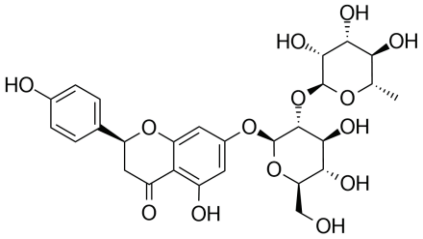


IV.6



IV.7

The extracts were screened for their antimicrobial (Gram positive, Gram negative bacteria and yeasts), antioxidant (DPPH method) and cytotoxic (MDA-MB-231 cancer

cell line) activities. Extracts from *P. madagascariensis* (acetone maceration **E5** and acetone ultrasound **E4**) and *P. neochilus* (acetone ultrasound **E13**) showed antimicrobial effects against Gram positive bacteria strains, namely, *Bacillus subtilis, Staphylococcus aureus* and *S. epidermidis* and a Gram negative bacteria strain, *Klebsiella pneumonia* (MIC values 1.95-250 μg/mL). No activity was verified against the tested yeast strains. The acetonic extract from *P. madagascariensis* prepared by ultrasound assisted extraction (**E4**) showed the more potent antimicrobial activity, comparable to reference antibiotics, against *S. aureus* strains (MIC < 3.91 μg/mL), including a MRSA strain (MIC < 0.98 μg/mL).

Considering the antioxidant activity, the methanolic extracts from the three plants showed the more potent radical scavenging activities at 100 ng/mL (60.8-89.0%). The remaing extracts tested showed moderated or low antioxidant properties (2.45-46.55%). The methanolic ultrasound extract of *P. madagascariensis* (**E6**) demonstrated the higher radical scavenging activity at 100 ng/mL (89.0%), which was comparable to the positive control, ascorbic acid (93.4%). Those antioxidant effects could be attributed to the presence of polyphenols (**IV.1** to **IV.3**) with known radical scavenging abilities.

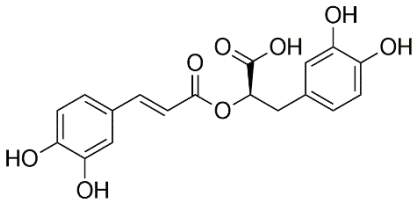
Considering the cytotoxic effects, all extracts tested showed low cytotoxic activity at 15 ug/mL (≥79.88% of cell viability) in the MDA-MB-231 breast cancer cell line. The maceration extract from *P. madagascariensis* (**E5**) showed moderate cytotoxic effects in the same cell line with IC_{50} of 64.52 μg/mL and was selected for further cytotoxicity studies.

The antibacterial and cytotoxic effects shown in the acetone extracts of *P. madagascariensis* (**E4** and **E5**) could explain the traditional uses in the treatment of respiratory and skin conditions related to infectious agents. The identified abietane diterpenes 7α-acetoxy,6β-hydroxyroyleanone (**IV.4**) and coleon U (**IV.5**) are described as antibacterial and cytotoxic compounds. The shown extract bioactivities could be explained by the presence of such compounds and thus validate their traditional uses.

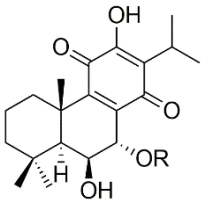
The more bioactive extracts from *P. madagascariensis* (organic solvent based extracts **E4**-**E9**) were characterized by HPLC-DAD with the identification and quantification of the major compounds. The remaining extracts were not included in the HPLC-DAD characterization due to their lack of bioactivity. Four compounds were generaly identified in the extracts using authentic standards overlay: rosmarinic acid (**IV.3**), 7α,6β-dihydroxyroyleanone (**IV.8**), 7α-acetoxy-6β-hydroxyroyleanone (**IV.4**) and coleon U (**IV.5**). A diterpenic compound, 7α-formyloxy-6β-hydroxyroyleanone (**IV.9**), was also isolated from the ultrasound acetonic extract of *P. madagascariensis* (**E4**) and characterized by ^{1}H and ^{13}C NMR. This compound was isolated for the first time from *P. madagascariensis*.

The cytotoxic effects of the pure compounds (**IV.3**-**IV.5** and **IV.8**-**IV.9**) were evaluated in a battery of cell lines including breast cancer (MDA-MB-231 and MCF-7), colon cancer (HCT116), non-small cell lung cancer (NCI-H460) and a normal lung bronchial (MCR-5) cell lines. All abietane diterpenes (**IV.4**-**IV.5** and **IV.8**-**IV.9**) originated growth inhibition of those cancer lines, with differences in potency and selectivity. The royleanones **IV.4**

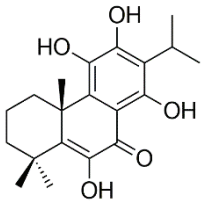
and **IV.8** displayed a high selectivity toward cancer cells (selectivity index of 4.3 and 3.2, respectively). Moreover, royleanones **IV.4** and **IV.8** were also evaluated in the NCI-H460/R cell line, which possess a multidrug resistant phenotype. The compounds showed similar cytotoxic effects in the multidrug resistant cell line (NCI-H460/R) and in the correspondent non-multidrug resistant cell line (NCI-H460). As the cytotoxic effects of those compounds were not reduced by the multidrug resistant phenotype, this showed that such compounds were not the target of efflux pumps. Thus, these diterpenes (**IV.4** and **IV.8**) were also active in the tested multidrug resistant cell lines.



IV.4



IV.8 R=H
IV.4 R=CHO
IV.9 R=$COCH_3$



IV.5

The structure-activity relationship of the royleanone abietanes, with modifications at the positions 6, 7 and 12, was established based on literature and experimental data. In this study, compounds possessing log P values between 2 and 5 correspond to compounds with improved cytotoxic effect. This observation revealed the relevance of the lipophilic properties for the cytotoxic effects. Also, the presence of an electron donating group in the position 6 and/or 7, seems to be important for high cytotoxic effects.

Compounds **IV.4**-**IV.5** and **IV.8**-**IV.9** may be considered promising compounds for further studies in order to fully understand the potential of the abietane diterpenoids as chemopreventive, chemoadjuvancts or chemotherapeutic agents.

To improve the bioactivity of the acetone ultrasound extract **E4**, a phytosomal formulation was prepared and characterized. This extract was selected due to its antibacterial potency and simple preparation. The phytophospholipidic complexation process was optimized to obtain the smallest and more uniform particles and followed by subsequent chitosan coating by ionotropic gelation using TPP as counter-ion. The obtained ChiPS were amorphous, uniform in shape, with an average size of 1082 ± 363 nm and zeta potential of +20.59 ± 12.02 mV. The phase contrast AFM imaging suggest the presence of a chitosan coating. This coating was also confirmed by the IR spectroscopy of the ChiPS. The signals for the phytosome were clearly diminished or even disappeared while the chitosan signals were still present, which should indicate the presence of chitosan coating over the phytosomal core. Those ChiPS showed a sustained release profile with a lower skin-like permeation fluxes in comparison to the non-encapsulated extract. This fact could be important for the retention of the active

compounds at the skin surface were they exert its antibacterial effects, with minor transdermal systemic absorption. The phytosomal formulation showed an up to 4-fold factor improvement of antibacterial effects against skin pathogens (*S. aureus* and *S. epidermidis*) with MIC values in the range of 0.98 to 31.3 µg/mL. Those values were comparable as those form potent antibiotics as vancomycin (MIC values in the range 0.98-7.81 µg/mL). Also, non relevant *in vitro* keratinocyte cytotoxicity and negligible *in vivo* mice skin irritation were verified, demonstrating the safety of this formulation for topical application.

The findings of this work allow the establishment of the formulated hypothesis to the category of thesis and state that:

- *Plectranthus* spp. plants, and specifically *P. madagascariensis*, *P. neochilus* and *P. porcatus*, are a source of bioactive compounds as indicated by their ethnomedicinal uses;

- The extracts and the isolated compounds from *P. madagascariensis* showed potent antibacterial and antiproliferative activities;

- The use of phytosome, and its further chitosan coating, correspond to an effective method for the potency improvement of the antibacterial extracts from *P. madagascariensis*.

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Annexes

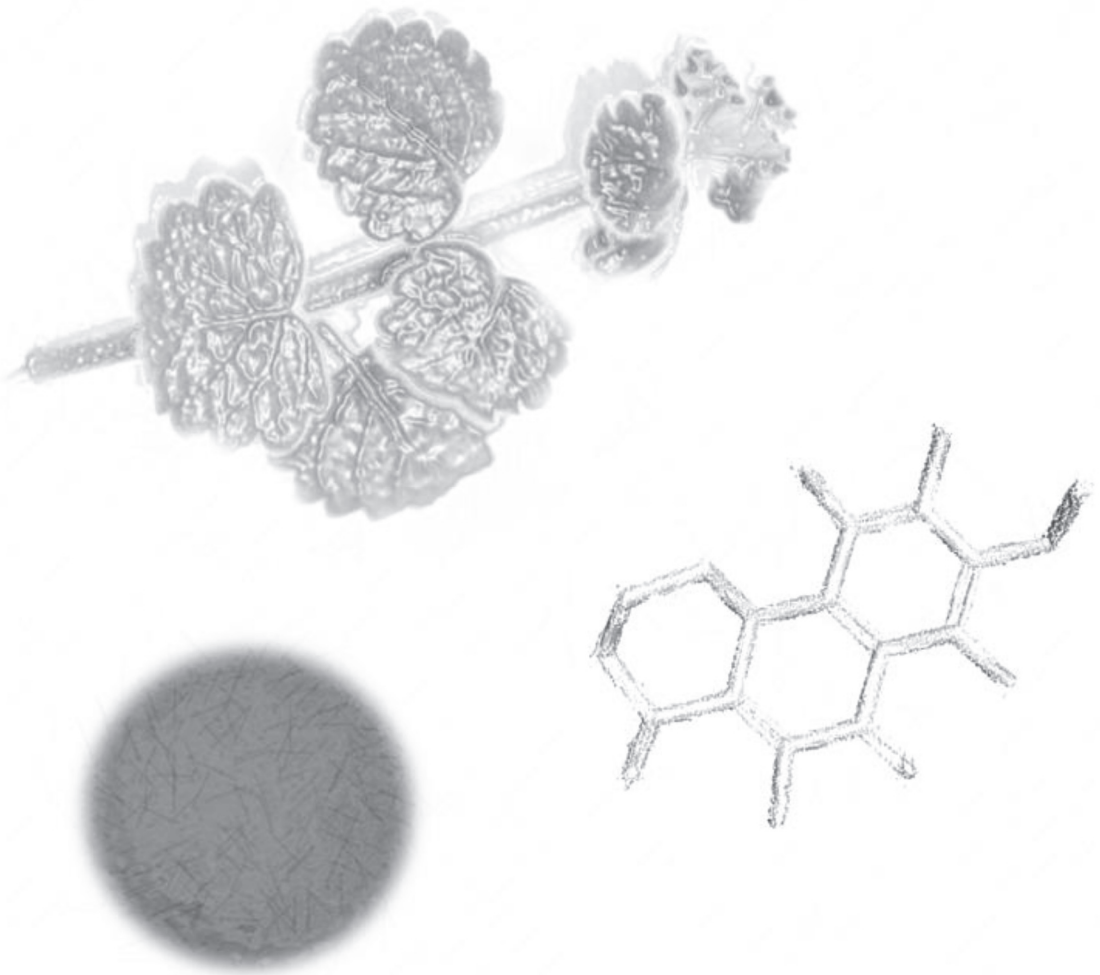


Table A in Annex 1. Phytosome preparation methodologies and used natural components published in the period 2006-2016.

Phytocomponent	Phospholipid	Proportion	Solvent	Time	Temperature	Method	Characterization	Ref.
Bacopa monnieri	Soya lecithin (30% PdC)	n/d	DCM	3h	60ºC	Anti-solvent (hexane)	SEM, DSC and FT-IR	[224]
Bergenin	PdC 80%	9:10	Ethanol	2h	60ºC	Solvent evaporation (vacuum)	SEM, TEM, XRD and FT-IR	[235]
Boswellic acid	Soy PdC 100%	1:1	DCM	2h	Room	Solvent evaporation	FT-IR	[247]
Calendula officinalis	Egg PdC 99%	3:1:1[†]	Chloroform	0*	Room	Solvent evaporation and thin film hydration	DLS and AFM	[257]
Catechin	Soy PdC 80%	1:1	DCM	3h	Δ	Anti-solvent (*n*-hexane)	SEM, DSC, XRD and NMR	[231]
Chlorogenic acid	HSPC	2:1	DCM	0*	Room	Anti-solvent (hexane)	DTA, FT-IR, DLS and SEM	[398]
Chrysophanol	Soy PdC 80%	1:1	DCM	5h	45-50ºC	Anti-solvent (hexane)	FT-IR, XRD, DSC and SEM	[399]
Curcumin	HSPC	1:1	DCM	2h	60ºC	Anti-solvent	DSC and OM	[227]
Curcumin	Soy PdC 100%	1:1	DCM	2h	Room	Solvent evaporation	DSC and FT-IR	[246]
Curcumin	Soy phospholipids	2:1 (w/w)	Ethanol	2h	50ºC	Anti-solvent (acetic acid 2%)	DLS, AFM, SEM, TEM, DSC and FTIR	[249]
Diosmin	Soy PdC 100%	1:2	DMSO:TBA	3h	room	Solvent evaporation (Lyo)	TEM, DSC and FT-IR,	[230]

Phytocomponent	Phospholipid	Proportion	Solvent	Time	Temperature	Method	Characterization	Ref.
Embelin	HSPC 90%	1:3	Ethanol	2h	60ºC	Solvent evaporation (SD)	FT-IR, RMN, DSC and XRD	375
Emodin	Soy PdC 80%	1:1	DCM	5h	45-50ºC	Anti-solvent (hexane)	FT-IR, XRD, DSC, SEM and NMR	228
Hesperetin	HSPC	1:1	DCM	2h	60ºC	Anti-solvent (hexane)	OM	400
Kaempferia galangal	Phospholipids	1:1	Ethanol	2h	60ºC	Solvent evaporation	DTA and SEM	374
Luteolin	Soy lecithin	2.5:1 (w/w)	THF	5h	Room	Solvent evaporation (vacuum)	UV, FT-IR, XRD and DSC	234
Luteolin	HSPC 90%	2.3:1	Ethanol	2h	45ºC	Solvent evaporation (vacuum, freeze drying)	UV, FT-IR, DSC, XRD and PCS	401
Mangiferin	Egg PdC 80%	1:1	Ethanol (80%)	2h	60ºC	Solvent evaporation	DSC, IR and SEM	240
Mangiferin	HSPC	2:1	DCM	2h	60ºC	Anti-solvent	DTA, FT-IR, DLS and SEM	402
Marsupsin	Soy phospholipids	2:1 (w/w)	Diethyl ether	0.25h	Room	Mechanical dispersion	TEM, FR-IR and NMR	245
Mitomycin C	Soy PdC 90%	1:3	THF	4h	40 °C	Solvent evaporation; nanoprecipitation	SEM, TEM, AFM, DSC, FTIR and XRD	229
Morin	Soy phospholipids	1:1.5	THF	2h	40°C	Solvent evaporation	TEM, FT-IR and XRD	225
Naringenin	Soy PdC 80%	1:1	DCM	3h	Room	Anti-solvent (hexane)	FTIR, DSC, NMR, XRD and SEM	226
Naringenin	HSPC	1:1	DCM	0*	Room	Anti-solvent (hexane)	n/d	403

Phytocomponent	Phospholipid	Proportion	Solvent	Time	Temperature	Method	Characterization	Ref.
Oxymatrine	PdC 60%	3:1	THF	3h	60◦C	Solvent evaporation (vacuum)	XRD and DSC	242
Puerarin	Soy phospholipids	1:1.2	$scCO_2$	n/a	n/a	SEDS	SEM, DSC, FT-IR and XDR	244
Quercetin	Soy lecithin	1:1	Ethanol	1h	Room	Solvent evaporation (vacuum)	DSC, FT-IR and XRD	404
Rutin	Egg lecithin	1:1	Methanol	3h	Δ	Solvent evaporation	DSC, FT-IR, XDR, SEM and TEM	355
Sinigrin	HSPC	1:1	DCM	0*	Room	Anti-solvent (hexane)	DLS, TEM, FT-IR and DSC	405
Silybin	PdC 80%	1:1	THF	2h	Room	Solvent evaporation (vacuum or SD)	ATR, NMR, XDR, DSC, SEM and TEM	239
Silybin	PdC 80%	n/d	Ethanol	0*	Room	Solvent evaporation (vacuum)	SEM, TEM and DSC	250

*until dissolves; †PdC : cholesterol : calendula (w/w); Δ – reflux; n/d – not disclosed. n/a – not applicable; AFM – atomic force microscopy; ATR - attenuated Total Reflection; DCM – dichloromethane; DLS – dynamic light scattering; DMSO – dimethylsufoxide; DTA – Differential thermal analysis; FT-IR – Fourier transform infrared spectroscopy; HSPC – hydrogenated soya PdC; Lyo – lyophilisation; NMR – nuclear magnetic resonance; OM – optical microscopy; PCS – photon correlation spectroscopy; SD – spray drying; SEM - Scanning electron microscopy; TBA - T-butyl alcohol; TEM - Transmission electron microscopy; THF – tetrahydrofuran.

Annex 2

Table 1 (Annex 2). Examples of commercially available phytosomal products with use as functional cosmetic ingredients.

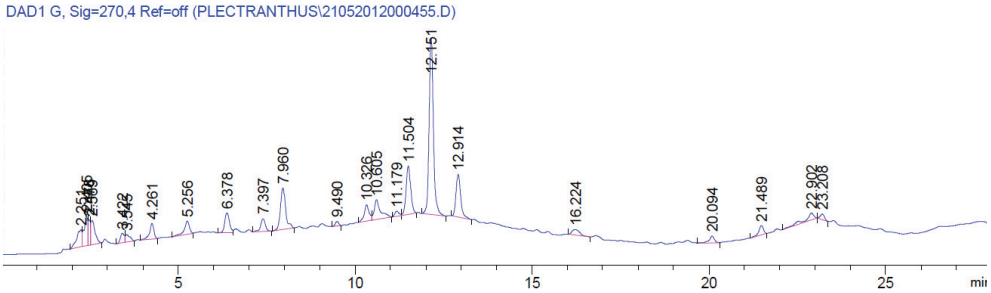
Designation	Content	Suggested uses[†]
Centella asiatica Selected Triterpenes®	Triterpenes including asiaticoside, asiatic acid and madecassic acid (30-35%) obtained from *Centella asiatica* leaf	Anti-wrinkles and Stretchmarks improver
Ginkoselect®	Ginkgoflavonglucosides (≥7%), ginkgoterpenes (≥2%), bilobalide (≥ 0.8%) and ginkgolides (≥0.8%) obtained from *Ginkgo biloba* leaf.	Antioxidant and Vasokinetic
Greenselect®	Polyphenols (25-30%) obtained from the young leaf of *Camellia sinensis*.	Antioxidant activity and whitening agent
Hawthorn®	Polyphenolic substances including Vitexin-2"-O-rhamnoside (≥3%) obtained from *Crataegus* spp. flowering top.	Antioxidant
Leucoselect®	Proanthocyanidins (25-30%) obtained from *Vitis vinifera* seed.	Antioxidant and capillarotropic
PA2®	Proanthocyanidin A2 (31-37%) obtained from *Aesculus hippocastanum* bark	UV protectant, trophodermic, firming and oval reshaping agent
Rexatrol®	Resveratrol (≥30%) obtained from *Polygonum cuspidatum* rhizome.	Antioxidant, Anti-ageing, Sirt1 modulator
Sericoside®	Sericoside (≥25%) obtained from *Terminalia sericea* root bark	Antioxidant, UV protectant, Anti-wrinkles, Soothing and Redensifier
Siliphos®	Silybin (about 30%) obtained from *Silybum marianum* fruit.	Anti-wrinkles and Retinoic acid-like activity
Silymarin®	Silybin like substances (15-20%) obtained from *Silybum marianum* fruit	Antioxidant and UV protectant

[†]Suggested uses claimed by Indena® SPA as described at www.indena.com/phytosome.

Annex 3

Original chromatograms from *Plectranthus* spp. extracts obtained according to methodology described at sub-section 2.2.5.

E1



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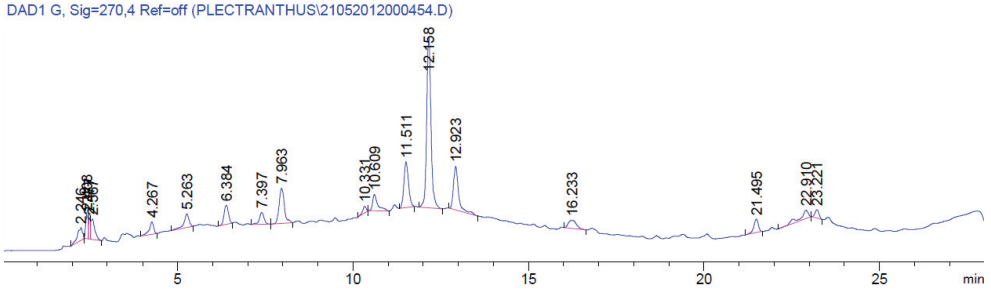

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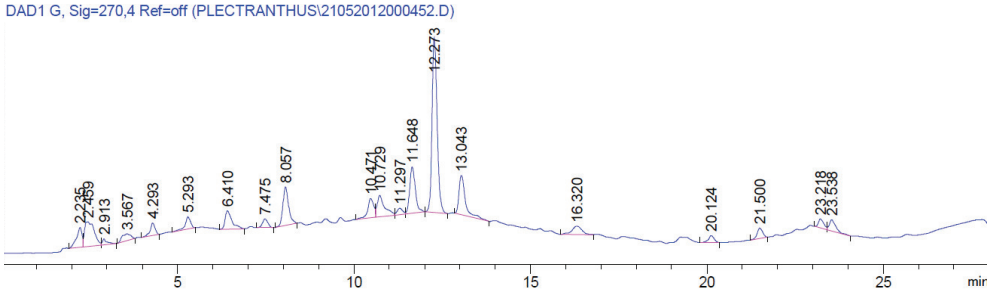

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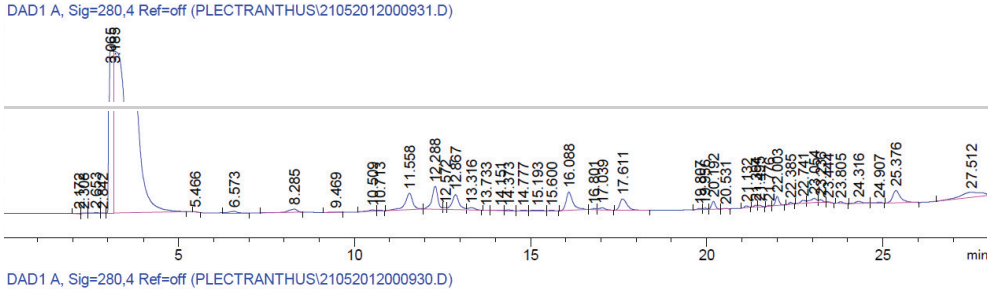

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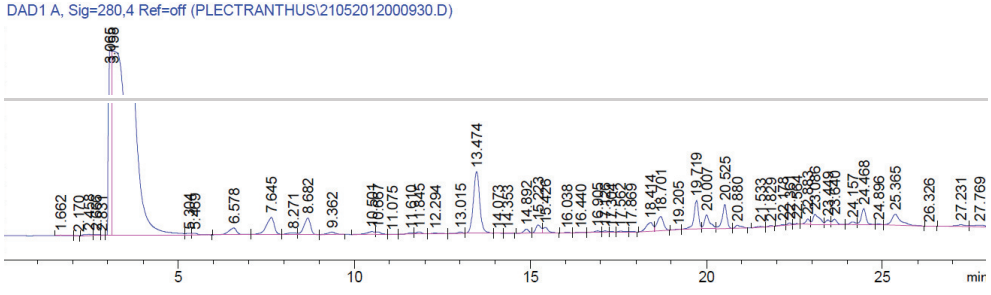

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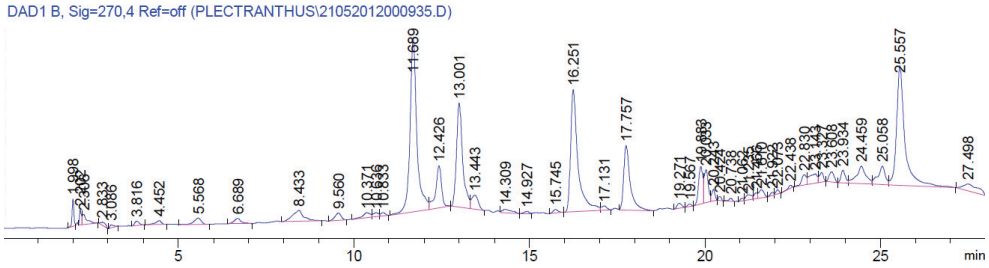

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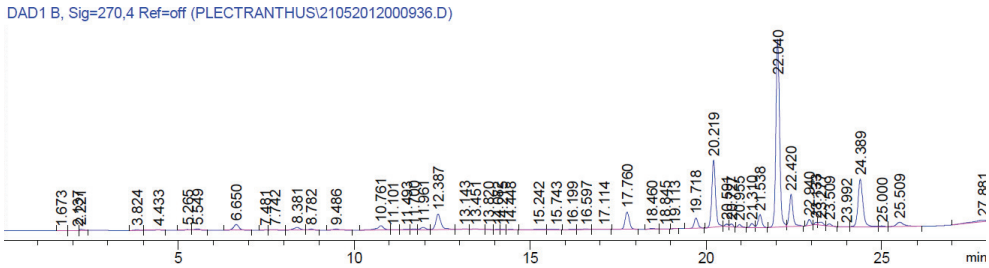

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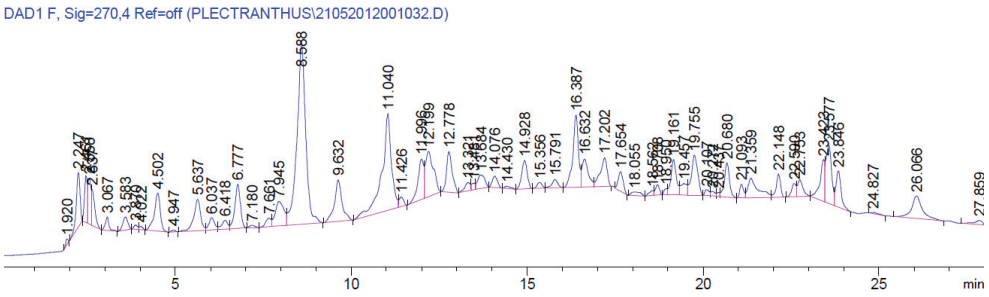

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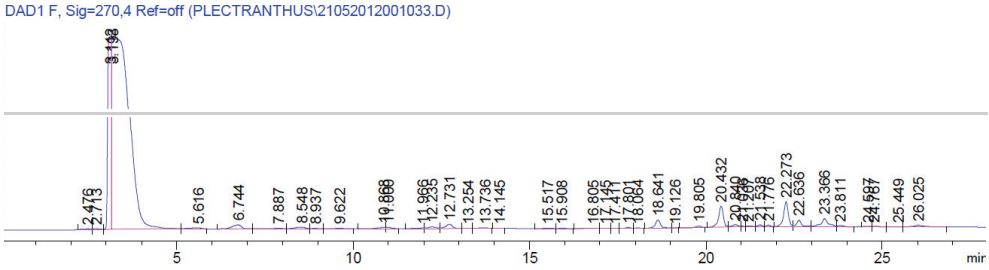

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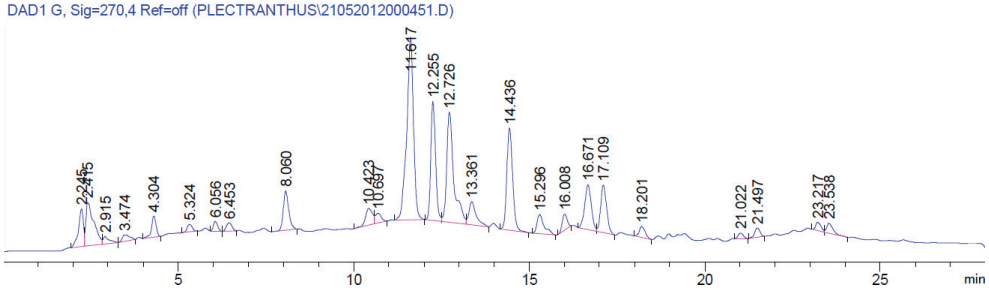

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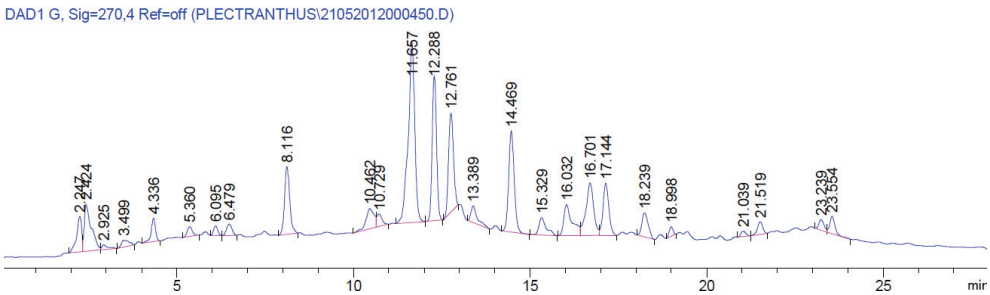

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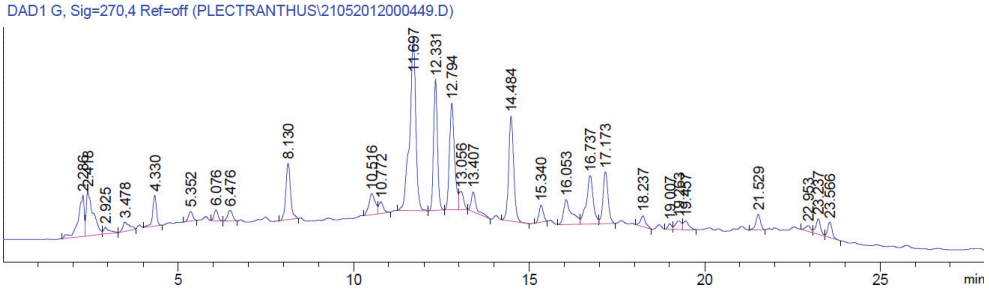

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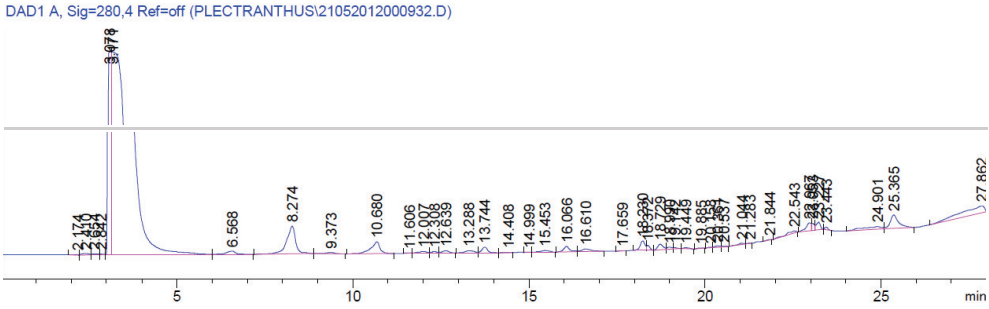

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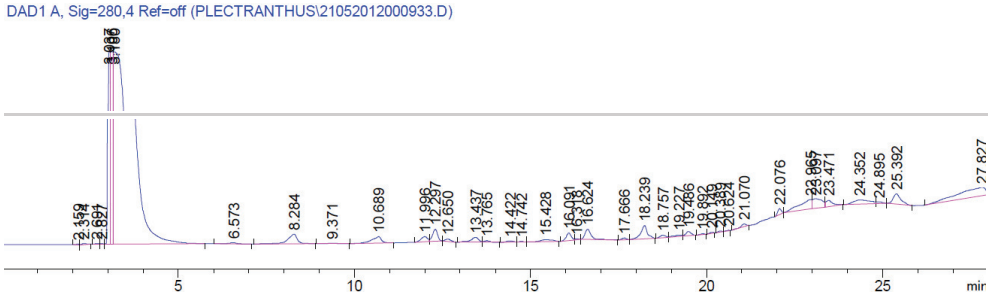

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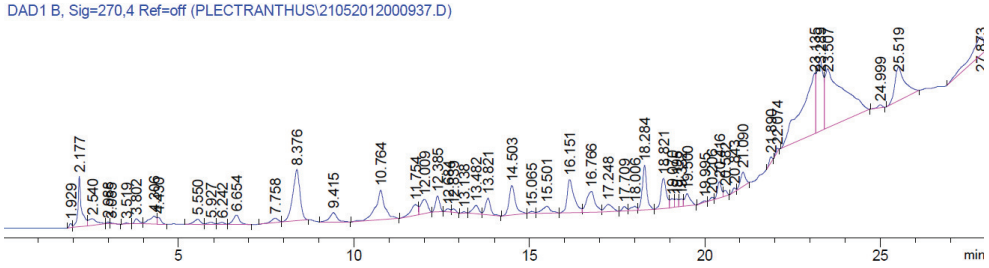

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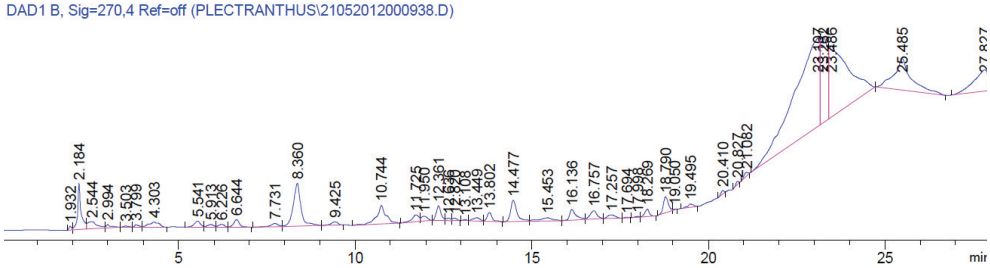

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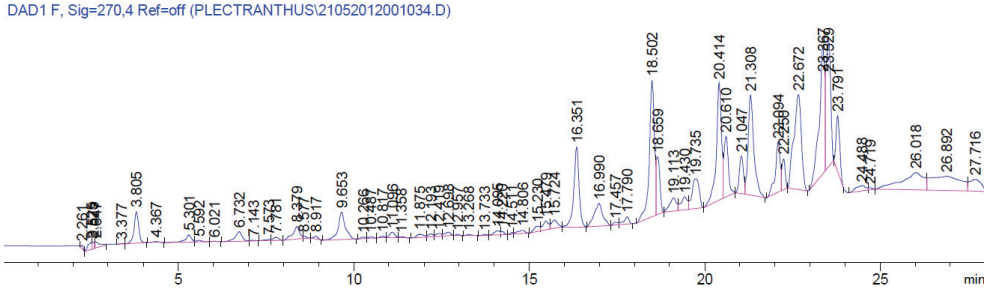

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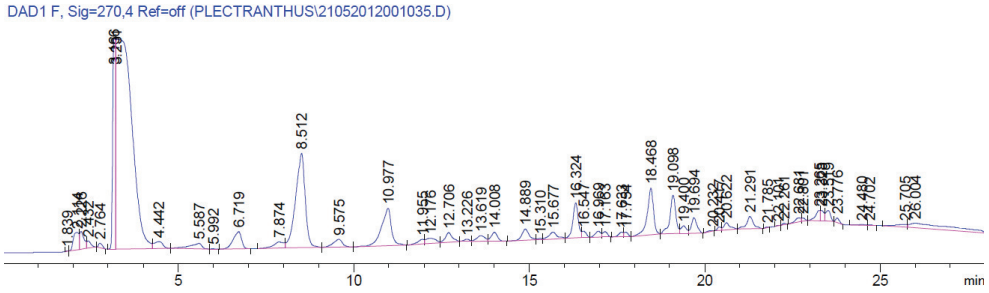

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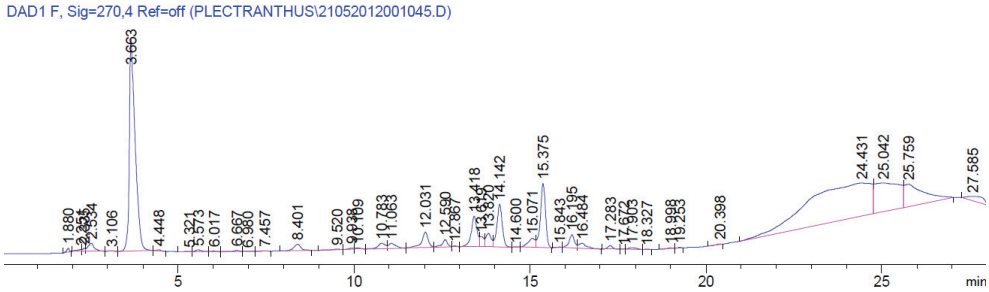

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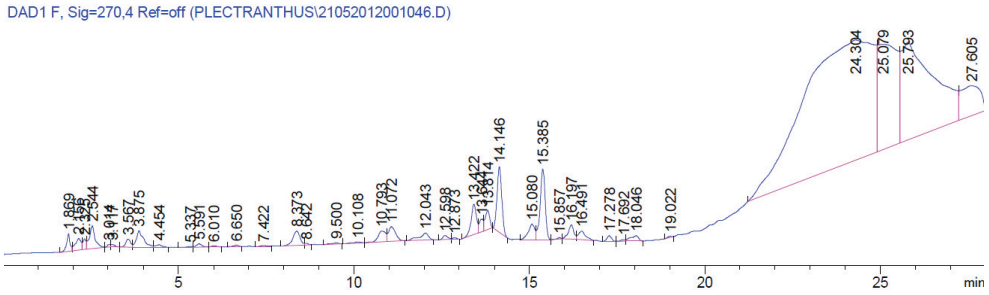

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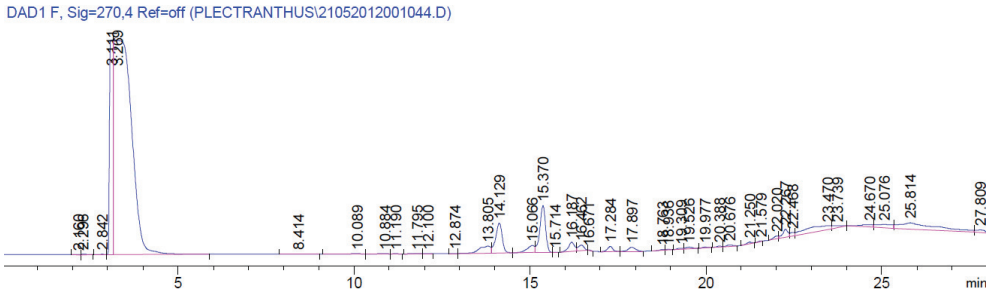

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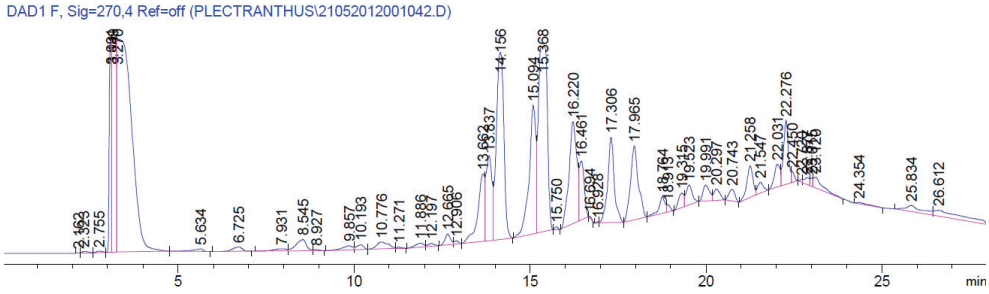

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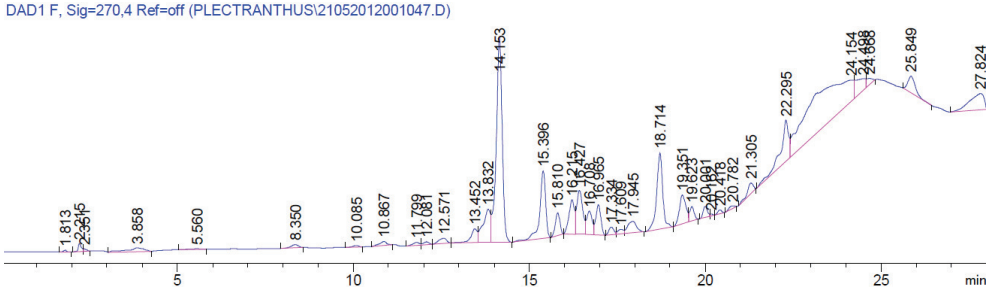

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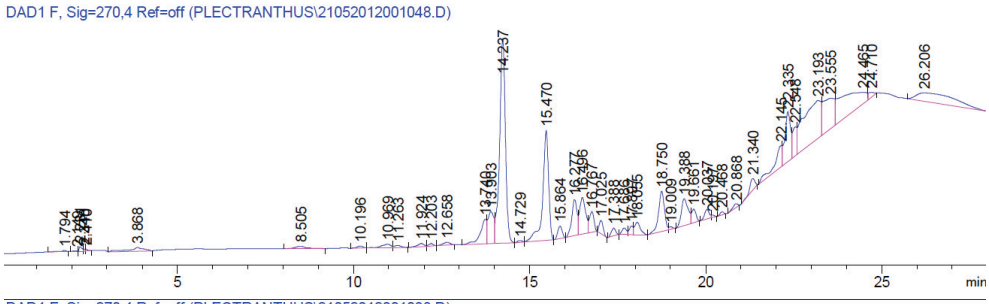
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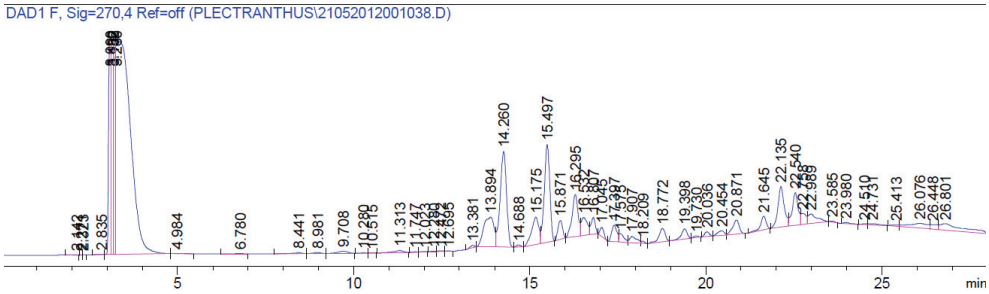
3.868
8.505
10.196
14.729
18.750
21.340
min

E25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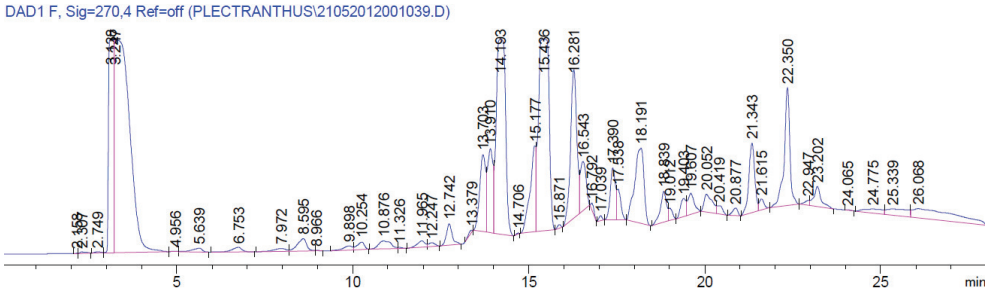
22.135
min

E26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5.639
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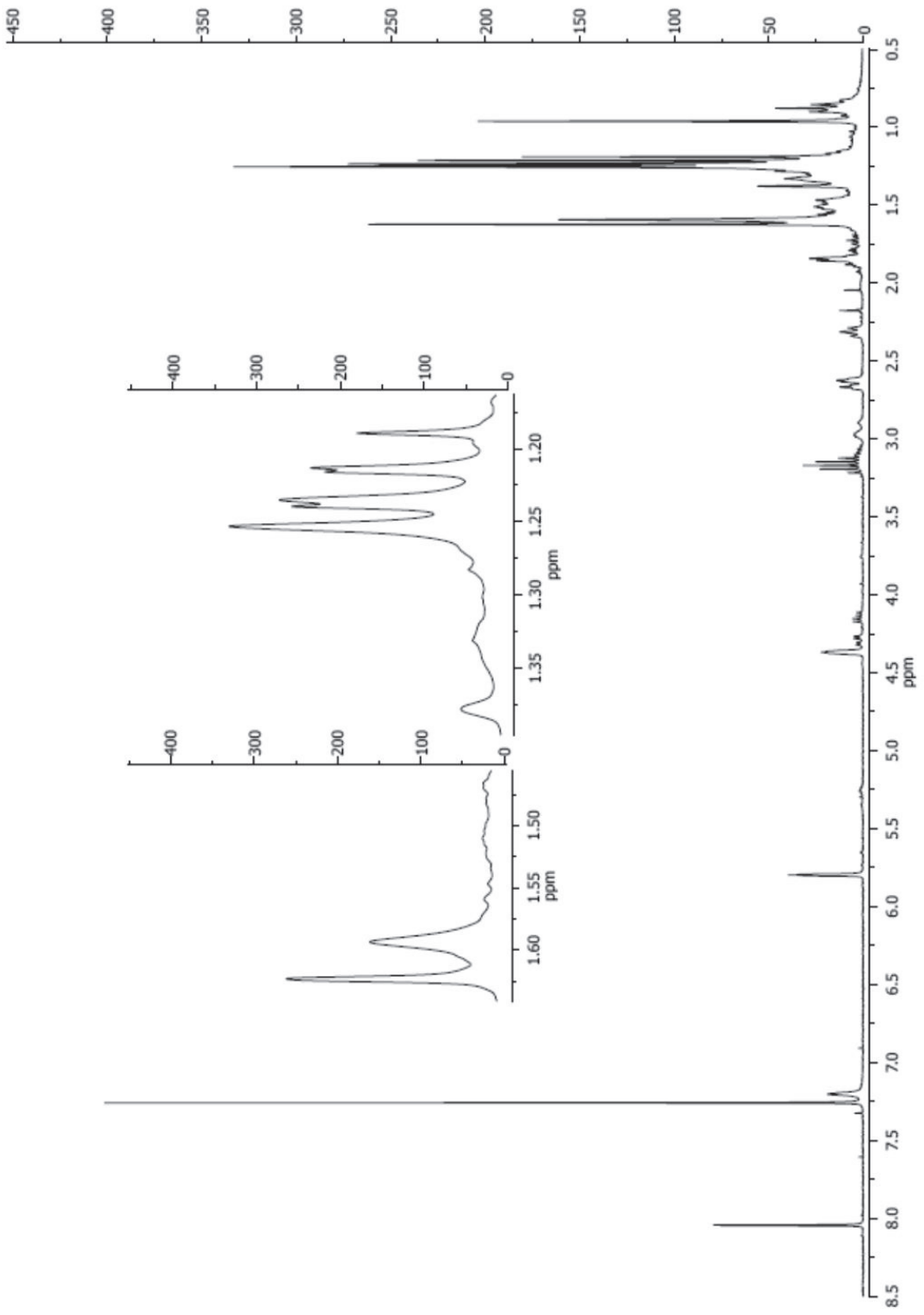
8.595
8.966
9.898
10.254
10.876
11.326
12.742
13.379
13.703
13.910
14.706
15.177
15.871
16.543
18.191
21.343
21.615
22.947
23.202
24.065
24.775
25.339
26.068
min

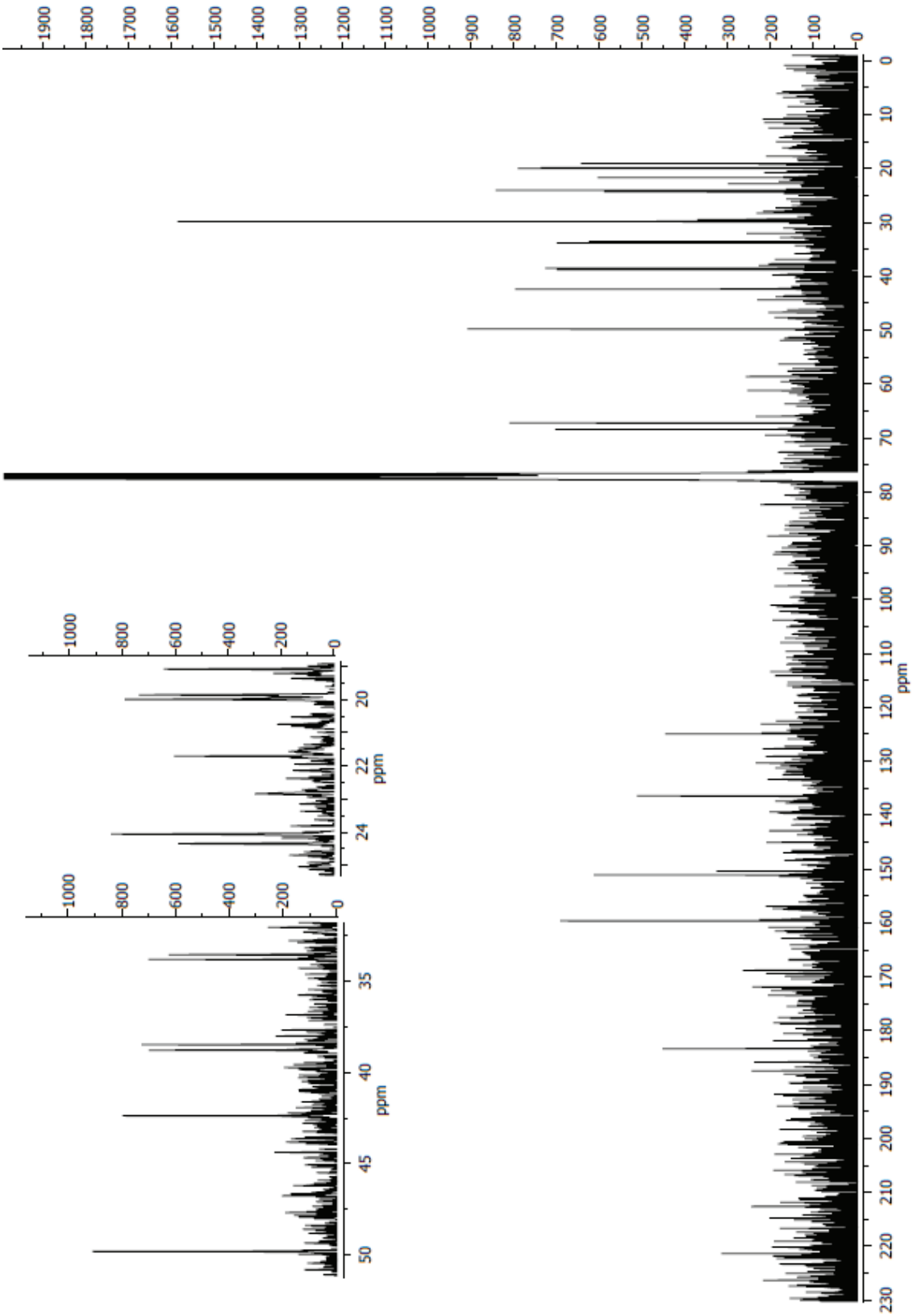
Annex 4

^{1}H-NMR spectrum of 7α-formyloxy-6β-hydroxyroyleanone (**III.3**) (300 MHz, $CDCl_3$).



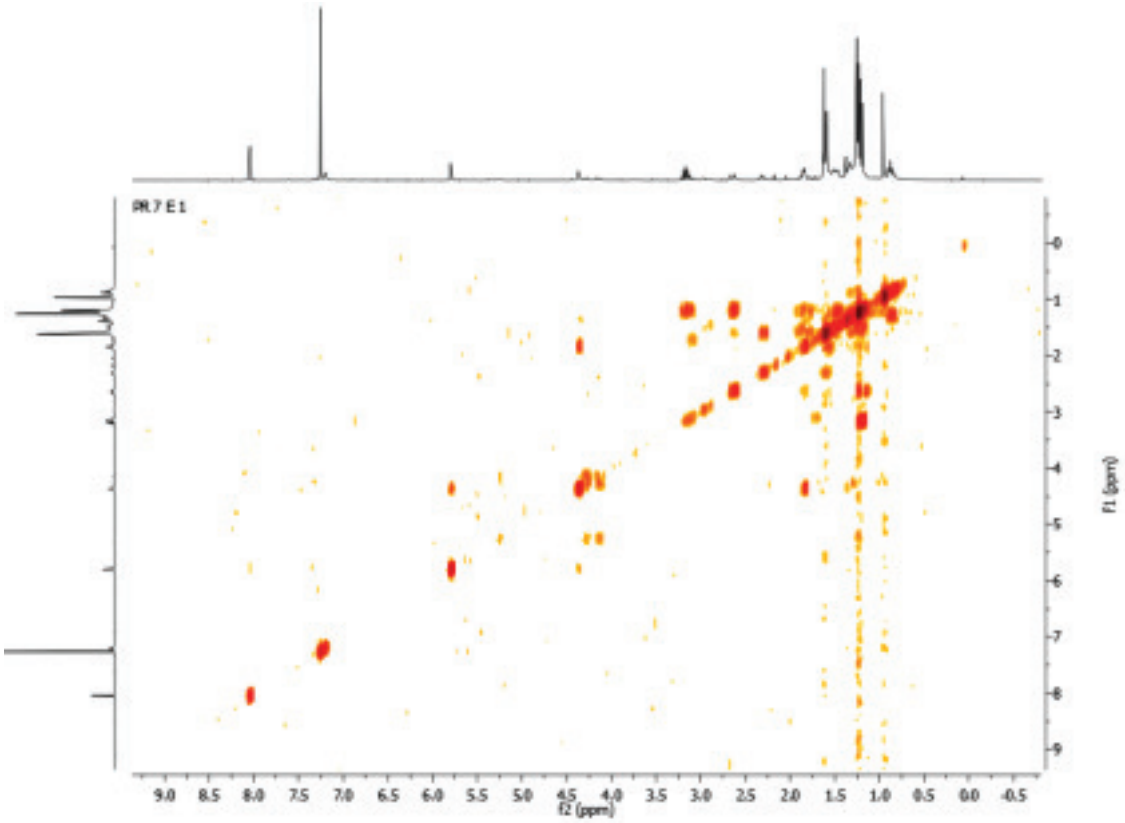
Annex 5

^{13}C-NMR spectrum of 7α-formyloxy-6β-hydroxyroyleanone (**III.3**) (75 MHz, $CDCl_3$).

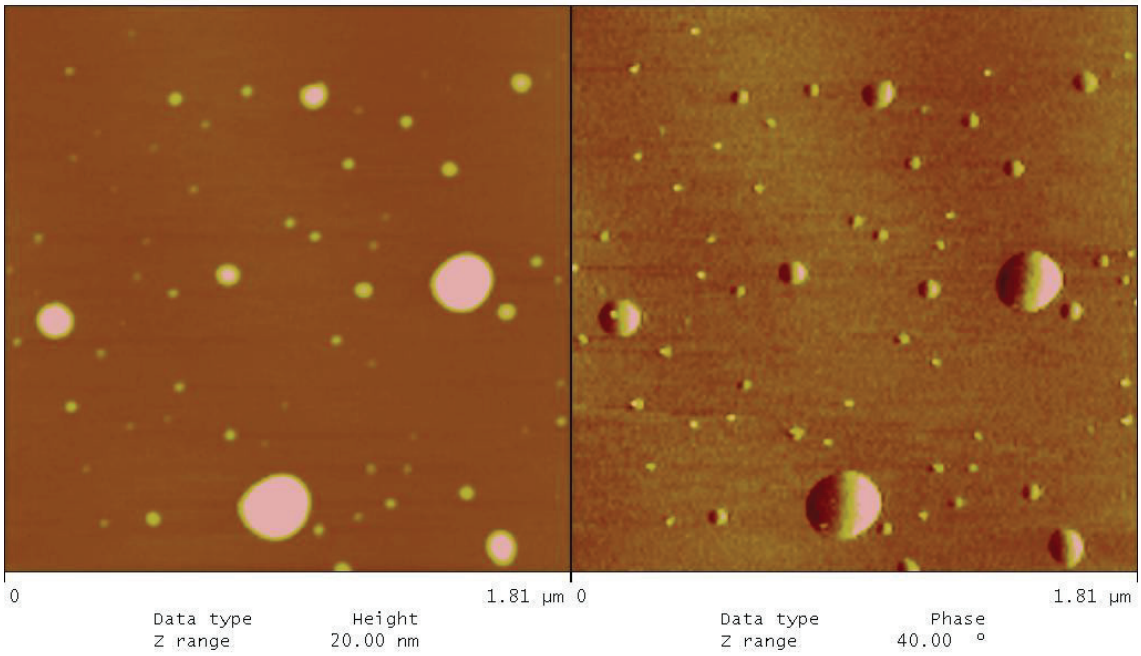


Annex 6

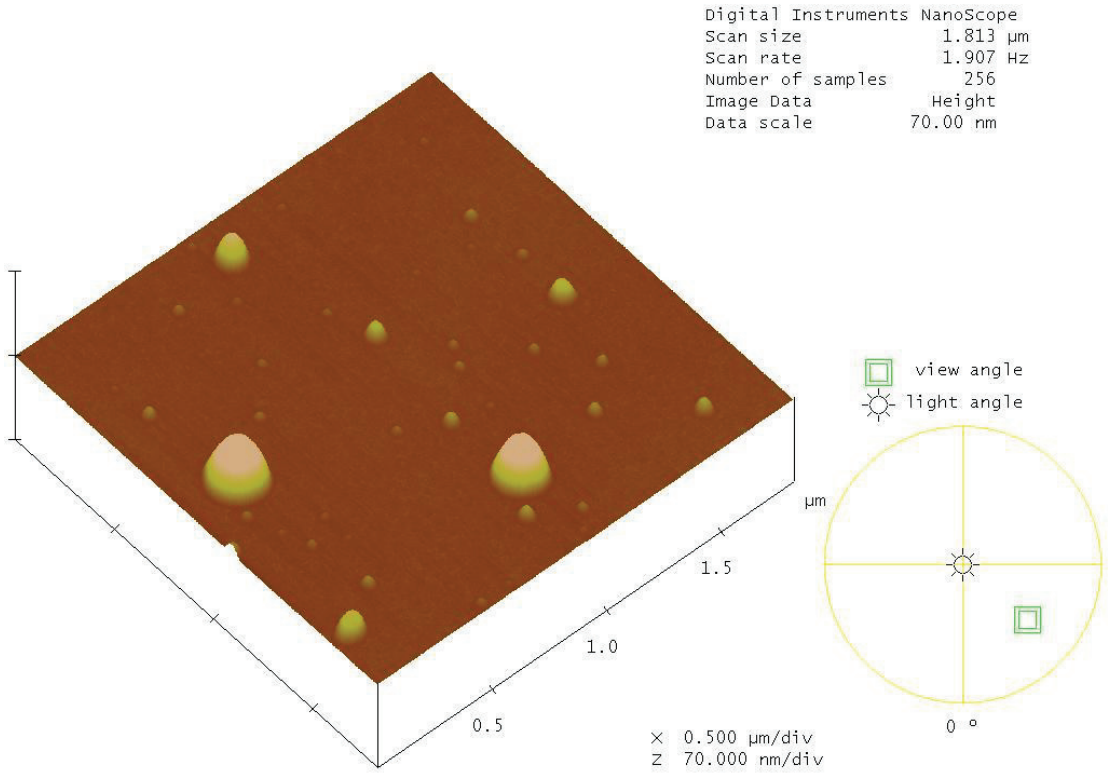
COSY-NMR spectrum of 7α-formyloxy-6β-hydroxyroyleanone (**III.3**) (300 MHz, $CDCl_3$).



Annex 7



fitossoma_3m.001



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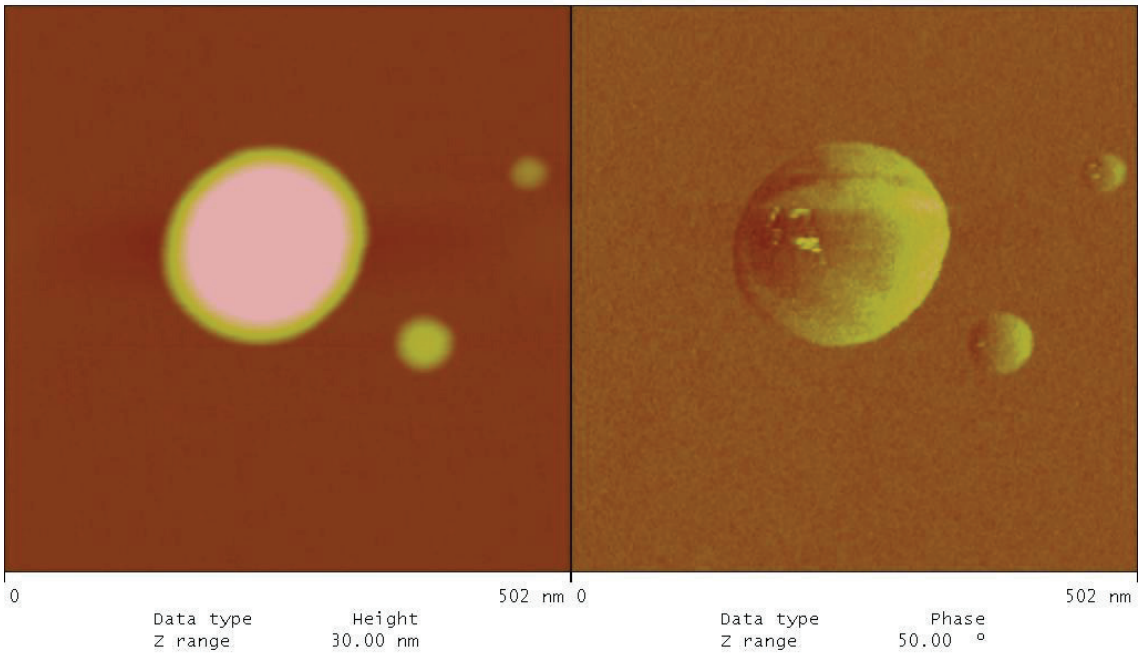

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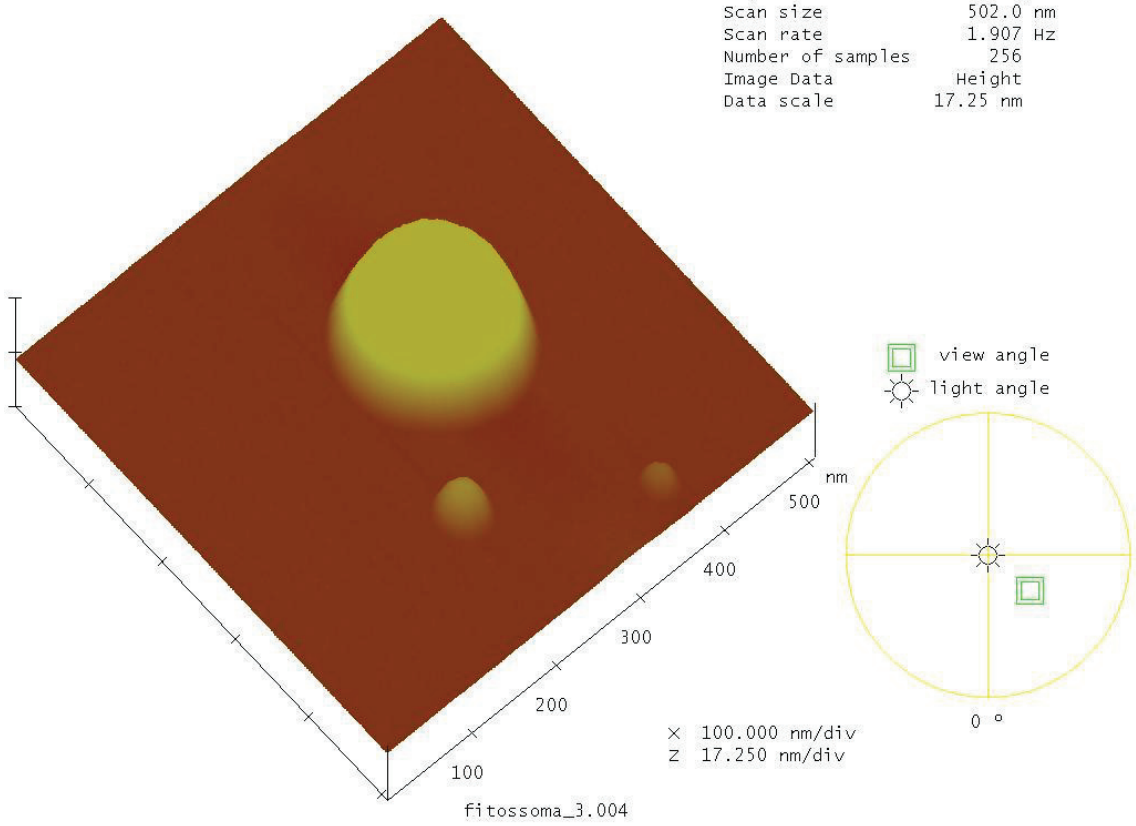

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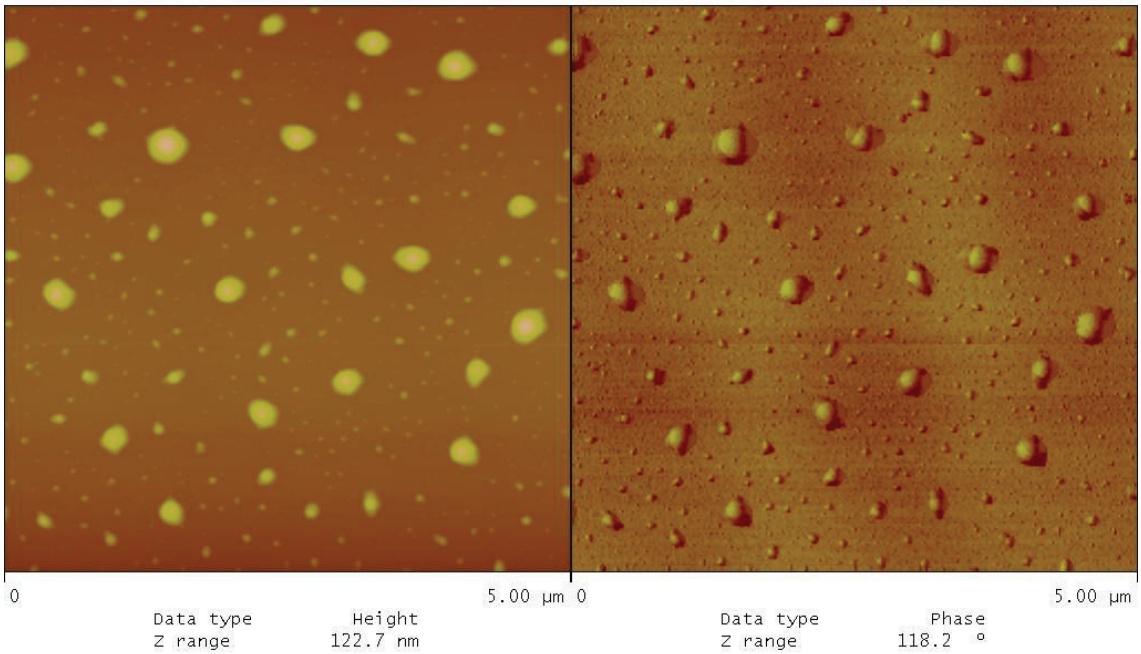

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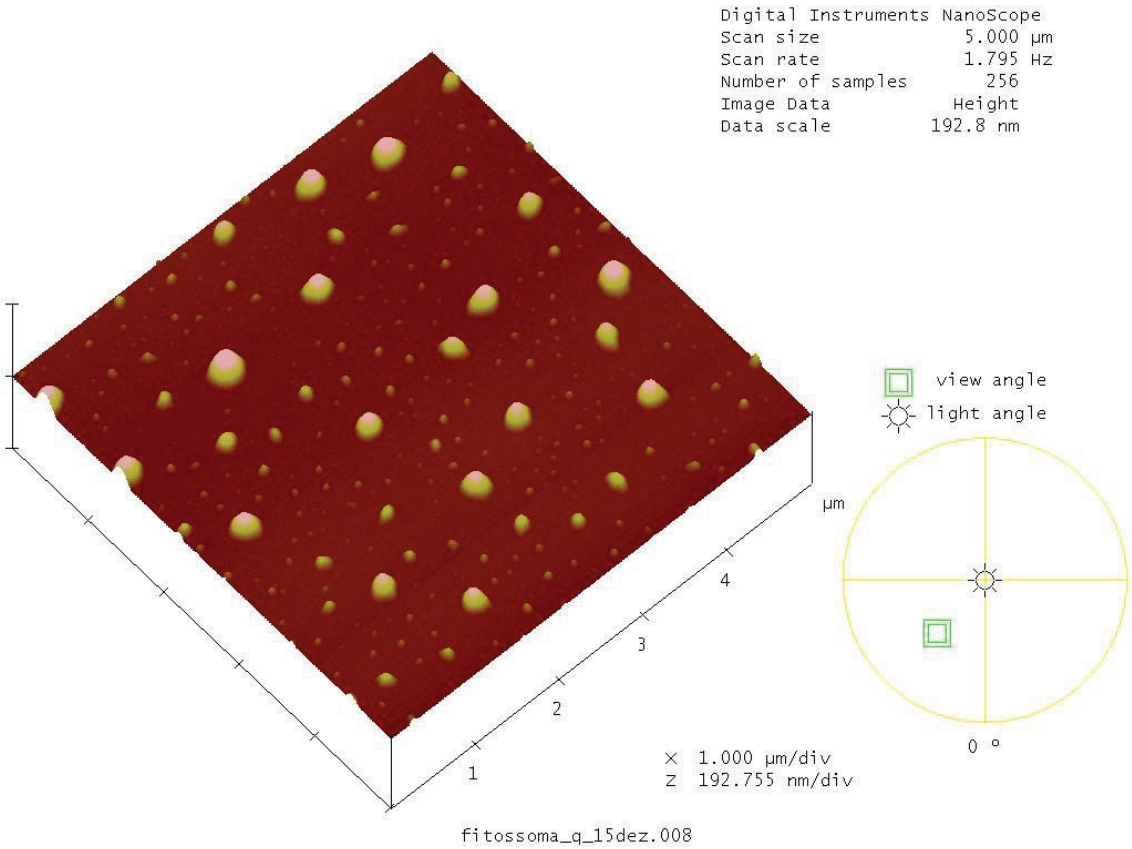

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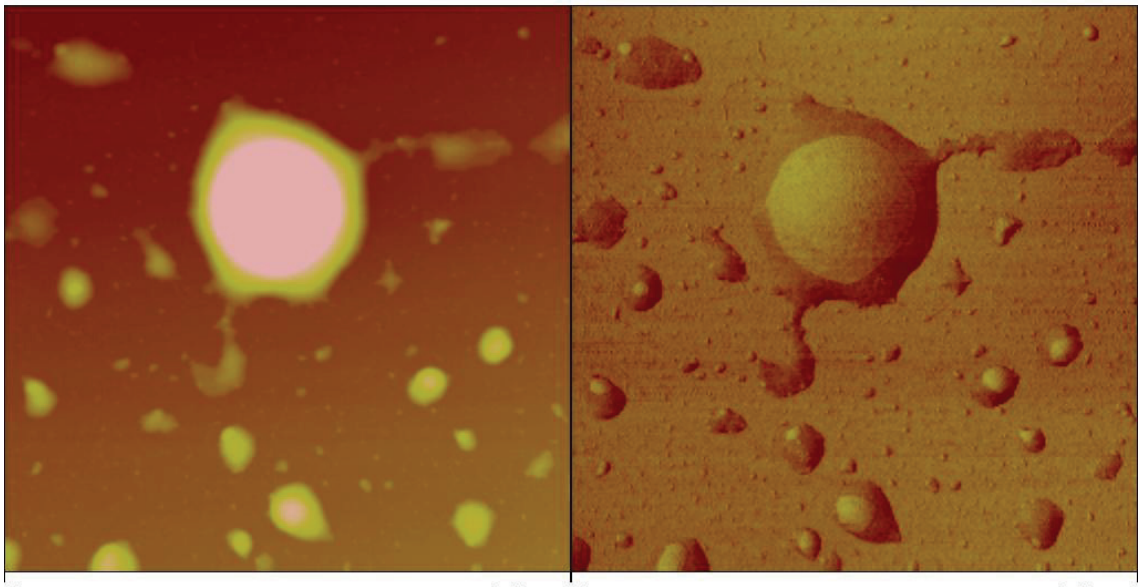

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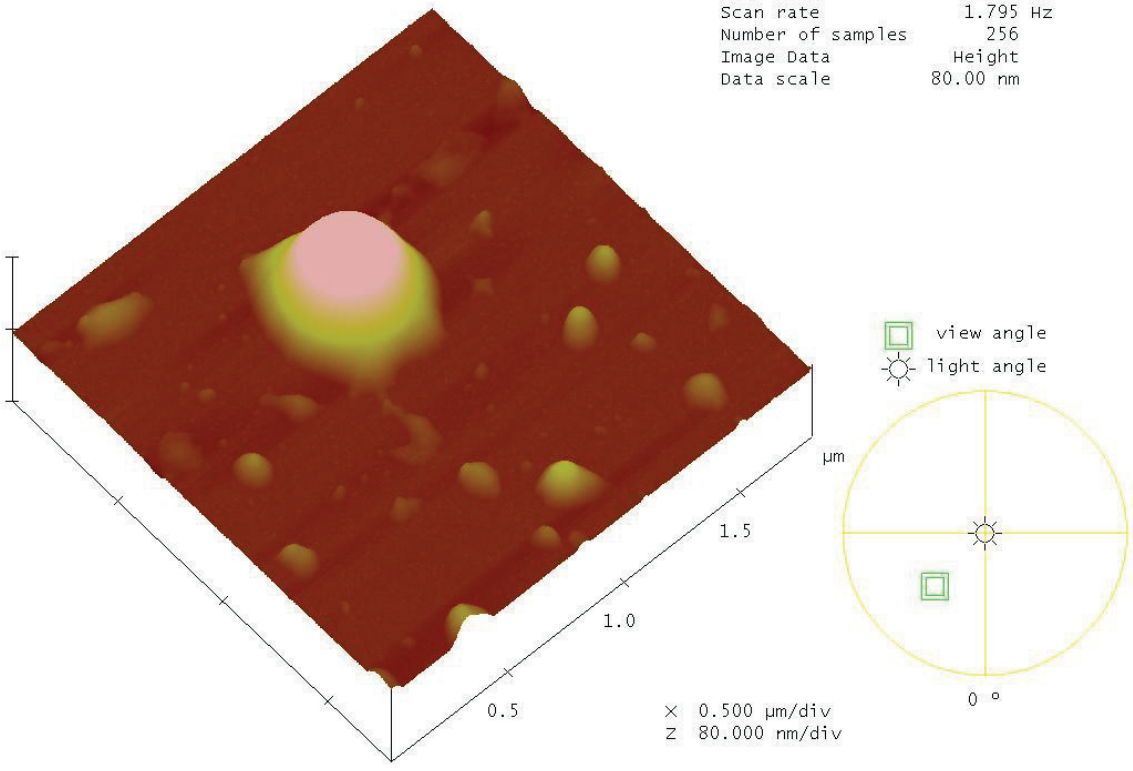
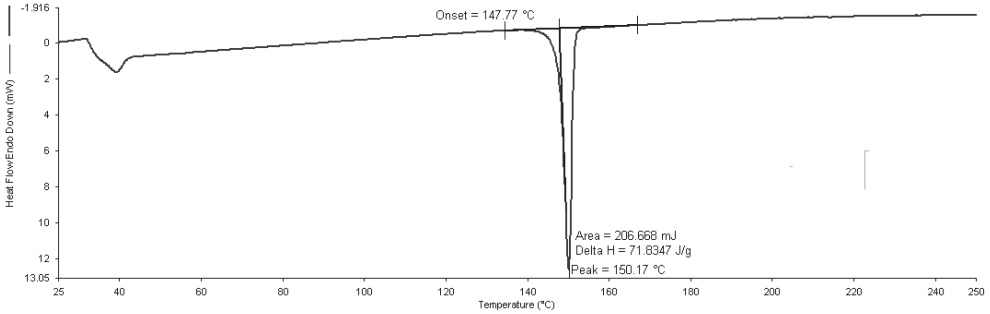


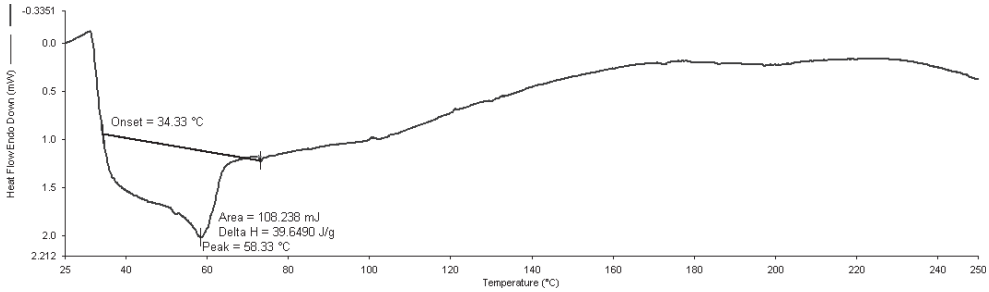
fitossoma_q_15dez.007

Annex 8

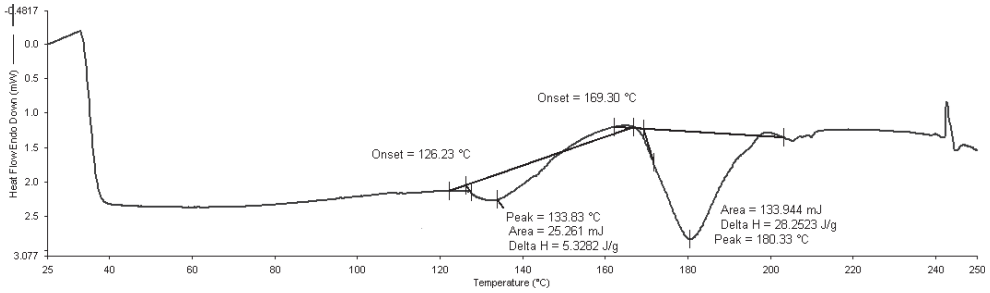
Original DSC thermograms of the ChiPS components.



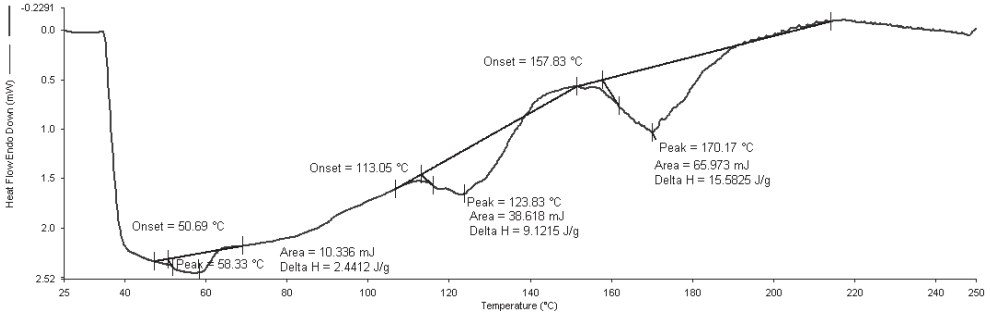
Graph A. DSC phase diagram of cholesterol. Onset = 147.77ºC; Peak = 150.17ºC.



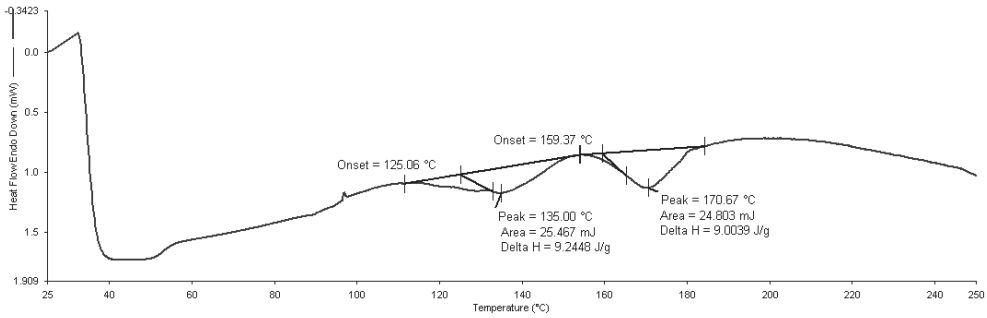
Graph B. DSC phase diagram of P. madagascariensis extract **E4**. Onset = 34.33ºC; Peak = 58.33ºC.



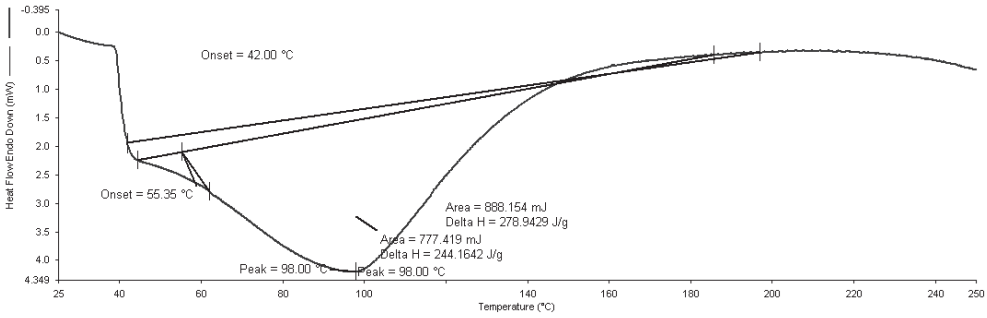
Graph C. DSC phase diagram of PdC from soybean (48%, Sigma-Aldrich, Germany). Two endothermic peaks were visible. Onset 1 = 126.23ºC; Peak 1 = 133.83ºC; Onset 2 = 169.30ºC; Peak 2 = 180.33ºC.



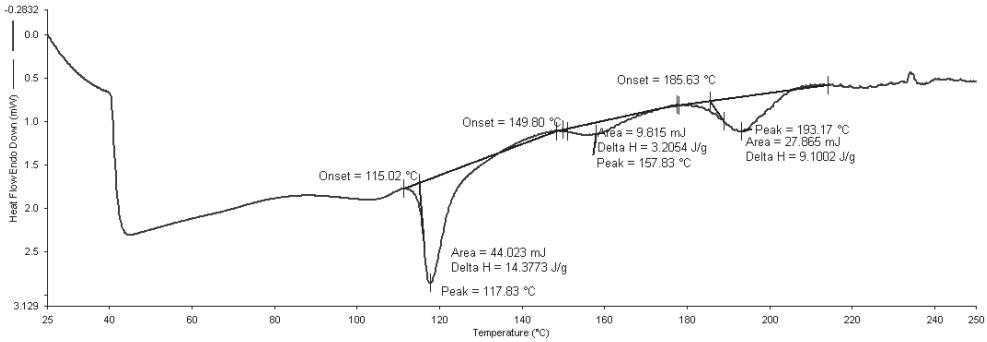
Graph D. DSC phase diagram of the physical mixture of cholesterol, extract and PdC. Three peaks were visible. Onset 1 = 50.69ºC; Peak 1 = 58.33ºC; Onset 2 = 113.05ºC; Peak 2 = 123.83ºC; Onset 3 = 157.83ºC; Peak 3 = 170.17ºC.



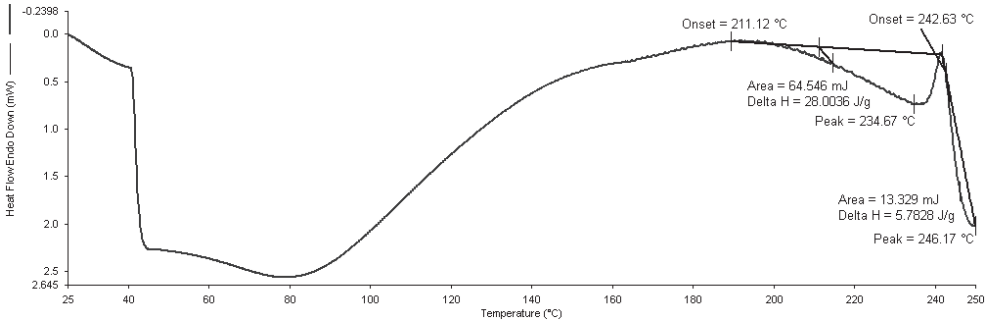
Graph E. DSC phase diagram of phytosome. Two endothermic peaks were visible. Onset 1 = 125.06ºC; Peak 1 = 135.00ºC; Onset 2 = 159.37ºC; Peak 2 = 170.67ºC.



Graph F. DSC phase diagram of low molecular weight chitosan (Sigma-Aldrich, Germany). A single broad endothermic peak was visible. Onset = 55.35ºC; Peak = 98.00ºC.

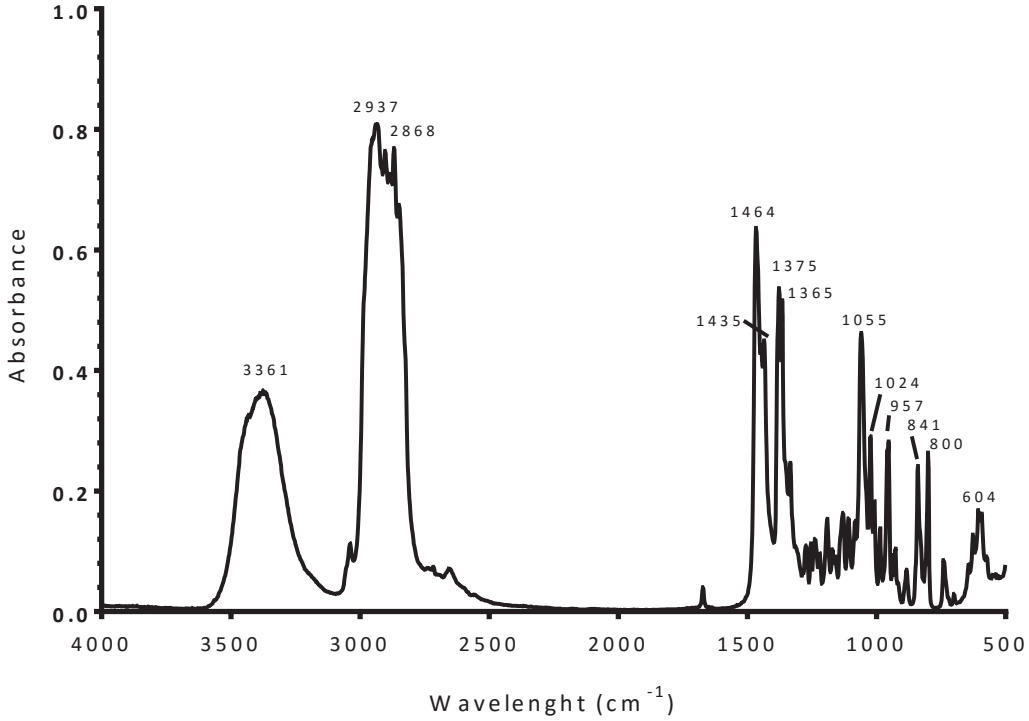


Graph F. DSC phase diagram of TPP.

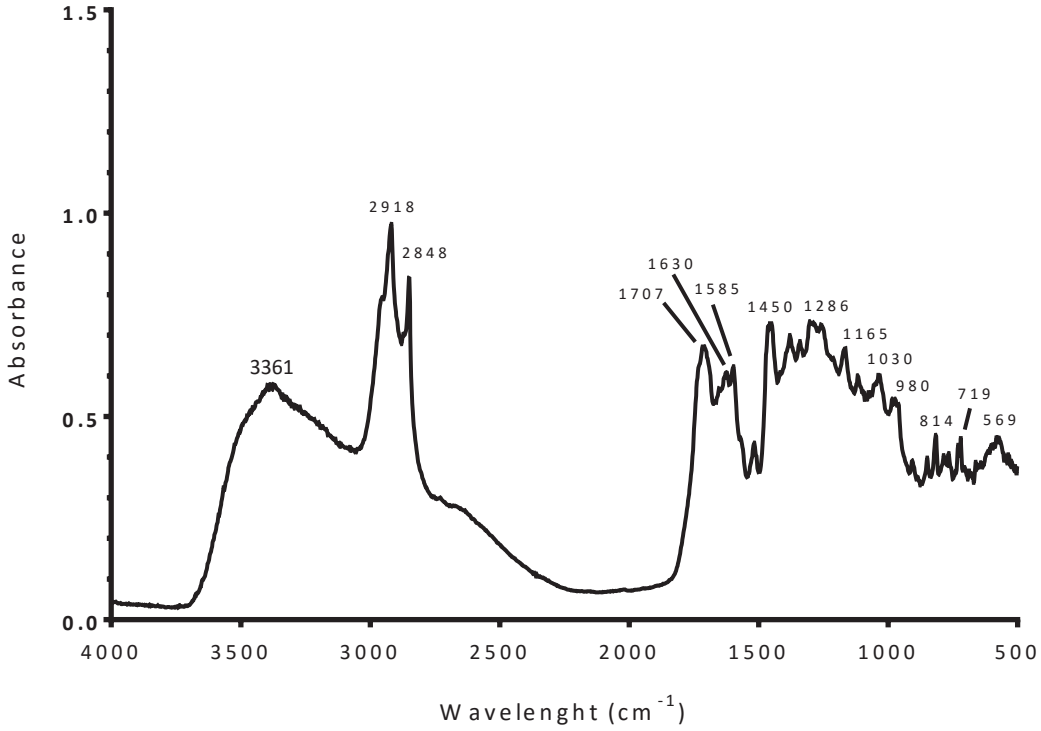


Graph F. DSC phase diagram of lyophilized ChiPS.

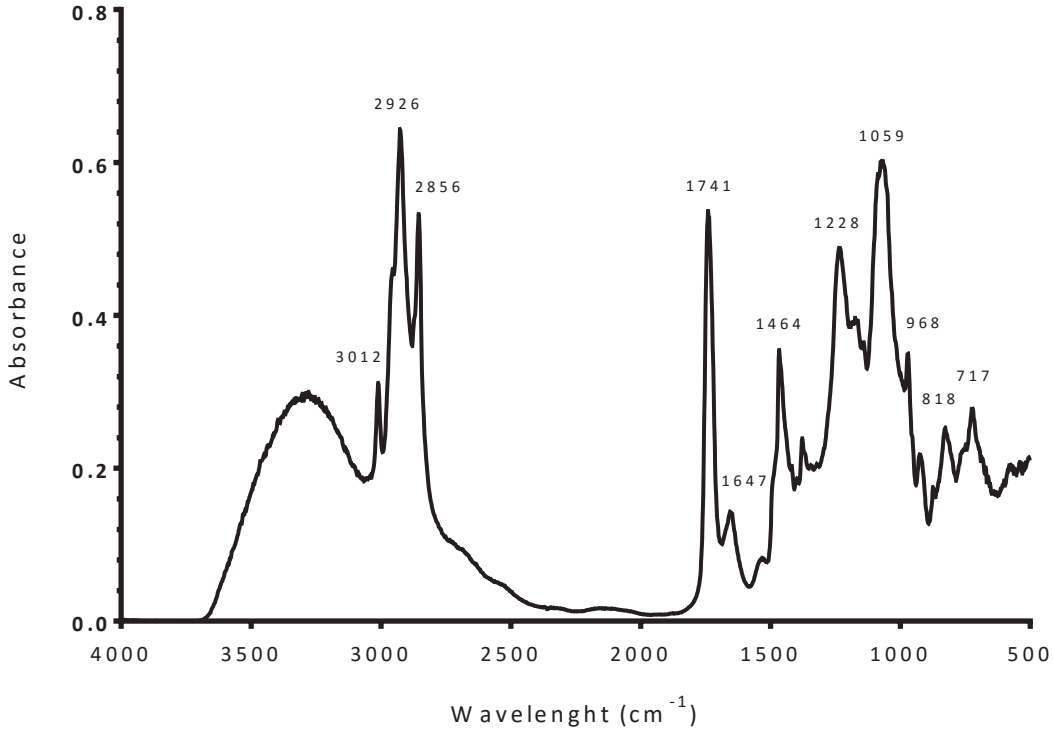
Annex 9



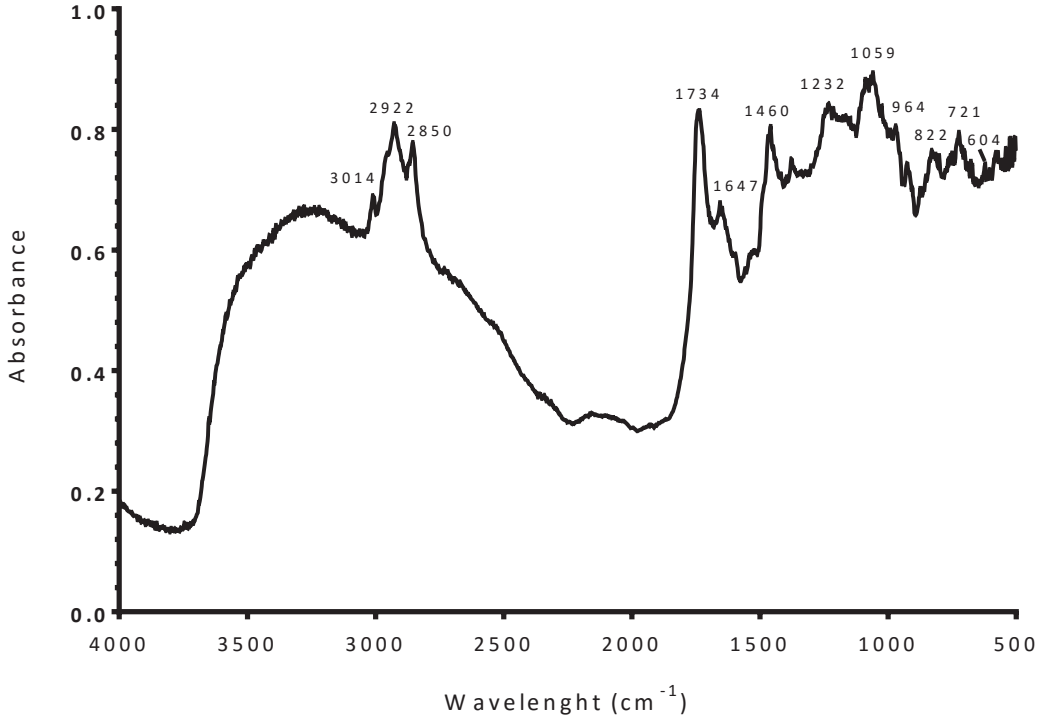
Graph A. DRIFTS of cholesterol (99%, Sigma-Aldrich).



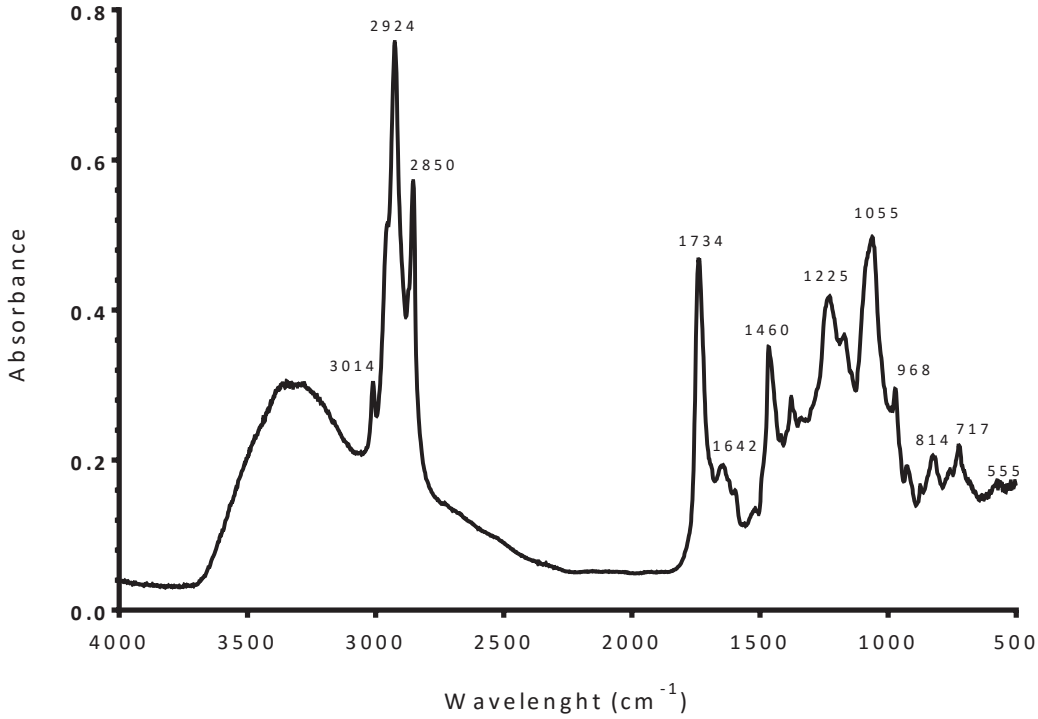
Graph B. DRIFTS of extract **E4**.



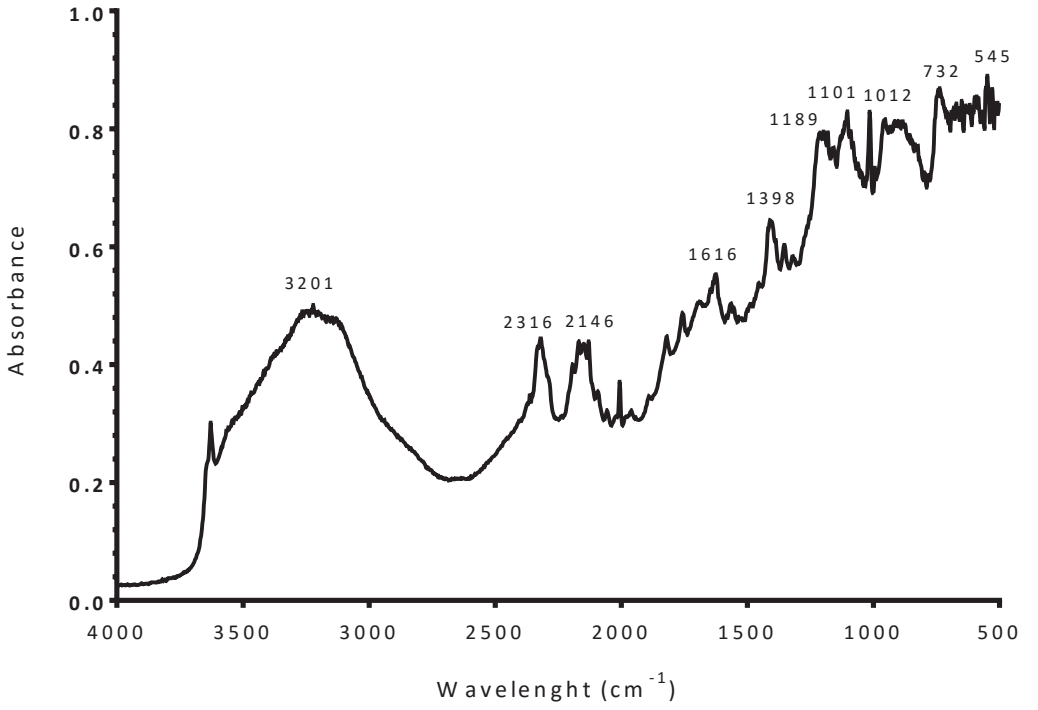
Graph C. DRIFTS of PdC (Sigma-Aldrich, 48% purity, from soybean).



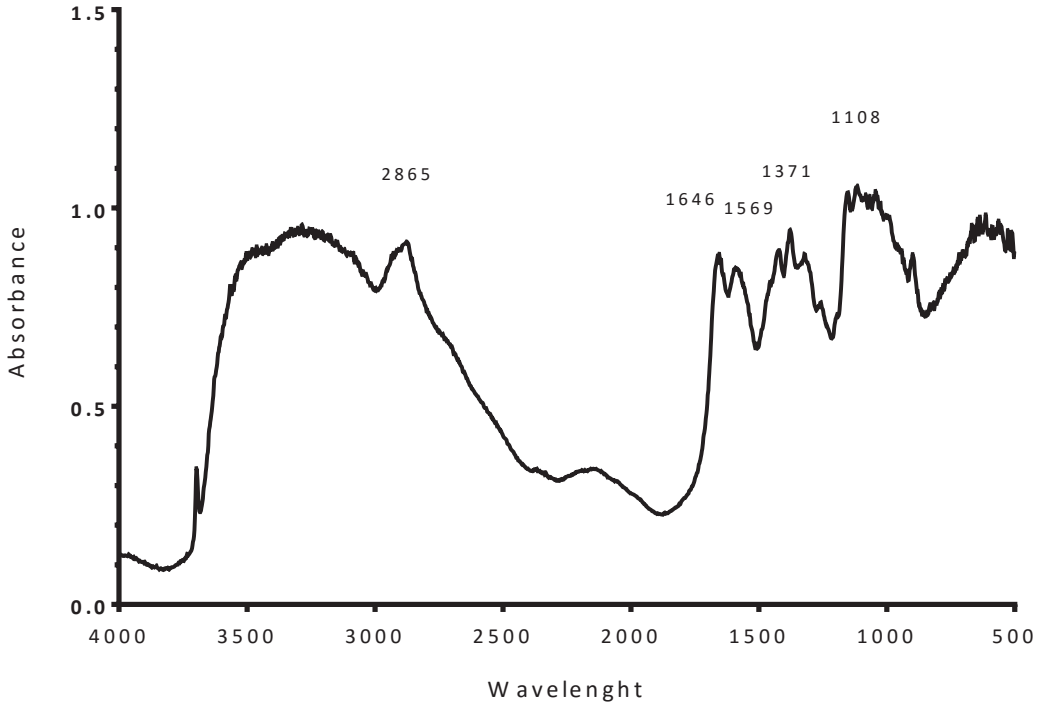
Graph D. DRIFTS of the physical mixture of extract **E4**, PdC and cholesterol.



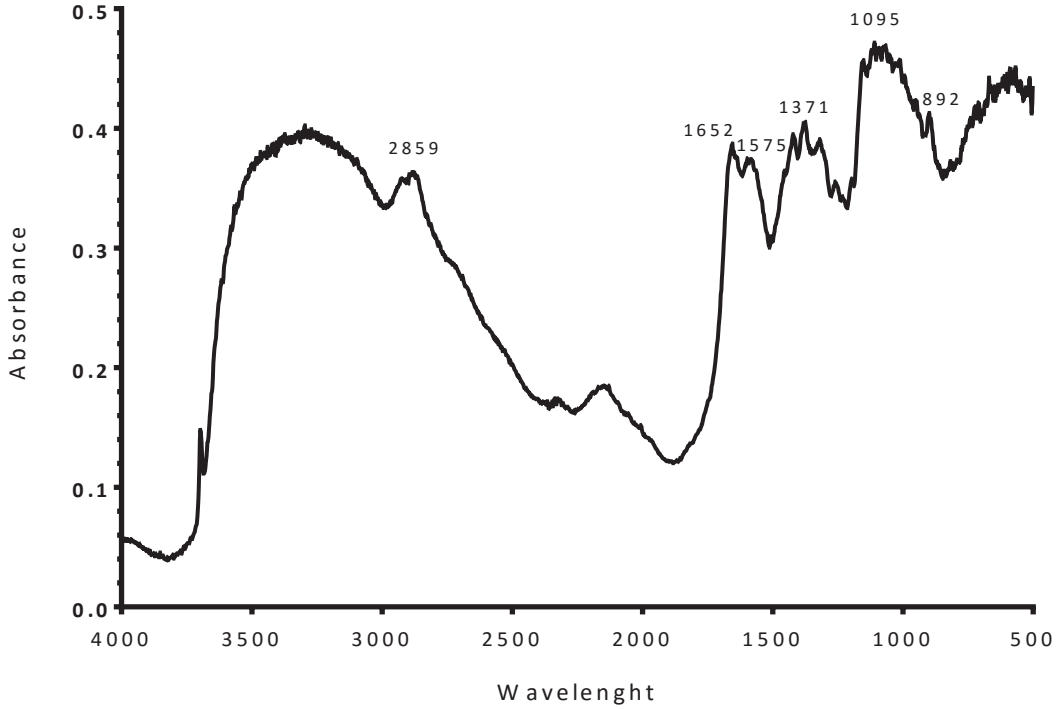
Graph E. DRIFTS of phytosomes.



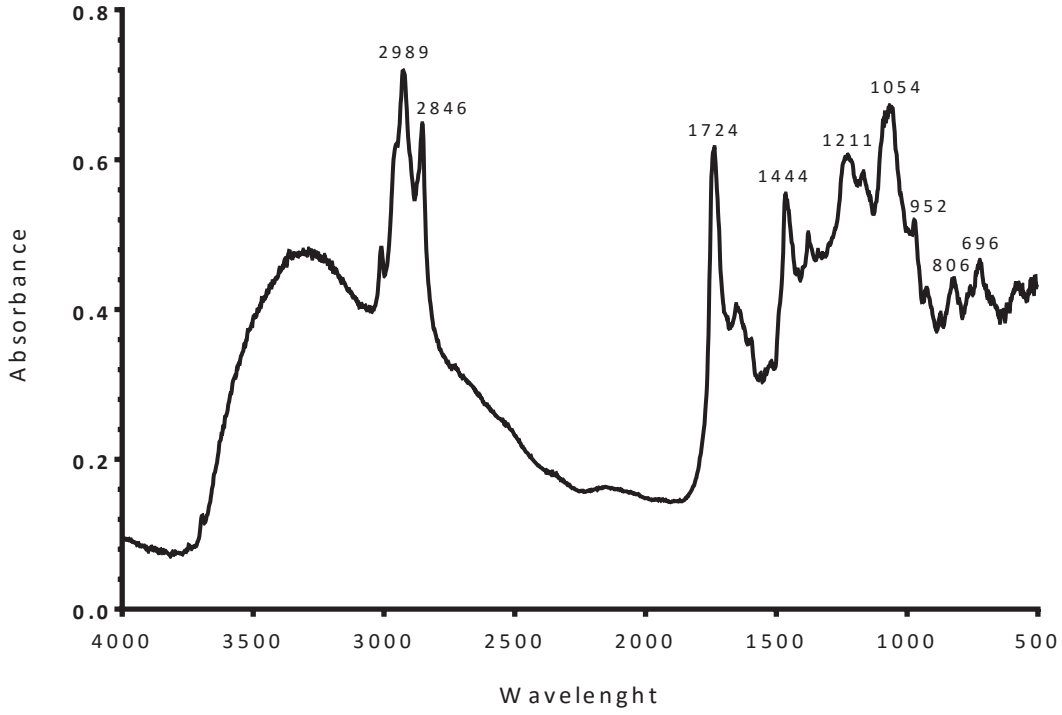
Graph F. DRIFTS of TPP.



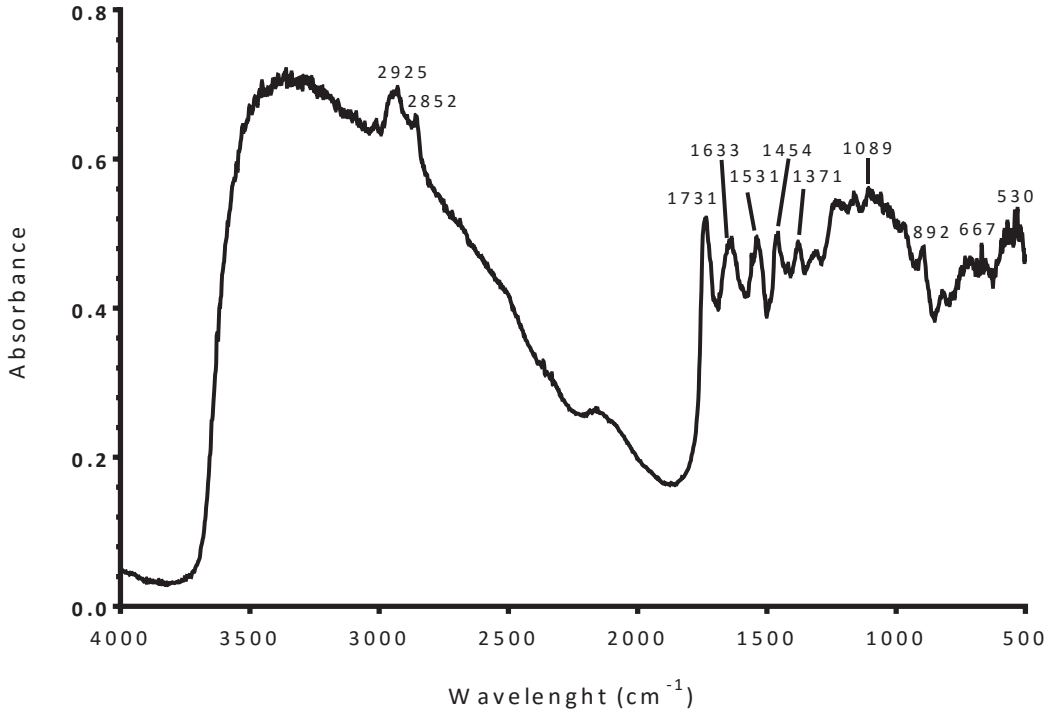
Graph G. DRIFTS of low molecular weight chitosan.



Graph H. DRIFTS of the physical mixture of TPP and chitosan.



Graph H. DRIFTS of the physical mixture of TPP, chitosan and phytosomes.



Graph I. DRIFTS of the ChiPS.

Annex 10

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Article

Antimicrobial Plant Extracts Encapsulated into Polymeric Beads for Potential Application on the Skin

Patrícia Rijo [1,2,*], Diogo Matias [1], Ana S. Fernandes [1,2], M. Fátima Simões [2], Marisa Nicolai [1] and Catarina Pinto Reis [1]

[1] CBiOS—Center for Research in Biosciences & Health Technologies, Universidade Lusófona de Humanidades e Tecnologias, Campo Grande 376, Lisboa 1749-024, Portugal;
E-Mails: diogohcmatias@gmail.com (D.M.); ana.fernandes@ulusofona.pt (A.S.F.);
mhfnicolai@gmail.com (M.N.); catarinapintoreis@gmail.com (C.P.R.)

[2] Research Institute for Medicines and Pharmaceutical Sciences (iMed.UL), Faculdade de Farmácia da Universidade de Lisboa, Av. Prof. Gama Pinto, Lisboa 1649-003, Portugal;
E-Mail: fatimaalfaiatesimoes@gmail.com

* Author to whom correspondence should be addressed; E-Mail: patricia.rijo@ulusofona.pt;
Tel.: +351-21-7515577/50; Fax: +351-21-7515598/79.

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Abstract: In this study, the *in vitro* bacterial growth inhibition, antioxidant activity and the content in bioactive components of *Plectranthus barbatus*, *P. hadiensis* var. tomentosus, *P. madagascarensis*, *P. neochilus* and *P. verticillatus* aqueous extracts were investigated and compared by three extraction methods (infusion, decoction and microwave extractions). The microwave extract of *P. madagascariensis* showed the higher antimicrobial activity against the *Staphylococcus epidermidis* strain with a minimum inhibitory concentration of 40 µg/mL. This extract also showed no toxicity in a general toxicity assay and no considerable cytotoxicity against a human keratinocyte cell line. The antioxidant activity of the extracts was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH method), and all showed antioxidant activity. The microwave extract of *P. madagascariensis* was the one with the highest antioxidant activity (IC_{50} value of 41.66 µg/mL). To increase extract stability, the microwave *P. madagascariensis* extract was then successfully encapsulated into alginate beads with high efficiency. This effective and low-cost strategy seems to be easy to extrapolate to an industrial scale with a future application on the skin.

Keywords: *Plectranthus*; antimicrobial activity; antioxidant activity; polymeric beads; alginate; extraction methods

1. Introduction

Natural products, widely used in traditional medicine, are a common source of bioactive molecules for the treatment of bacterial infections. Particularly, plants of the *Plectranthus* genus (Lamiaceae) have several ethnobotanical documented uses, including the treatment of various infections [1,2]. These traditional uses are well supported by the isolation of diverse antimicrobial metabolites, namely diterpenoids and polyphenols [3–5]. The use of herbal extracts as therapeutic agents may be an interesting area for developing countries and has aroused the interest of the population in developed countries. However, aqueous extracts generally present some formulation problems, such as long-term instability, low bioavailability and a significant burst release.

Biodegradable and biocompatible polymers can be used as carriers of pharmaceutical active ingredients and are a useful strategy for the enhancement of their stability characteristics. Alginate is a low-cost and biodegradable polymer. "Alginate" is the term typically used for the salts of alginic acid, but it can also refer to all the derivatives of alginic acid and alginic acid itself. Alginic acid is an algal polysaccharide and a species of polycarboxylic acid. It consists of α-L-guluronic acid (G) and β-D-mannuronic acid (M), and their relative proportion (M/G) ratio determines the biofunctional properties of alginic acid [6]. The gelling properties of alginate depend strongly upon its monomeric composition, sequential arrangements and the lengths of the G-blocks [7]. Nowadays, alginates are widely used in food, textile industry and other fields, including paper coating, pharmaceuticals and welding rods. In the pharmaceutical industry, alginates are generally applied as the thickening, gelling, stabilizing, suspending, controlled release and emulsifying agents [8]. Products originated by this way can have several applications, such as drug delivery [9,10]. Different techniques could be used in the encapsulation process, such as internal and external gelations [8], ultrasonic assisted atomization [11] and the spray drying process [12]. In some encapsulation methods, the active ingredient is encapsulated into calcium alginate beads and slowly released from the polymer matrix when the bead is exposed in the appropriate environment [13,14]. In general, alginate solutions are stable in the pH range 5.5–10 at room temperature for a long time, and one approach to improve the calcium alginate bead stability may be obtained with the medium enrichment with calcium ions, where the beads are suspended [15].

2. Experimental Section

2.1. Chemicals

Alginic acid sodium salt from brown algae [low glucuronic content (named as Alginate Low G) with low viscosity (4–12 cPs) 1% in H_2O at 25 °C], dimethyl sulphoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,6-di-*tert*-butyl-4-hydroxytoluene (BHT), chlorogenic and rosmarinic acids, phosphate buffered saline (PBS; 0.01 M, pH 7.4), trypsin, Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin solution, fetal bovine serum and thiazolyl blue

tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Steinheim, Germany). All chemicals were of analytical grade.

2.2. Plant Material

The whole plant material of *Plectranthus barbatus* Andrews, *P. hadiensis* (Forssk.) Schweinf. ex Spreng. var. tomentosus (Benth.) Codd, *P. madagascarensis* (Lam.) Benth., *P. neochilus* Schltr. and *P. verticillatus* (L.F.). Druce grown in the Parque Botânico da Tapada da Ajuda from cuttings provided by the Kirstenbosch National Botanical Gardens, South Africa, were collected between 2007 and 2008, always in June and September, and voucher specimens were deposited in the Herbarium "João de Carvalho e Vasconcellos" of the "Instituto Superior de Agronomia", Lisboa (LISI), Portugal.

2.3. Extract Preparation

Aqueous extracts of each plant were prepared using 10 mg of dried and powdered plant material in 100 mL of distilled water and then filtered through Whatman N.° 5 paper (Whatman, Inc., Clifton, NJ, USA.) by different extraction techniques: (1) decoction extracts were prepared by boiling the plant material in distilled water for 10 min; (2) infusion extracts were prepared by adding freshly boiled distilled water to plant material for 10 min; (3) microwave extracts were prepared by using the plant material in distilled water in a conventional microwave oven for 2 min at a continuous irradiation of 2.45 GHz. From every prepared extract, an aliquot of 1 mL was freeze-dried (Freezone 2.5 L, Freeze-dryer Labconco, Kansas City, MO, USA) and the dry weight determined on the average of three independent measures (Table 1).

2.4. High-Performance Liquid Chromatography (HPLC) Analysis

The determination of the extracts' main components was done by HPLC analysis and was carried out in an Agilent Technologies 1200 Infinity Series LC System equipped with diode array detector (DAD), ChemStation*Software* and a LiChrospher® 100 RP-18 (5 mm) column from Merck (Darmstadt, Germany). Extracts were analyzed by injecting 20 µL and using a gradient composed of Solution A (methanol), Solution B (acetonitrile) and Solution D (0.3% trichloroacetic acid in water) as follows: 0 min, 15% A, 5% B and 80% D; 20 min, 80% A, 10% B and 10% D; 25 min, 80% A, 10% B and 10% D; and 28 min, 15% A, 5% B and 80% D. The flow rate was set at 1 mL/min. The standards were run under the same conditions in methanol, and the detection was carried out between 200 and 600 nm with a diode array detector (DAD). All analyses were performed in triplicate.

2.5. Microorganisms and Well Diffusion Method

The antimicrobial activity of each prepared extract was evaluated against six bacterial species obtained from the American Type Culture Collection (ATCC), namely *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *S. epidermidis* ATCC 12228. The well diffusion assay was used to screen the extracts with antimicrobial activity.

Previously prepared extracts were freeze-dried using the same equipment as Section 2.3 and reconstituted in DMSO to a 1 mg/mL concentration. Stock solutions of reference antibiotics (vancomycin and norfloxacin) were also prepared to 1 mg/mL in DMSO.

In aseptic conditions, Petri dishes containing 20 mL of solid Mueller-Hinton culture medium were inoculated with 0.1 mL of bacterial suspension matching a 0.5 McFarland standard solution and uniformly spread on the medium surface using a sterile swab. Wells of approximately 5 mm in diameter were made in the medium with a sterile glass Pasteur pipette and 50 µL of each extract were added into the well. A positive control of vancomycin for Gram-positive bacteria, norfloxacin for Gram-negative bacteria and a negative control of DMSO were used. Plates were incubated at 37 °C for 24 h. The antibacterial activity was evaluated by measuring the diameter (mm) of the inhibition zone formed around the wells and compared to controls.

2.6. Determination of Minimum Inhibitory Concentrations (MICs)

Antibacterial activity was also determined using the microplate broth microdilution method. In aseptic conditions, 100 µL of liquid Mueller-Hilton medium was distributed in each well of a 96-well plate. To the first well of each row was added 100 µL of extract, the positive control or negative control solutions at a 1 mg/mL concentration. Using a multichannel micropipette, a serial dilution was made to 1:2 proportion between each row of wells (1.95–500 µg/mL range). Lastly, 10 µL of bacterial suspension were added to every well and plates were covered and incubated at 37 °C for 24 h. The bacterial growth was measured with an absorbance microplate reader (Thermo Scientific Multiskan FC, Loughborough, UK) set to 620 nm. Assays were carried out in triplicate for each tested microorganism.

2.7. Determination of Antioxidant Activity

To evaluate the radical scavenging activity, the DPPH method was used [16]. Ten microliters of plant extract were added to a 990 µL solution of DPPH (0.002% in methanol). The mixture was incubated for 30 min at room temperature. The absorbance was measured at 517 nm against a corresponding blank and the antioxidant activity was calculated as:

$$AA\% = \frac{A_{\mathrm{DPPH}} - A_{\mathrm{sample}}}{A_{\mathrm{DPPH}}} \times 100 \quad (1)$$

where AA is the antioxidant activity, A_{DPPH} is the absorption of DPPH against the blank and A_{sample} is the absorption of the extract or control against the blank. Tests were carried out in triplicate, and the sample concentration providing 50% of antioxidant activity (IC_{50}) was obtained by plotting antioxidant activity against the sample concentration.

2.8. Screening of General Toxicity

To evaluate general eukaryotic toxicity, the *Saccharomyces cerevisiae* model developed by Roberto and Caetano was applied [17]. An inoculum of *S. cerevisiae* was added to 20 mL of YPD liquid medium (YPD means yeast extract 1%, peptone 0.5% and dextrose 2%) in a sterile Erlenmeyer

where *EE*% corresponds to the encapsulation efficiency, C_i to the initial concentration of rosmarinic acid and C_f to the final concentration of rosmarinic acid (non-encapsulated or free rosmarinic acid) present in the supernatant.

2.12. Stability Studies of Plectranthus *spp. Extract-Loaded Calcium Alginate Beads*

2.12.1. Stability over the Time

Hydrated and filtered beads were left at room temperature for 7 days and then liquefied in 50 mL of 55 mM of sodium citrate. The amount of rosmarinic acid in the supernatant and in the remaining beads was quantified using the same HPLC method described before. Fresh extracts were used as the controls.

2.12.2. Stability against UV Radiation

Two hundred milligrams of freeze-dried calcium alginate beads were placed in vials containing 10 mL of water under UV light (274 nm) and left for magnetic stirring for 2 h. The amount of rosmarinic acid in the supernatant and remaining in beads was quantified using the same HPLC method. Fresh extracts were used as the controls.

3. Results and Discussion

3.1. Extraction Yields

Aqueous extracts of *P. barbatus*, *P. hadiensis* var. tomentosus, *P. madagascarensis*, *P. neochilus* and *P. verticillatus* were prepared by three methods: decoction, infusion and microwave. Aliquots of the resulting extracts were freeze-dried to determine the dry weight of each extract (Table 1). The best yield was verified for the *P. barbatus* infusion extract with a mean value of 33 ± 3.0 mg/mL. It could also be identified that the trend of the infusion technique led to a higher dry weight and in the microwave extracts, the lowest dry weight, except for *P. verticillatus*, where only the microwave was determined.

Table 1. Aqueous extract dry weights for each *Plectranthus* species and extraction method.

Plant	Extraction method	Weight of dry extract (mg/mL)
P. barbatus	Decoction	26 ± 2.0
	Infusion	33 ± 3.0
	Microwave	22 ± 1.0
P. hadiensis var. Tomentosus	Decoction	32 ± 2.0
	Infusion	26 ± 1.0
	Microwave	17 ± 1.0
P. madagascarensis	Decoction	22 ± 1.0
	Infusion	26 ± 4.0
	Microwave	15 ± 4.0
P. neochilus	Decoction	22 ± 2.0
	Infusion	23 ± 10.0
	Microwave	11 ± 4.0
P. verticillatus	Decoction	nd
	Infusion	nd
	Microwave	12

Note: nd, not determined.

3.2. Phytochemical Composition of Extracts

The prepared *Plectranthus spp.* aqueous extracts were analyzed by HPLC-DAD and their main constituents identified by comparison of their UV absorbance spectra with standard UV absorbance spectra. Chlorogenic and rosmarinic acids were identified in all extracts (Table 2), and in *P. madagascariensis* extracts, the rosmarinic acid yield was quantified (Figure 1).

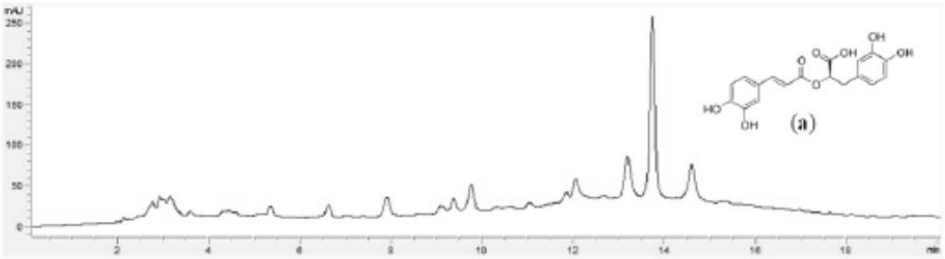
The calculated calibration curve for rosmarinic acid was $y = 5175.9x - 127.46$ ($R^2 = 0.999$).

Table 2. Identified polyphenols and antioxidant activity of prepared aqueous *Plectranthus* extracts.

Plant	Extraction method	Identified polyphenols (*w*/*w*% of extract)	Antioxidant activity (AA%)
P. barbatus	Decoction	CA, RA	10.30
	Infusion	CA, RA	15.70
	Microwave	CA, RA	10.10
P. hadiensis var. tomentosus	Decoction	CA, RA	17.9
	Infusion	CA, RA	40.0
	Microwave	CA, RA	32.6
P. madagascarensis	Decoction	CA, RA (15.72)	20.60
	Infusion	CA, RA (10.93)	10.20
	Microwave	CA, RA (18.64)	17.20
P. neochilus	Decoction	CA, RA	12.1
	Infusion	CA, RA	14.7
	Microwave	CA, RA	14.0
P. verticillatus	Microwave	CA, RA	19.5

Notes: CA, chlorogenic acid; RA, rosmarinic acid and *w*/*w*, weight/weight.

Figure 1. Example of a chromatogram of *P. madagascariensis* extract (5 mg/mL) obtained by the microwave technique. (**a**) Rosmarinic acid structure.



3.3. Antibacterial Activity

The antibacterial activities of the prepared aqueous *Plectranthus* extracts were screened initially by well diffusion assay, and only the *P. madagascariensis* microwave extract showed a 10 mm of inhibition zone against *S. epidermidis*. This extract showed a potent antimicrobial activity against *S. epidermidis* with a minimum inhibitory concentration (MIC) value of 40 µg/mL.

3.4. Antioxidant Activity

All extracts in this study showed antioxidant activity at 10 µg/mL (Table 2) and the *P. madagascariensis* extracts were the most active (10.2% to 20.6% of antioxidant activity). Thus, the *P. madagascariensis* microwave extract was elected for further studies, considering the results obtained on antimicrobial activity (Section 3.3) and the IC_{50} of 41.66 µg/mL.

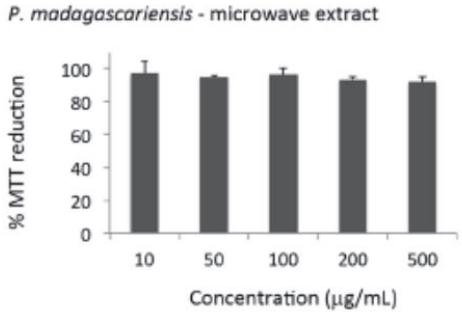
3.5. General Toxicity

The *S. cerevisiae* model is a simple and effective model to predict general eukaryotic toxicity. This technique was applied to the *P. madagascariensis* extract and no toxicity was verified.

3.6. Cytotoxicity

The cytotoxicity profile of *P. madagascariensis* microwave extract was studied in a human keratinocyte cell line (HaCaT) using the MTT assay. For concentrations up to 500 µg/mL and an incubation period of 24 h, no considerable cytotoxic effects were observed, as shown in Figure 2.

Figure 2. The effect of the *P. madagascariensis* extract on the viability of human keratinocytes. Results are the average values ± SD of two independent experiments, each comprising four replicate cultures. MTT, thiazolyl blue tetrazolium bromide.

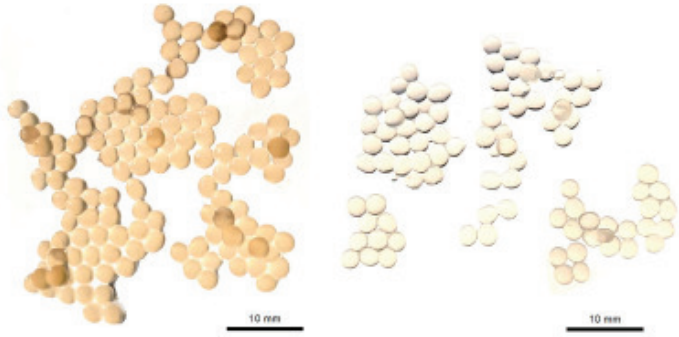


3.7. Production and Characterization of Plectranthus *spp. Extract-Loaded Calcium Alginate Beads*

Calcium alginate beads with herbal extracts were successfully prepared using the extrusion/external gelation method. This method was very rapid and can be easily adapted to the industrial scale. In general, there are two main techniques for producing calcium alginate beads, based on the source of the calcium. In external gelation method, alginate droplets are gelled in a calcium chloride solution, and thus, the calcium source is external to the droplet. Gelation is initiated from the surface and works toward the inner core. In contrast, the internal gelation method uses an internal source of calcium, where the alginate solution is preloaded within soluble calcium salt, releasing calcium ions with pH adjustment [8,14]. In this study, calcium alginate beads were spherical in shape and showed a mean diameter of 2.57 ± 0.21 and 2.33 ± 0.19 mm for loaded and empty particles, respectively, in the digital images (Figure 3). These images confirmed the encapsulation of the herbal extracts inside the calcium

alginate beads since empty calcium alginate beads were transparent; meanwhile, when the extract was presented, the beads changed to yellow.

Figure 3. Digital images of (**a**) the herbal extract-loaded calcium alginate beads and (**b**) the empty calcium alginate beads.



(**a**) (**b**)

Alginate is a polyelectrolyte hydrocolloid, so it interacts with water, reducing its diffusion and stabilizing its presence. Such water may be held specifically through direct hydrogen bonding or the structuring of water within extensive, but contained, inter- and intra-molecular voids. In this study, an aqueous microwave *P. madagascariensis* extract was successfully encapsulated into calcium alginate beads, where the encapsulation efficiency value was very high (around 88%). Alginate and the encapsulated extracts have the same hydrophilic properties, and thus, a high value of encapsulation efficiency was expected. The gelling properties of alginate strongly depend upon its monomeric composition, sequential arrangements and the lengths of the G-blocks [7]. As previously described, alginate forms gels with calcium, and this is an auto-cooperative reaction that occurs between calcium and the homopolymeric blocks of G on the alginate chain [7,19]. Calcium alginate gels shrink during gel formation. This leads to the loss of water and an increase in the concentration of polymer in the beads relative to that in the alginate solution and may be reducing some of the encapsulant diffusion to outside of the polymeric matrix. Herein, the physical characteristics of calcium alginate gel are also influenced by the alginate concentration and molecular size, the calcium concentration and gelling time [20]. In addition, in the extrusion/external gelation method, sodium alginate solution is forced into a bath of a calcium chloride solution, and then, calcium alginate is formed as fibers (gel). If low viscosity alginates are used, a strong solution is generally formed without any viscosity problems, and the calcium bath is not diluted as rapidly. In general, low G alginates, like we used in this study, produced very flexible gels [15]. Gel fibers have very good strength in both wet and dry forms, and as with most polymer fibers formed by extrusion, stretching while forming increases the linearity of the polymer chains and the strength of the fiber.

3.8. Stability Studies

Microencapsulation is a very useful process utilized in the incorporation of active ingredients within polymers aiming at, among other objectives, the prolonged release of pharmaceutical compounds and protection from atmospheric agents (moisture, light, heat and/or oxidation) [21]. The extract contents of chlorogenic acid (CA) and rosmarinic acid (RA) were maintained in calcium alginate beads over time (for seven days) and against UV in contrast to free extracts (or non-encapsulated). Similarly to previous studies [8,22,23], alginate proved to be a very good encapsulant material. Future studies are now on-going and include the incorporation of this system into a topical dosage form and its potential application on the skin.

4. Conclusions

In this study, a *P. madagascariensis* aqueous microwave extract was identified as possessing relevant anti-*S. epidermidis* activity along with antioxidant capacity and low toxicity in the models tested. Rosmarinic acid was identified as the main component of this extract. These properties may be beneficial for the healing of a variety of skin conditions, this extract being a potential antibacterial skin active ingredient. To prevent the long-term instability of the extract, the encapsulation of the *P. madagascariensis* microwave extract into a polymeric matrix was successfully obtained. This encapsulation method produced several and spherical particles, homogenous in color and particle size and with very high encapsulation efficiency. The long-term stability studies also demonstrate the viability of this system to the improvement of the stability of this extract and its biological activities.

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Conflicts of Interest

The authors declare no conflict of interest.

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Annex 11

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Biopharmaceutical Sciences | *Ciências Biofarmacêuticas* *Biomed Biopharm Res.*, 2015; (12) 1: , 127-138

Antimicrobial screening of *Plectranthus madagascariensis* and *P. neochilus* extracts

Pesquisa da actividade antimicrobiana de extratos de Plectranthus madagascariensis e P. neochilus

Margarida Pereira, Diogo Matias, Filipe Pereira, Catarina P. Reis, M. Fátima Simões and Patrícia Rijo
CBIOS, Universidade Lusófona de Humanidades e Tecnologias, Campo Grande, 376, 1749-024 Lisboa, Portugal
Email: patricia.rijo@ulusofona.pt

Abstract

Natural products are widely used as traditional medicines and are a common source of bioactive molecules for the treatment of bacterial infections. In particular, some plants of the genus Plectranthus (Lamiaceae) have demonstrated several applications, including the treatment of various infections. In this work, aqueous, acetonic and methanolic extracts of P. madagascariensis and P. neochilus were prepared using several extraction methods (infusion, decoction, maceration, microwave- and ultrasound-assisted and supercritical fluids). All extracts were screened for their antimicrobial activity against Gram positive bacteria (Enterococcus faecalis, Staphylococcus aureus, Bacillus subtilis and Mycobacterium smegmatis) and Gram negative bacteria (Pseudomonas aeruginosa, Klebsiella pneumoniae and Escherichia coli) and two yeast (Candida albicans and Saccharomyces cerevisiae) strains. The P. madagascariensis acetonic extracts obtained using ultrasound and maceration methods and P. neochilus acetonic extract obtained by ultrasound technique showed activity against the five tested Gram positive bacteria (5–24 mm of inhibition zone using the well diffusion test). The antimicrobial activity was further evaluated by the microdilution method (MIC values were between 250–0.49 μg/mL) and the bioautography assay against S. aureus.
The P. madagascariensis ultrasound acetonic extract was the most active extract against all the tested Gram positive bacteria (MIC values ranged between 31.25 - 0.49 μg/mL). It was also active against resistant MRSA and VRE strains (MIC values ranged between 31.25-0.98 μg/mL). The S. aureus bioautography assay showed that the more polar compounds were the responsible for the antimicrobial activity.

Keywords: Antimicrobial activity; natural products; *Plectranthus spp*; extraction methods.

Resumo

Os produtos naturais são amplamente utilizados como remédios tradicionais e são uma fonte comum de moléculas bioativas para o tratamento de infeções bacterianas. Em particular, algumas plantas do género Plectranthus (Lamiaceae) demonstraram diversas aplicações, incluindo no tratamento de várias infeções. Neste trabalho, foram preparados extratos aquosos, acetónicos e metanólicos de P. madagascariensis e P. neochilus utilizando vários métodos de extração (infusão, decocção, maceração, extração assistida por micro-ondas e por ultrassom e com fluidos no estado supercrítico). Todos os extratos foram testados quanto à atividade antimicrobiana face a bactérias de Gram-positivo (Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis Bacillus subtilis e Mycobacterium smegmatis) e de Gram-negativo (Pseudomonas aeruginosa, Klebsiella pneumoniae e Escherichia coli) e duas estirpes de leveduras (Candida albicans e Saccharomyces cerevisiae). O extrato acetónico de P. madagascariensis obtido pelas técnicas de ultrassom e maceração e o extrato acetónico de P. neochilus obtido pela técnica de ultrassom demonstraram atividade contra as cinco bactérias Gram-positivas testadas (5-24 mm de zona de inibição no teste de difusão). A atividade antimicrobiana foi ainda avaliada pelo método da microdiluição (valores de CMI entre 250-0,49 μg/mL) e pelo ensaio de bioautografia usando o S. aureus. O extrato acetónico de P. madagascariensis foi o mais ativo contra todas as bactérias de Gram positivo testadas (valores de CMI entre 31,25-0,49 μg/ml). Foi também ativo em bactérias resistentes a antibióticos: MRSA e VRE (valores de CMI entre 31,25-0,98 μg/mL). Os ensaios de bioautografia com S. aureus demonstraram que os compostos mais polares são os responsáveis pela sua atividade antimicrobiana.

Palavras-chave: Actividade antimicrobiana; Produtos naturais; *Plectranthus spp*; Métodos de extração.

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Introduction

The medicinal plants, which are the basis of the traditional medicine, have been intensely studied in the recent decades. Many of those works were dedicated to the antimicrobial action of the plants and, lately, to the discovery of potential and new antimicrobial agents[1,2].

The antimicrobials of natural origin usually present complex chemical structures which may be important for specific interactions and recognition of potential macromolecular targets in the pathogenic microorganisms. These active natural products are, generally, plant secondary metabolites biosynthesized to fight and prevent the proliferation of plant pathogenic microorganisms[1].

In the developing countries it is estimated that about 80% of the population depends on traditional medicine for the primary health care[2-3]. Thus, there is an increased interest for screening the medicinal plants to identify the bioactive components of potential therapeutic value for the discovery and development of new drugs[3].

The family of Lamiaceae contains various genera, such as *Mentha* (mint), *Salvia* (sage) and *Ocimum* (basil), which have a great commercial importance mainly due to their aromatic characteristics. *Plectranthus* is another important genus of this plant family that is widely found in Africa, Asia and Australia. It comprises about 300 species, 62 of which are reported as having food usage and therapeutic use, namely as antimicrobial agents against mycobacteria, bacteria, viruses, protozoa and fungi[2, 4-5].

The phytochemical studies on some species of *Plectranthus* revealed the presence of a large number of diterpenes and triterpenes[2].

Diterpenes are natural compounds with a hydrocarbon backbone with twenty carbon atoms, corresponding to four isoprene units (a chemical structure of five carbon atoms). They may have an acyclic structure but most of them are cyclic compounds. Diterpenes are the second major group of terpenes resulting from the plant metabolism. They have several significant roles in the growth and development of the plant and are also important in the resistance to environmental stress[2].

More than two thousands of diterpenoid compounds are known with diverse structures. They show a wide array of pharmacological activities including bronchodilator, anti-hypertensive, anti-inflammatory and platelet aggregation inhibitory properties. These properties are likely related to the hydrocarbon backbone cyclization (bicyclic, tricyclic, tetracyclic or macrocyclic) combined with a wide range of oxidized functional groups (e.g., hydroxyl and carbonyl groups)[2].

Several phytochemical studies have reported the isola-

Introdução

As plantas medicinais que constituem a base da medicina tradicional têm sido, nas últimas décadas, objeto de intenso estudo farmacológico. Muitos destes trabalhos dedicaram-se ao estudo da ação antimicrobiana das plantas com o objetivo de contribuir para a descoberta de novos antimicrobianos. [1][2]

Os metabolitos antimicrobianos de origem natural apresentam geralmente estruturas químicas complexas eventualmente importantes para as interações específicas e reconhecimento dos alvos macromoleculares das bactérias patogénicas. Estes produtos naturais correspondem, geralmente, a metabolitos secundários biossintetizados para combater e prevenir a proliferação de microrganismos patogénicos para as plantas. [1]

Nos países em desenvolvimento estima-se que cerca de 80% da população dependa da medicina tradicional para os cuidados de saúde primários.[2][3] Por conseguinte, a pesquisa de compostos bioactivos, com eventual interesse terapêutico, a partir das plantas medicinais é do maior interesse para a descoberta e o desenvolvimento de novos fármacos. [3]

A família de plantas *Lamiaceae* é constituída por vários géneros, tais como a *Mentha* (menta), *Salvia* (sálvia) e *Ocimum* (manjericão), que possuem uma grande importância comercial especialmente devido às suas características aromáticas. Outro género, também muito importante desta família de plantas, é o género *Plectranthus* que se encontra amplamente distribuído por África, Ásia e Austrália. Este género inclui mais de 300 espécies estando 62 destas descritas como tendo uso alimentar e uso terapêutico, nomeadamente, como agentes anti-infecciosos para combater micobactérias, bactérias, vírus, protozoários e fungos.[2][4-5]

Os estudos fitoquímicos em algumas espécies de *Plectranthus* revelaram a presença de uma grande variedade de componentes incluindo diterpenos e triterpenos. [2]

Os diterpenos são compostos naturais com um esqueleto hidrocarbonado constituído por vinte átomos de carbono, o que corresponde a quatro unidades de isopreno (uma estrutura química de cinco carbonos). Podem possuir uma estrutura acíclica, mas na maior parte são compostos cíclicos e são o segundo maior grupo de terpenos resultante do metabolismo das plantas. Têm diversos papéis relevantes, nomeadamente, no crescimento e desenvolvimento da planta e são importantes na resistência ao stress ambiental.[2]

São conhecidos mais de dois mil compostos diterpénicos com estruturas distintas, apresentando uma ampla gama de propriedades farmacológicas, incluindo a broncodilatadora, anti-hipertensora, anti-inflamatória e

tion and characterization of diterpenes, mostly abietanes with a royleanone substructure, showing antimicrobial activities. However, only a few studies have related the diterpene activity with the traditional use of the plant species[6-7].

Some species of the *Plectranthus* genus have ethnopharmacological applications, with antimicrobial activity often cited[8]. Indeed, it is known that both the metabolites and extracts of various *Plectranthus* spp. have exhibited activity against Gram-positive and Gram-negative bacteria and yeasts[3-4, 9].

This work aimed to study the microbial properties of *Plectranthus madagascariensis* and *Plectranthus neochilus*. Thus, the different antimicrobial activities of the extracts obtained by different solvents (water, acetone or methanol) and using different extraction methods (infusion, decoction, maceration, microwave or ultrasound) were examined. These activities were evaluates using the well diffusion assay in solid medium, the bioautography method and through the determination of the minimum inhibitory concentration (MIC) in liquid medium.

inibidora da agregação plaquetária. A multiplicidade de ações farmacológicas dos diterpenos está relacionada com a diversidade de ciclização do esqueleto (bicíclico, tricíclico, tetracíclico ou macrocíclico) aliada a uma vasta gama de grupos funcionais com oxigénio (por exemplo grupos hidroxilo e grupos carbonilo).[2]

A maioria dos estudos fitoquímicos realizados teve como finalidade o isolamento e a caracterização destes compostos. Embora alguns diterpenos tenham demonstrado uma potente actividade antimicrobiana, como por exemplo os abietanos com estrutura de roileanona, poucos foram os estudos efectuados que relacionassem esta característica com o uso tradicional de certas espécies vegetais.[6][7]

Algumas espécies de *Plectranthus* com aplicações etnofarmacológicas são usadas pela sua acção antimicrobiana. Muitas atividades biológicas têm sido atribuídas a plantas deste género e a sua atividade antimicrobiana é frequentemente citada [8]. Com efeito, sabe-se que tanto os extratos como os metabolitos de vários *Plectranthus* spp. apresentam frequentemente actividades contra bactérias de Gram-positivo, Gram-negativo e leveduras. [3][4][9]

Este trabalho teve como objectivo estudar as características antimicrobianas das plantas *Plectranthus madagascariensis e Plectranthus neochilus*. Assim, pesquisaram-se as diferenças na actividade biológica resultantes da utilização de diferentes solventes de extracção (água, acetona e metanol) e diferentes métodos de extracção (infusão, decocção, maceração, microondas e ultrassons). A actividade antibacteriana foi determinada através do ensaio de difusão em poços através de meio sólido, do método de bioautografia e do método da determinação da concentração inibitória mínima (MIC) em meio líquido.

Material and Methods

Plant material

Plectranthus madagascarensis and Plectranthus neochilus were provided by the Faculty of Pharmacy of the University of Lisbon.

Extraction methodologies of the plant material

The aqueous plant extracts were prepared using 10 g of powdered plant material dissolved in 100 mL of distilled water, subsequently filtered using Whatman paper n.° 5 (Whatman, Inc., Clifton, NJ.). These aqueous extracts were obtained using three extraction methods: infusion, decoction and microwave-assisted.

The acetone and methanol extracts were prepared using

Materiais e Métodos

Material vegetal

Plectranthus madagascarensis e *Plectranthus neochilus,* foram fornecidos pela Faculdade de Farmácia da Universidade de Lisboa.

Metodologias de extracção do material vegetal

Os extratos vegetais aquosos foram preparados usando 10 g de material vegetal em pó colocado em 100 mL de água destilada, sendo posteriormente filtrado utilizando um papel de filtro Whatman n.° 5 (Whatman, Inc., Clifton, NJ, EUA). Estes extratos foram obtidos através de três métodos de extracção distintos: infusão, decocção e assistidos por micro-ondas.

10 g of powdered plant material dissolved in 200 mL of the respective solvents. These extracts were obtained using maceration extraction method and sonication.
In the decoction method the extracts were prepared boiling the plant material in distilled water for 10 minutes. In the infusion method, the extracts were prepared by addition of boiling distilled water to the plant for 10 minutes. The extracts prepared by the microwave-assisted method were obtained mixing the plant material with distilled water into a microwave oven for 2 minutes at a continuous irradiation at 2.45 GHz. In the maceration method, the plant material and the solvent were left under stirring at room temperature. After 24 hours, the plant material was filtered into a round bottom flask and then evaporated (vacuum at 40 °C). In the ultrasonic method, the Erlenmeyer with the plant material and the solvent was placed in an ultrasonic bath at room temperature for 2 hours.

Microorganisms

The microorganisms used throughout this study were antibiotic sensitive (*Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 10231; *Mycobacterium smegmatis* ATCC 607; *Klebsiella pneumoniae* ATCC 9997; *Saccharomyces cerevisiae* ATCC 9763 and *Staphylococcus epidermidis* ATCC 12228) and antibiotic resistant (methicillin-resistant *S. aureus* CIP 106760 and FFHB 29593 (MRSA) and low-level vancomycin-resistant *E. faecalis* ATCC 51299 (VRE).

Antimicrobial activity

Well diffusion method

Initially, the microorganisms were cultured on Muller-Hinton agar for bacteria and Sabouraud agar for yeasts. Then, 100 µL of a standardized microorganism suspension, corresponding to 0.5 McFarland, was used to inoculate a Petri dish of solid Mueller-Hinton medium (Sabouraud for yeasts). These suspensions were spread over the medium surface using a sterile swab[10-11].
Subsequently, agar wells were made of approximately 5.0 mm in diameter with a Pasteur pipette. Then, 50 µL of each sample, negative control (DMSO) and positive controls (vancomycin, amphotericin B or norfloxacin) for Gram positive bacteria (S. *aureus, E. faecalis, B. subtilis ; M. smegmatis and S. epidermidis*), Gram-negative (*K pneumoniae, P. aeruginosa and E. coli*) and yeasts (*S. cerevisiae and C. albicans*), were added on each of the wells. Plates were incubated at 37 °C for 24

Os extratos de acetona e de metanol foram preparados usando 10 g de material vegetal em pó colocado em 200 mL dos respectivos solventes. Estes extratos foram obtidos utilizando o método de extracção de maceração e ultrassons.
No método de extracção por decocção, os extratos foram preparados por ebulição do material vegetal em água destilada durante 10 minutos. No método de infusão, os extratos foram preparados através da adição de água destilada fervente durante 10 minutos. Os extratos preparados pelo método de extracção de micro-ondas resultaram da junção do material vegetal utilizado com água destilada colocados num aparelho de micro-ondas convencional durante 2 minutos a uma irradiação contínua de 2,45 GHz. No método da maceração, o material vegetal e o solvente foram deixados sob agitação à temperatura ambiente. Após 24 horas, o macerado foi filtrado para um balão de fundo redondo e, em seguida, evaporado a pressão reduzida a 40 °C dando origem a um resíduo. No método de extracção por ultrassons, o matraz com o material vegetal e o solvente foi colocado num aparelho de ultrassons à temperatura ambiente durante 2 horas, após as quais a mistura foi filtrada e solvente evaporado (pressão reduzida e 40 °C).

Micro-organismos

Os micro-organismos utilizados ao longo deste estudo foram sensíveis aos antibióticos (*Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 10231; *Mycobacterium smegmatis* ATCC 607; *Klebsiella pneumoniae* ATCC 9997; *Saccharomyces cerevisiae* ATCC 9763 e *Staphylococcus epidermidis* ATCC 12228) e bactérias resistentes (*S. aureus* resistente à meticilina CIP 106760 e FFHB 29593 (MRSA) e de baixo nível de resistência à vancomicina *E. faecalis* ATCC 51299 (VRE)).

Actividade Antimicrobiana

Método de difusão em poços

Inicialmente, os micro-organismos foram inoculados em meio de Mueller-Hinton para as bactérias e Sabouraud para as leveduras. De seguida, foram utilizados 100 µL de uma suspensão de microorganismo padronizado, correspondente a 0,5 McFarland, para inocular uma placa de Petri com meio de Mueller-Hinton sólido (Sabouraud para as leveduras). Estas suspensões foram espalhadas sobre uma superfície usando uma zaragatoa estéril.[10,11]
Posteriormente, os poços foram feitos no agar com aproximadamente 5,0 mm de diâmetro, com uma pipeta de Pasteur. Em seguida, 50 µL de cada amostra, do controlo negativo (DMSO) e dos controlos positivos (vancomicina, anfotericina B ou norfloxacina) para as bactérias Gram-positivas (*S. aureus, E. faecalis, B. subtilis, M. smegmatis* e *S. epidermidis*), Gram-negativa (*K. pneumoniae, P. aeruginosa* e *E. coli*) e leveduras (*S. cerevisiae* e *C. albicans*), foram adicionados em

hours. After this period, the diameters of the inhibition zones were measured (no growth) and the results were expressed in millimeters (mm).
The assay was performed under aseptic conditions and in triplicate[11].

Bioautography Method

The bioautography is a technique that evaluates qualitatively the antimicrobial activity of mixtures of natural or synthetic substances. This technique is widely used mainly due to the high sensitivity and method ease[12].
This method was only used for the acetonic extracts of *P. madagascariensis* obtained by ultrasound and maceration methods and the acetonic extracts of *P. neochilus* obtained by ultrasound. This selection was based on the activity previously examined in the diffusion method. A Gram-positive reference bacterium, *S. aureus*, was chosen. The extracts were applied on a TLC plate (10x10 cm) and then developed using a mixture of *n*-hexane: ethyl acetate (8:2; v/v). The chromatogram was allowed to dry completely and was placed on a solution of Mueller-Hilton inoculated with *S. aureus* containing tetrazolium chloride (10 % w/v), and was incubated overnight at 37°C.
The development of the plate showed clear zones against a red background indicating inhibition of the bacterial growth by the extract bioactive compounds. The assay was performed four times[13].

Determination of minimum inhibitory concentration (MIC)

The assay was performed using the microplate broth microdilution method according to the Clinical and Laboratory Standards Institute (2011). Briefly, 100 µL of Mueller-Hilton broth (Sabouraud for yeasts) was placed into each well of a 96 microplate, under aseptic conditions.100 µL of each extract sample, the appropriate positive control of each microorganism and negative controls at a concentration of 1 mg/mL, were added on the first well. Using a multichannel micropipette, a 1:2 microdilution series was made. 10 µL of a standardized bacterial suspension, corresponding to 0.5 McFarland of each microorganism were then placed in all wells. Finally, the plates were incubated at 37 °C for 24 hours. The microbial growth was evaluated in a microplate absorbance reader (Multiskan FC Thermo Scientific, Loughborough, UK) at 620 nm. The assays were performed in triplicate for all microorganisms[3].
The antimicrobial activity of the extracts was also tested against antibiotic-resistant bacteria *S. aureus* (MRSA - Methicillin-resistant *Staphylococcus aureus*; FFHB - clinical isolate) and *E. faecalis* (VRE - Vancomycin-low resistant *Enterococcus*)[14].

cada um dos poços. As placas foram incubadas a 37 °C durante 24 horas. Após este período, os diâmetros das zonas de inibição foram medidos (ausência de crescimento) e os resultados foram expressos em milímetros (mm). Os ensaios foram realizados sob condições assépticas e em triplicado.[11]

Método da Bioautografia

A Bioautografia é uma técnica que avalia qualitativamente a actividade antimicrobiana de substâncias de origem sintética ou natural contidas numa mistura que são previamente separadas por cromatografia em camada delgada (ccd). Esta técnica é amplamente utilizada devido à sua sensibilidade e facilidade de visualização dos resultados de inibição do crescimento de microrganismos. [12]
Este método foi apenas utilizado para os extratos acetónicos do *P. madagascariensis* obtidos por ultrassons e maceração e para os extratos acetónicos do *P. neochilus* obtidos por ultrassons, que foram escolhidos por apresentarem maior actividade no método da difusão. A bactéria Gram-positiva de referência escolhida para este estudo foi S. *aureus*.
Os extratos foram aplicados numa placa de ccd (10x10 cm), esta foi desenvolvida utilizando uma mistura de *n*-hexano: acetato de etilo (8:2; v/v) e foi deixada a secar completamente. O cromatograma foi colocado numa solução de Mueller-Hilton inoculada com *S. aureus* contendo cloreto de tetrazólio (10 % m/v) e foi incubada durante a noite a 37° C. Após a incubação a placa apresentou zonas claras contra um fundo vermelho o que indica a inibição do crescimento bacteriano dos compostos bioactivos do extrato. O ensaio foi realizado quatro vezes.[13]

Método da determinação da Concentração Mínima Inibitória (CMI)

O ensaio foi realizado pelo método de microdiluição em poços de microplacas de acordo com o Clinical and Laboratory Standards Institute (2011). Resumidamente, 100 µL de meio de Mueller-Hilton (Sabouraud para as leveduras) foram colocados em cada poço de uma microplaca de 96 poços, sob condições assépticas. No primeiro poço foram adicionados 100 µL de cada amostra de extrato, de igual modo, do apropriado controlo positivo de cada microrganismo e do controlo negativo, a uma concentração de 1 mg / mL. Microdiluições de 1:2 em série foram realizadas, usando uma micropipeta multicanal. Em todos os poços foram colocados 10 µL de uma suspensão bacteriana padronizada, correspondente a 0,5 McFarland de cada micro-organismo. Finalmente, as placas foram incubadas a 37 °C durante 24 horas. O crescimento microbiano foi avaliado num leitor de microplacas de absorvância (Multiskan FC Thermo Scientific, Loughborough, Reino Unido) a 620 nm. Os ensaios foram realizados em triplicado para todos os micro-organismos.[3] A atividade antimicrobiana dos extratos também foi testada contra bactérias resistentes a antibióticos *S. aureus* (MRSA - *Staphylococcus aureus* resistente à meticilina; FFHB - isolado clínico) e *E. faecalis* (VRE - *Enterococcus* de baixa resistência à vancomicina).[14]

Results

Well Diffusion method

The antibacterial activity of the several extracts of the two species of the *Plectranthus* genus was evaluated by the well diffusion method. Considering all extracts, the acetonic extracts of *P. madagascariensis* obtained by the ultrasound and the maceration methods and the acetonic extract of *P. neochilus* obtained by the ultrasound technique were the more active against the five Gram-positive bacteria tested (5-24 mm of inhibition zone). The highest antibacterial activity was found against bacteria *S. aureus* (Table 1).

None of the extracts showed significant antibacterial activity against Gram negative bacteria or against the yeasts tested, with inhibition zones identical to that obtained with the negative control DMSO (5 mm).

It was also noted that none of the extracts showed significant antibacterial activity against Gram-negative bacteria and yeasts.

Table 1 - Antimicrobial activity of the acetonic extracts obtained by the well diffusion method.

Microrganisms		*Plectranthus madagascariensis*		*Plectranthus neochilus*
		Ultrasound (mm)	Maceration (mm)	Ultrasound (mm)
Gram Positive Bacteria	*S. aureus*	20	24	8
	E. faecalis	15	5	5
	B. subtilis	24	20	12
	M. smegmatis	26	26	20
	S. epidermidis	25	10	n.d.
Gram Negative Bacteria	*K.pneumoniae*	22	25	5
	P. aeruginosa	5	5	5
	E. coli	5	5	5
Yeasts	*C. albicans*	5	5	5
	S. cerevisiae	5	5	5

n.d. – not determined

Resultados

Método de difusão em poços

A actividade antimicrobiana dos vários extratos das duas espécies do género *Plectranthus* foi avaliada pelo método de difusão em poços. De todos os extratos estudados, os extratos acetónicos de *P. madagascariensis* obtidos por ultrassons e pelo método de maceração e do extrato acetónico do *P. neochilus* obtido pela técnica de ultrassons mostraram atividade contra as cinco bactérias Gram-positivas testadas, com 5-24 mm de zona de inibição. A maior actividade antibacteriana encontrada foi em relação à bactéria *S. aureus* (Tabela 1).
Nenhum dos extratos mostrou actividade antibacteriana significativa contra bactérias Gram negativas nem nas leveduras testadas, apresentando zonas de inibição iguais à obtida com o controlo negativo DMSO (5 mm).

Tabela 1 - Actividade antimicrobiana dos extratos acetónicos pelo método de difusão em poços.

Micro-organismo		*Plectranthus madagascariensis*		*Plectranthus neochilus*
		Ultrassons (mm)	Maceração (mm)	Ultrassons (mm)
Bactérias Gram Positivo	*S. aureus*	20	24	8
	E. faecalis	15	5	5
	B. subtilis	24	20	12
	M. smegmatis	26	26	20
	S. epidermidis	25	10	n.d.
Bactérias Gram Negativo	*K. pneumoniae*	22	25	5
	P. aeruginosa	5	5	5
	E. coli	5	5	5
Leveduras	*C. albicans*	5	5	5
	S. cerevisiae	5	5	5

n.d. – não determinado

In this last case, the zones of inhibition were similar to the negative control DMSO (5 mm).

Bioautography Method

Concerning the bioautography assays, the three bioactive extracts previously selected (acetonic extracts of *P. madagascariensis* obtained by ultrasound and the maceration method and the acetonic extract of *P. neochilus* obtained by the ultrasound technique) have also shown antibacterial activity against the *S. aureus* bacteria (Figures 1- 3). The plates revealed that the antimicrobial compounds were the more polar compounds (<Rf) corresponding to clear zones against a red background, thus indicating inhibition of the bacterial growth.

Método de Bioautografia

Relativamente aos ensaios de bioautografia, nos três extratos bioactivos selecionados anteriormente (os extratos acetónicos de *P. madagascariensis* obtidos por ultrassons e pelo método de maceração e do extrato acetónico do *P. neochilus* obtido pela técnica de ultrassons) também se observou atividade antibacteriana em relação à bactéria *S. aureus* (Figuras 1 a 3). Os cromatogramas monstraram que a atividade antimicrobiana corresponde aos compostos mais polares, pois foi na parte inferior do cromatograma (< Rf) que não se observou coloração vermelha característica do crescimento bacteriano.

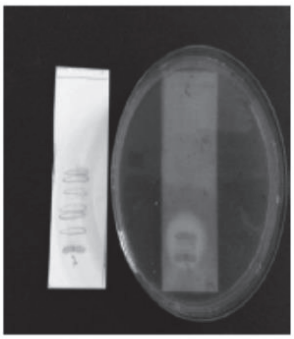

Figure 1/ Figura 1.
Antimicrobial activity observed by the bioautography method of the *P. madagascariensis* acetonic extract obtained by the ultrasound technique.
Atividade antimicrobiana observada pelo método de bioautografia do extrato acetónico de *Plectranthus madagascariensis* obtido por ultrassons

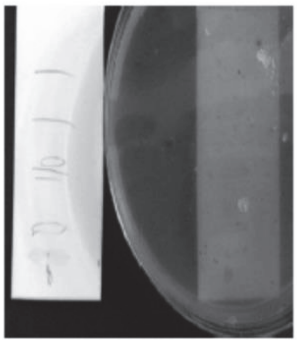

Figure 2/ Figura 2.
Antimicrobial activity observed by the bioautography method of the *P. madagascariensis* acetonic extract obtained by the maceration technique.
Atividade antimicrobiana obtida pelo método de bioautografia do extrato acetónico do *Plectranthus madagascariensis* obtido por maceração.

Figure 3/ Figura 3.
Antimicrobial activity observed by the bioautography method of the *P. neochilus* acetonic extract obtained by the ultrasound technique.
Atividade antimicrobiana obtidaatravés do método de bioautografia do extrato acetónico do *Plectranthus neochilus* obtido por ultrassons.

Microdilution method: minimum inhibitory concentration evaluation (MIC)

The antimicrobial activity of bioactive extracts previously selected by the well diffusion method and bioautography was further evaluated by the broth microdilution method. The values of minimum inhibitory concentration (MIC) were determined ranging from 250 to 0.49 µg/mL The acetone extract of *P. madagascariensis* obtained by ultrasound method was the most active against all Gram positive bacteria tested (Table 2) (MIC values ranging from 31.25 to 0.49 µg/mL) and the Gram-negative bacteria *K. pneumoniae* (<0.49 µg/mL against <7.81 for P. neochilus ; Table 2). The acetone extract of *P. madagascariensis* obtained by ultrasound method was also tested against three strains of the antibiotic resistant bacteria MRSA and VRE and the corresponding MIC values were between 31.25 to 0.98 µg/mL (Table 3).

Método de microdiluição: Determinação da concentração mínima inibitória (CMI)

A atividade antimicrobiana dos extratos bioativos selecionados pelos métodos de difusão em poços e por bioautografia foi ainda avaliada pelo método de microdiluição. Os valores de concentração mínima inibitória (CMI) foram determinados e foram obtidos entre 250 e 0,49 µg/mL. O extrato acetónico de *P. madagascariensis* obtido por ultrassons foi o extrato mais ativo contra todas as bactérias de Gram positivo (Tabela 2) testadas (valores de CMI entre 31,25-0,49 µg/mL) e a bactéria de Gram negativo *K. pneumoniae* (7,81-<0,49 µg/mL; Tabela 2). O extrato acetónico de *P. madagascariensis* obtido por ultrassons foi também testado em três estirpes de bactérias resistentes a antibióticos MRSA e VRE e os correspondentes valores de CMI foram entre 31,25-0,98 µg/mL (Tabela 3).

Table 2/ Tabela 2 - Antimicrobial activity of the acetone extracts by the microdilution method (MIC values ranging between 250-0.49 µg/mL)
Atividade antimicrobiana dos extratos acetónicos pelo método de microdiluição (Valores de MIC entre 250-0,49 µg/mL)

Bacteria		*P. madagascariensis*		*P. neochilus*
		Ultrasound method/ Ultrassons	Maceration Method/ Maceração	Ultrasound Method/ Ultrassons
Gram positive/ Gram positivo	*B.subtilis*	3.91	62.5	125
	S.aureus	3.91	250	250
	S.epidermidis	7.81	62.5	62.5
	M.smegmatis	31.25	62.5	15.62
	E.faecalis	n.d	15.62	n.d
Gram negative/ Gram negativo	*K.pneumoniae*	<0.49	3.91	<7.81

n.d. – not determined/ não determinado

Table 3/ Tabela 3 - Antimicrobial activity of acetonic extract of P. madagascariensis obtained by ultrasound method by the microdilution method against resistant bacteria (MIC values ranging between 250-0.49 ug/mL) Atividade antimicrobiana do extrato acetónico de P. madagascariensis obtido por ultrassons pelo método de microdiluição contra bactérias resistentes (valores de CMI entre 250-0,49 µg/mL).

Bacteria/ Estirpe Bacteriana	*S. aureus* (MRSA)	*S. aureus* FFHB	*E. faecalis* (VRE)
MIC	1.95	0.98	31.25

Discussion

In the present study, the antimicrobial activities of two species of the genus *Plectranthus* (*P. neochilus* and *P. madagascarensis*) were investigated. Different extraction methods were carried out including infusion, decoction and microwave methods for aqueous extracts and ultrasonic and maceration for acetonic and methanolic extracts. The extraction solvents used were water, acetone and methanol. The objective of this study was to investigate the antimicrobial characteristics of both species and evaluate the bioactivity differences between the different extraction solvents and methods.

In vitro tests for antimicrobial activities were performed using Gram-positive bacteria (*S. aureus, E. faecalis, B. subtilis, M.smegmatis* and *S. epidermidis*), Gram-negative bacteria (*K. pneumoniae, P. aeruginosa* and *E .coli*) and yeasts (*C. albicans* and *S. cerevisiae*). The antimicrobial activity was performed by the well diffusion method to select and identify the bioactive extracts. Only the acetone extracts of *P. madagascarensis* (obtained by maceration and ultrasound methods) showed inhibition activity against the Gram positive bacteria (Table 1) and showed no significant activity against the Gram negative bacteria and yeasts. This is in agreement with other studies previously published[7]. Aqueous and methanolic extracts were shown to be inactive against all the microorganisms tested.

The previously selected active extracts were evaluated by the broth microdilution method to determine the MIC values. The antimicrobial activity against three antibiotic-resistant bacteria, MRSA and VRE, was also evaluated by the same method in the most active acetonic extract previously selected (*P. madagascarensis* obtained by ultrasound methods). Through the bioautography method it was observed that the most polar compounds are responsible for the antimicrobial activity. Other studies in the genus *Plectranthus* species[2,15,16]

Discussão

No presente estudo foram pesquisadas as atividades antimicrobianas de duas espécies do género *Plectranthus (P. neochilus* e *P. madagascarensis)*. Foram realizados diferentes métodos de extração tendo sido utilizado os métodos de infusão, decocção e microondas para os extratos aquosos e o método de ultrassons e maceração para os extratos acetónicos e metanólicos, utilizando como solventes de extração a água, a acetona e o metanol. O objetivo deste estudo foi pesquisar as características antimicrobianas das duas espécies e observar as diferenças na bioatividade entre os diferentes solventes e métodos de extração.

Os ensaios *in vitro da* atividade antimicrobiana foram realizados utilizando bactérias de Gram positivo (*S. aureus, E. faecalis, B. subtilis, M. smegmatis* e *S. epidermidis*), bactérias de Gram negativo (*K. pneumoniae, P. aeruginosa* e *E. coli*) e leveduras (*C. albicans e S. cerevisiae*). A atividade antimicrobiana foi avaliada pelo método de difusão em poços, de modo a selecionar e identificar os extratos bioativos. Apenas os extratos acetónicos de *P. madagascarensis* (obtidos por maceração e ultrassons) demostraram resultados positivos em relação às bactérias Gram positivo (Tabela 1) mas não apresentaram atividade significativa contra as bactérias de Gram negativas e leveduras, o que está de acordo com outros estudos publicados anteriormente.[7] Os extratos aquosos e metanólicos mostraram ser inativos em todos os micro-organismos testados.

Os extratos que demostraram ser ativos foram avaliados pelo método de microdiluição de modo a determinar os valores de CMI. A atividade antimicrobiana contra três bactérias resistentes a antibióticos MRSA e VRE foi também avaliada pelo mesmo método, no extrato mais ativo seleccionado anteriormente (*P. madagascarensis* obtido por ultrassons). Através do método de bioautografia foi observado que os compostos mais polares são

describe compounds such as abietane-royleanone type, phenolic compounds (such as rosmarinic acid), essential oils and flavonoids as responsible for the antimicrobial activity. The cytotoxicity of the extracts that have shown antimicrobial activity should be evaluated in order to verify that the inhibition of microbial growth is due to its antimicrobial activity and not to its constituents toxicity. Future phytochemical studies must be performed to reveal the structural identification (by spectroscopic methods) of the compounds responsible for the antimicrobial activity. Thus, from this study *Plectranthus* genus and in particular the species *P. madagascariensis* and *P. neochilus* demonstrate to be important sources of antimicrobial compounds with potential for developing new antibiotics.

os responsáveis pela atividade antimicrobiana. Outros estudos em espécies do género *Plectranthus* [2][15][16] citam os compostos do tipo abietano-roileanona, compostos fenólicos (como o ácido rosmarínico), óleos essenciais e flavonóides como os responsáveis pela atividade antimicrobiana.
A citotoxicidade dos extratos que demostraram atividade antimicrobiana deverá ser avaliada de modo a verificar que a inibição do crescimento microbiano se deve à sua atividade antimicrobiana e não à toxicidade dos seus constituintes.
Outros estudos fitoquímicos deverão ser realizados, no futuro, para identificar (através de métodos espectroscópicos) os compostos bioativos responsáveis pela atividade antimicrobiana observada. Do presente estudo concluiu-se que o género *Plectranthus* nomeadamente as espécies *P. madagascariensis* e *P. neochilus* demonstram ser fontes importantes de compostos antimicrobianos com potencial para o desenvolvimento de novos antibióticos.

Conclusion

In this study, only acetonic extracts of the *P. madagascariensis* and *P. neochilus* showed activity against Gram-positive bacteria. The aqueous and methanolic extracts were inactive.
In the study, we may conclude that extracts of *P. madagascariensis* and *P.neochilus* may be considered a potential source of new bioactive chemicals and thus promising drug candidates for therapeutic critical areas such as infectious diseases.

Conclusão

No presente estudo, foram preparados extratos aquosos, acetónicos e metanólicos de *P. madagascariensis* e *P. neochilus* utilizando vários métodos de extração (infusão, decocção, maceração, extração assistida por micro-ondas e por ultrassom e com fluidos no estado supercrítico). Todos os extratos foram testados contra bactérias de Gram-positivo, de Gram-negativo e leveduras, mas apenas os extratos acetónicos de *P. madagascariensis* (obtidos por maceração e ultrassons) e *P. neochilus* (obtidos por ultrassons) mostraram atividade em relação às bactérias de Gram positivo testadas. Com este estudo é possível concluir que os extratos de *P. madagascariensis* e *P. neochilus* poderão ser uma potencial fonte de novas substâncias bioativas importantes sendo, por conseguinte, candidatos promissores a fármacos em áreas terapêuticas fundamentais como as doenças infecciosas.

Conflict of interests

The author declares that there are no financial or personal relationships that could be viewed as potential conflict of interest.

Conflitos de Interesse

O autor declara que não existem relações financeiras ou pessoais que pudessem ser vistas como potenciais conflitos de interesse.

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Annex 12

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Plectranthus madagascariensis phytosomes: formulation optimization

Fitossomas de Plectranthus madagascariensis: otimização da formulação

Diogo Matias[1], Luís Roque[1], Maria de Fátima Simões[1,3], Ana Diaz-Lanza[2], Patrícia Rijo*[1,3] and Catarina P. Reis*[1,4]
[1]Universidade Lusófona Research Center for Biosciences & Health Technologies (CBIOS), Campo Grande, 376, 1749-024 Lisboa, Portugal
[2]Dpto. Ciencias Biomédicas, Univesidad de Alcalá, Plaza de San Diego, s/n, 28801 Alcalá de Henares, Madrid, Espanha
[3]Instituto de Investigação do Medicamento (iMed.ULisboa), Faculdade de Farmácia da Universidade de Lisboa, Av. Professor Gama Pinto 1649-003 Lisboa, Portugal
[4]IBEB - Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal
*Shared senior authorship
E-mail: patricia.rijo@ulusofona.pt

Abstract

Medicinal plants have been a reliable source of bioactive natural products with potential pharmaceutical applications. The delivery of those bioactive agents into nanosystems is considered a promising strategy for the optimization of their pharmacologic effects. This work describes the preparation of phytosomes containing a bioactive extract from Plectranthus madagascariensis and optimization of the preparation method. Different formulations and process parameters were studied. It was observed that smaller and more uniform particles were obtained using acetone as solvent, a reaction time of two hours, and the addition of 2.5% molar concentration of cholesterol. The optimally prepared phytosomes had a diameter of 191.3 ± 75.3 nm with a polydispersity index of 0.243 ± 0.18, and a spherical shape with amorphous appearance. These nanosystems were able to encapsulate 92.8% of the extract, as evaluated by HPLC, relative to 7α,6β-dihydroxyroyleanona, the main extract component. This study suggests a future application of those phytosomes in the delivery of bioactive agents with therapeutic interest.

Keywords: Phytosome, Plectranthus, Lamiaceae, Diterpenes, Formulation optimization

Resumo

As plantas medicinais são uma fonte interessante de produtos naturais bioactivos com potenciais aplicações farmacêuticas. A veiculação destes agentes bioactivos em nanossistemas é considerada uma estratégia promissora para a optimização dos seus efeitos farmacológicos. O objectivo deste trabalho incide na preparação de fitossomas contendo um extracto bioactivo de Plectranthus madagascariensis e a sua optimização no método de preparação. Foram estudados vários parâmetros de formulação e processo. Observou-se que partículas menores e mais uniformes foram obtidas utilizando acetona como solvente, um período de reacção de 2h e com a adição do colesterol a uma concentração molar de 2,5%. Os fitossomas apresentaram um diâmetro de 191,3 ± 75,3 nm e com um índice de polidispersividade de 0,243 ± 0,18. Os fitossomas apresentaram uma forma esférica mas com aspecto amorfo. Estes nanossistemas foram capazes de encapsular 92,8% do extracto avaliado por HPLC em relação ao composto 7α,6β-dihidroxi-roileanona, o principal composto do extracto. Este estudo sugere uma futura aplicação destes fitossomas na veiculação de agentes bioactivos com interesse terapêutico.

Palavras-chave: Fitossoma, Plectranthus, Lamiaceas, Diterpenos, Optimização da formulação

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Introduction

Medicinal plants have been one of the most valuable sources of new drug leads. Recent reviews have shown that for modern clinically used drugs, about 90% were from natural origin, were derived from natural products, or their chemical structure was inspired from natural products[1].

Plants of the *Plectranthus* genus (Lamiaceae) have diverse ethnopharmacological applications including gastro-intestinal, infection and skin conditions[2]1 and 2. The members of Clade 1 (corresponding to the formally recognized genus Coleus. These uses may be related to the presence of bioactive diterpenes, namely abietanes[3]. Some physico-chemical characteristics of those molecules impair their bioavailability, however, namely an inadequate partition coefficient (log P) for solubilization in aqueous medium and/or to biological membrane crossing and the intrinsic instability of those molecules in physiological fluids (gastric pH effect, gut microflora metabolization, etc.)[4].

New drug delivery systems as the Phytosome® (Indena, Milan, Italy), have proven to be effective in surpassing those limitations[5]. A phytosome is complex formed between phospholipids such as phosphathydilcholine and the phytocomponents in the natural product. As main components of the cell membrane, the phospholipids possess an outstanding biocompatibility. Additionally, the phospholipids were notable for their amphiphilic structures. This characteristic leads to an self-assembling ability which can easily originate emulsions and confer humectant characteristics, valuable from the cosmetic point of view. In aqueous medium, the phospholipids create supramolecular structures which are naturally dependent on their properties and specific conditions. There are several phytosome based products already in the market[6].

They differ from liposomes as they have a similar proportion between phospholipid and drug (1:1 or 2:1) allowing higher entrapment of drug in the complex. Additionally, in liposomes the drug is placed into an internal cavity of the particle with limited interaction with surrounding medium, while in phytosomes the drug is dispersed in the phospholipid medium[7].

The first reports of phyto-phospholipid complex preparation used essentially organic aprotic solvents as dichloromethane, or dioxane as the reaction medium-[8]catalase and thiobarbituric acid reactive substances with respect to carbon tetrachloride treated group ($P < 0.05$ and <0.01. Those harmful solvents have been progressively substituted by less toxic solvents as acetone or ethanol[9]. The phytosomes were generally obtained

Introdução

As plantas medicinais são consideradas excelentes fontes de novos protótipos de fármacos. Recentes revisões bibliográficas demonstraram que, de todos os fármacos utilizados na clínica moderna, cerca de 90% apresentaram origem natural, isto é, são derivados de produtos naturais ou a sua estrutura química foi inspirada em produtos naturais[1]. As plantas do género *Plectranthus* (Lamiaceae) apresentam um conjunto diverso de usos etnofarmacológicos com aplicações no tratamento de sintomas gastrointestinais, infeções e problemas de pele[2]1 and 2. The members of Clade 1 (corresponding to the formally recognized genus Coleus. Esta utilização tradicional poderá estar relacionada com a presença de diterpenos e, em particular, diterpenos com esqueleto do tipo de abietano[3]. No entanto, algumas características físico-químicas destas moléculas limitam a sua biodisponibilidade como, por exemplo, o coeficiente de partição (log P) inadequado à solubilidade em meio aquoso e/ou à passagem através das membranas biológicas e a instabilidade intrínseca dessas moléculas em alguns fluidos fisiológicos (efeito do pH gástrico, metabolização pela microflora intestinal, etc)[4]. Novas estratégias de veiculação de fármacos como acontece com os fitossomas® (Indena, Milão, Itália) têm demonstrado inúmeras vantagens para ultrapassar essas limitações[5]. Os fitossomas são complexos moleculares formados por fosfolípidos, como a fosfatidilcolina, e os fitocomponentes existentes num produto natural. Como principais componentes da membrana celular, os fosfolípidos possuem uma excelente biocompatibilidade. Além disso, os fosfolipídos são conhecidos pelas suas estruturas anfifílicos. Esta característica confere uma capacidade de auto-organização, para fácil formação de emulsões e características humectantes muito interessantes do ponto de vista cosmético. Em meio aquoso, os fosfolipídos geram diferentes estruturas supramoleculares que são naturalmente dependentes das suas propriedades e condições específicas. Existem já no mercado inúmeros produtos baseados em fitossomas[6].

Eles diferem dos lipossomas pois têm um teor de fosfolípido similar ao do fármaco (proporção 1:1 ou 1:2), que permite uma maior proporção de fitocomponentes no complexo. No caso dos lipossomas, o composto ativo é colocado no interior de uma cavidade interna da partícula, verificando-se uma interação limitada com o meio circundante. No fitossoma, existe uma dispersão do fármaco no meio fosfolípidico[6]. As primeiras referências da preparação destes complexos referem-se ao uso essencialmente de solventes orgânicos no meio reaccional[7] como por exemplo, o diclorometano ou o

by solvent evaporation (drying at reduced pressure or freeze-drying), *salting out* or using supercritical fluids as the reaction medium[10].
This study describes the production optimization process of phytosomes containing a bioactive extract of *Plectranthus madagascariensis* based in two important parameters for their future clinical application: smaller particle size and more uniform size distribution.

dioxano[7]. Estes solventes apresentam grandes limitações de segurança e têm sido progressivamente substituídos por solventes menos tóxicos como a acetona ou o etanol[7]. As técnicas mais frequentes de preparação de fitossomas são a evaporação de solvente (secagem, a pressão reduzida ou liofilização), *salting out* ou ainda a utilização de fluídos supercríticos como meio reacional[7].
Neste artigo, é descrito o processo de otimização da produção de fitossomas contendo um extrato bioactivo de *Plectranthus madagascariensis*, baseado em dois parâmetros importantes para a sua futura aplicação clínica: menor tamanho possível e distribuição de tamanho mais uniforme.

Material and Methods

Chemicals

Phosphatidylcholine (48% purified from soy lecithin) and cholesterol were purchased from Sigma-Aldrich, Co (St. Louis, MO, USA). The chromatography solvents were HPLC-grade (Merck, Darmstadt, Germany) and remaining reagents were analytic grade.

Plant extract preparation

Dried and pulverized *Plectranthus madagascariensis* plant was sonicated (USC1200TH 45 kHz, VWR, Radnor, PA, USA) into acetone (100 mg plant/mL solvent). The resultant was filtered through Whatman paper nº4, vacuum-dried (RV06, IKA, Staufen, Germany) and an aliquot was analyzed using a validated HPLC-DAD method (1200 Infinity, Agilent Technologies, Santa Clara, California, USA), identifying the main components of the extract by comparison to authentic standards[11].

Phytosome preparation: research parameters

Different solvents (acetone, dichloromethane and ethanol), reaction times (60, 120 and 240 minutes) and cholesterol concentrations in the formulation (molar percentage 0, 2.5 and 5 %) were tested in order to obtain the smallest and most uniform nanoparticles. Briefly, molar equivalent concentrations of phosphatidylcholine and extract were solubilized in 20 mL of solvent, with constant stirring at 50ºC during the reaction time. Then, 40 mL of reverse osmosis water was added and the organic solvent was eliminated in a rotary evaporator, after which the pellet was recovered by centrifugation (23540G, 10 min).

Material e Métodos

Reagentes

Fosfatidilcolina (48% purificada a partir da lecitina de soja) e colesterol foram adquiridos à Sigma-Aldrich, Co (St. Louis, MO, EUA). Os solventes de cromatografia são de grau HPLC (Merck, Darmstadt, Alemanha) e os restantes reagentes foram de grau analítico.

Preparação do extracto vegetal

Material vegetal, seco e moído, de *Plectranthus madagascariensis* foi sonicado (USC1200TH 45 kHz, VWR, Radnor, PA, USA) em acetona (100 mg planta/mL solvente). O resultante foi filtrado em filtro Whatman n.º 4, seco a pressão reduzida (RV06, IKA, Staufen, Alemanha) e uma alíquota foi analisada usando um método validado de HPLC-DAD (1200 Infinity, Agilent Technologies, Santa Clara, Califórnia, EUA) sendo identificados os componentes maioritários do extracto por comparação com amostras autêntitcas[11].

Preparação dos Fitossomas: parâmetros a investigar

Diferentes solventes (acetona, diclorometano e etanol), tempos de reação (60, 120 e 240 min) e a concentrações de colesterol na formulação (percentagem molar de 0; 2,5 e 5%) foram testadas de modo a obter fitossomas de tamanho reduzido e uniformes. Resumidamente, as concentrações de equivalente molar de fosfatidilcolina e extrato foram solubilizados em 20 mL de solvente e mantidos em agitação a 50°C durante o tempo de reação. Posteriormente, 40 mL de água bidestilada foram adicionados e o solvente orgânico foi eliminado por evaporação (utilizando o evaporador rotativo), sendo o precipitado recuperado por centrifugação (23540G, 10 min).

Determination of encapsulation efficiency

Encapsulation efficiency (EE, %) of the extract into phytosomes was determined by evaluation of the non-encapsulated fraction. After centrifugation, the supernatant was collected, freeze-dried and dissolved into methanol prior to analysis by HPLC-DAD. The encapsulation efficiency was determined based on 7α,6β-dihydroxyroyleanone (the major compound in the extract) for which a calibration curve was established using pure synthetic standards.

Physical and morphological characterization

Phytosomes were diluted with distilled water and analysed in a Delsa Nano C Particle Analyzer (Beckman Coulter, CA, USA). The mean particle size and polydispersity index (PI) were evaluated at ambient temperature and 165°C. Morphology was assessed by surface electronic microscopy (SEM 5200LV, JEOL Ltd., Tokyo, Japan) at20 Kv after drying and gold cover (500 nm) of the samples.

Determinação da eficiência de encapsulação

A eficiência de encapsulação (EE, %) do extracto nos fitossomas foi determinada pela avaliação da fração do extracto não-encapsulado. Após centrifugação, o sobrenadante foi recolhido, liofilizado e dissolvido em metanol, antes da análise realizada em HPLC-DAD. A eficiência de encapsulação foi determinada tendo por base o pico de 7α,6β-dihidroxiroyleanona (o composto maioritário do extrato), para o qual tinha sido estabelecida previamente uma curva de calibração utilizando amostras autênticas.

Caracterização física e morfológica

Os fitossomas foram diluídos com água destilada e analisados num Delsa Nano C (Coulter, CA, EUA). O tamanho médio, o índice de polidispersividade (PI) foram avaliados neste equipamento à temperatura ambiente com 165°C. A morfologia foi avaliada por microscopia electrónica de varrimento (SEM 5200LV, JEOL, Tóquio, Japão) a 20 Kv após secagem e revestimento de ouro (500 nm) das amostras.

Results

Extract preparation and characterization

Extract was obtained as a yellowish gum with a yield of 0.22% ($m_{extract}/m_{dry\ plant}$). The HPLC-DAD analysis showed that this extract was rich in polyphenols and diterpenes, including 7α,6β-dihydroxyroyleanone (**1**) and coleon U (**2**) among the main components (Figure 1).

Resultados

Preparação e caracterização do extrato

O extrato apresentou uma coloração amarelada com um rendimento de extração de 0,22% ($m_{extrato}/m_{plantas\ secas}$). A análise por HPLC-DAD demonstrou que o extrato apresentou um elevado teor em polifenóis e diterpenos, sendo os componentes maioritários a 7α,6β-dihidroxiroyleanona (**1**) e a coleona U (**2**) (figura 1).

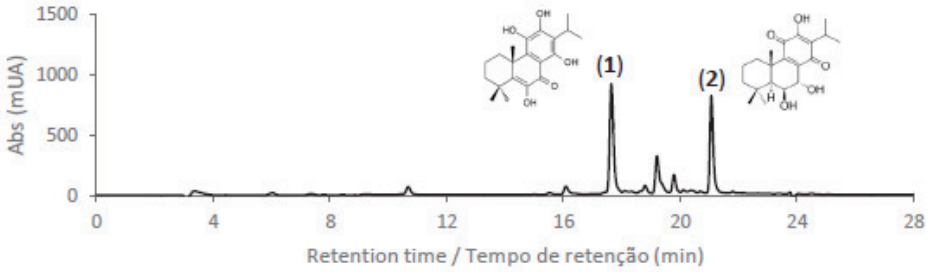


Figura 1/ Figure 1. HPLC profile of the prepared Plectranthus madagascariensis extract (280 nm) and identified major compounds /Perfil de HPLC do extracto de Plectranthus madagascariensis (280 nm) e identificação dos compostos maioritários

Phytosome complexes optimization

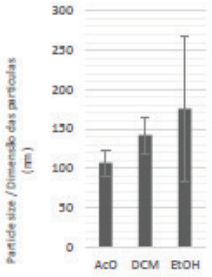
The results from the optimization process were presented in the Tables 1-3. Under the process conditions studied, the smallest and most uniform particles were produced using acetone, a reaction time of two hours and adding 2.5% of cholesterol.

Optimização dos fitossomas

Os resultados obtidos durante o processo de optimização estão apresentados nas tabelas 1-3. Os fitossomas de tamanho reduzido e uniformes foram produzidos utilizando a acetona, com um período de reação de 2h e adicionando 2,5% de colesterol.

Table 1/ Tabela 1. Influence of solvent choice in phytosome properties
Influência do solvente nas propriedades dos fitossomas.

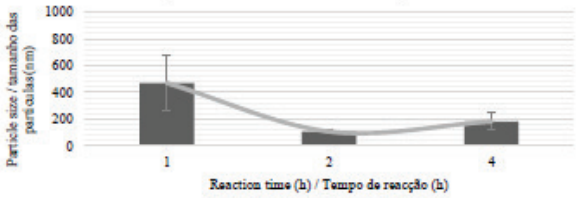
Reaction solvent / Solvente reação	**Size ± SD / Dimensão ± DP**	**PI / IP**
Acetone / Acetona	107.2 ± 16.55	0.419 ± 0.06
Dichloromethane / Diclorometano	142.2 ± 23.3	0.511 ± 0.12
Ethanol / Etanol	175.7 ± 91.65	0.359 ± 0.001



PI / IP: Polydispersivity index / Indice de polidispersividade; AcO: Acetone / Acetona; DCM: Dichloromethane / Diclorometano; EtOH: Ethanol / Etanol

Table 2 / Tabela 2. Influence of reaction time in phytosome properties
Influência do tempo de reação nas propriedades dos fitossomas.

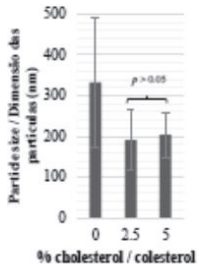
Time / Tempo (h)	**Size / Tamanho**	**PI / IP**
1	469.85 ± 201.95	0.772 ± 0.04
2	107.2 ± 16.55	0.410 ± 0.06
4	182.1 ± 64.35	0.458 ± 0.01



PI / IP: Polydispersivity index / Indice de polidispersividade

Table 3/ Tabela 3. Influence of cholesterol concentration in phytosome properties
Influência da concentração de colesterol nas propriedades dos fitossomas.

Cholesterol / Colesterol (%, M)	Size / Dimensão	PI / IP
0 %	332.2 ± 159.7	0.247 ± 0.03
2.5 %	191.3 ± 75.3	0.243 ± 0.18
5 %	203.3 ± 55.8	0.263 ± 0.15



PI / IP: Polydispersivity index / Índice de polidispersividade; %M: molar percentage / percentagem molar.

Encapsulation efficiency of optimized formulation

In order to quantify the amount of non-encapsulated main compound of the extract (**2**), a calibration curve was developed in the 0.005-1 mg/mL range (y = 25442x – 85.131; LOD: 0.002; LOQ: 0.006) with high linearity (R^2 = 0,9999). The encapsulation efficiency was 92.83 ± 0,520 % which is in accordance with other methods of production of phyto-phospholipidic particles[9].

Morphologic characterization

The nanoparticles were formed after the addition of water, verified by a change in the coloration of the reaction medium from translucent yellow to a yellowish opaque solution (Figure 2). The SEM images show that the phytosomes present a relatively amorphous spherical shape (Figure 2).

Eficiência de encapsulação da formulação otimizada

Para a determinação da EE foi construída uma curva de calibração utilizando uma gama de concentrações entre 0,005-1 mg / mL (y = 25442x - 85,131; LDD: 0,002; LOQ: 0,006) com alta linearidade (R^2 = 0,9999), tendo por base o composto maioritário do extracto não encapsulado. A eficiência de encapsulação foi de 92,83 ± 0,52%, o que está em conformidade com os resultados verificados em outros métodos de preparação de partículas fito-fosfolipídicas[8].

Morfologia

Após a adição de água, verificou-se uma mudança na coloração do meio da reação de amarelo translúcido para uma solução opaca e com uma coloração amarelada (Figura 2). As imagens SEM demonstraram que os fitossomas apresentam uma forma esférica relativamente amorfa (Figura 2).

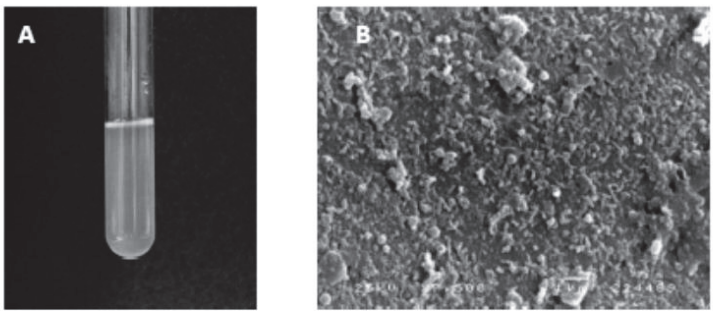


Figure 2/ Figura 2. Macro-(A) and microscopic (B) aspect of the optimized phytosomes / Aspecto macro- (A) e microscópica (B) dos fitossomas optimizados.

Discussion

This study demonstrates that the selection of formulation production parameters could be important factors in the final characteristics of the drug delivery system. The extract was successfully encapsulated into phytosomes, which are an excellent option for the delivery of natural compounds with low stability. Previously published studies have shown that the complexation of natural compounds with phospholipids could enhance the stability of a formulation[8]. The use of acetone for this formulation was more effective, producing smaller and more uniform particles than dichloromethane or ethanol, which are more frequently found in literature[8,9]catalase and thiobarbituric acid reactive substances with respect to carbon tetrachloride treated group ($P < 0.05$ and <0.01. This could be related to the enhanced solubility of the extract components in this solvent, which is the same used for the extraction. This is solvent is also considered less toxic than dichloromethane in pharmaceutic formulations (ICH class 2 *vs* ICH class 3). Related to the time of reaction, the literature describes that an extension of the reaction time does not always produce advantages at the granulometric level. The phenomena related to the higher aggregation probability could be responsible for this observation[12]. Cholesterol has been be used in phytosomes as stability enhancer of the nanosystems[13]. In this study it was shown that the inclusion of cholesterol results in smaller particles but without statistically significant difference between the use of a molar percentage of 2.5 or 5% ($p > 0.05$, t-student test, MS Excel). The smaller size of those nanosystems could be crucial for their absorption at the oral or transdermal level, if applicable. Previous studies have shown that a reduction on the size of the drug delivery systems improved their intestinal absorption [14,15]. This could mean that this size reduction improves the bioavaliability of the encapsulated phytocomponents.

The inclusion of the extract into phytosomes could be useful to obtain a stable pharmaceutic formulation with possible application as antibacterial or antitumor treatments. The acetonic extract prepared from *P. madagascariensis* possess as main compounds two diterpenes with interesting antibacterial[11,16] and anticancer[12] activities, 7α,6β-dihydroxyroyleanone and coleon U, respectively. The abietane diterpenes present in this extract possess conjugated quinone systems which contribute to their cytotoxicity by the induction of free radicals[18]. The coleon U has also shown to be a selective inhibitor of the protein kinase C delta[19]11,12,14-tetrahydroxy-abieta-5,8,11,13-tetraene-7-one (coleon U. This mechanism of action could be useful for the modulation of molecular pathways that are altered in some types of cancer.

Discussão

Este estudo demonstrou que a seleção dos parâmetros do processo e formulação poderão ser fatores importantes nas características finais do sistema de veiculação de fármacos. O extrato foi encapsulado com sucesso em fitossomas, representando aqui uma excelente alternativa para a veiculação de compostos de origem natural relativamente instáveis. Os estudos anteriores descritos na literatura, demonstraram que a complexação de produtos naturais com fosfolípidos melhora a estabilidade das formulações[8]. A utilização de acetona como meio reacional permitiu a obtenção de fitossomas de tamanho mais uniforme e de menor dimensão quando comparado com os solventes mais frequentemente referidos na literatura, diclorometano ou etanol[8,9]catalase and thiobarbituric acid reactive substances with respect to carbon tetrachloride treated group ($P < 0.05$ and <0.01. Este facto poderá estar relacionado com a melhor solubilidade dos componentes do extrato neste solvente, uma vez que este foi o mesmo solvente extrator. Este solvente é também considerado menos tóxico que por exemplo o diclorometano para uso em preparações farmacêuticas (ICH classe 2 *versus* ICH classe 3). Em relação ao tempo de reação, é descrito na literatura que a extensão do tempo reacional nem sempre apresenta vantagens do ponto de vista granulométrico. Os fenómenos relacionados com a maior probabilidade de aglomeração poderão ser os responsáveis por esta observação[12]. O uso de colesterol nos fitossomas está geralmente relacionado com o seu efeito estabilizador dos nanossistemas[13]. No presente estudo, observou-se uma diminuição no tamanho após adição do colesterol mas sem diferenças estatisticamente significativas entre a percentagem molar de 2,5 ou 5% ($p > 0.05$, teste t-student). O pequeno tamanho destes nanossistemas, poderá ser crucial para a sua absorção quer ao nível transdérmico ou oral, se aplicável. Estudos anteriores demonstraram que uma redução de tamanho dos sistemas de veiculação de fármacos conduz a uma melhoria da absorção intestinal dos mesmos[14,15]. Deste modo, a redução do tamanho destas nanoparticulas poderá conduzir a uma melhoria da biodisponibilidade dos fitocomponentes encapsulados.

A inclusão do extrato em fitossomas poderá ser extremamente útil para obter uma forma farmacêutica estável com aplicação terapêutica que poderá será ser a nível de actividade antibacteriana ou antitumoral. O extrato acetónico preparado a partir de *P. madagascariensis*, possuí como componentes maioritários, dois diterpenos com interessante actividade antibacteriana[11,16] e antitumoral[17,18], a 7α,6β-dihydroxyroyleanone e a coleona U, respectivamente. Os diterpenos abietânicos presentes neste extrato, possuem na sua estrutura uma quinona conjugada que pode contribuir para a sua citotoxicidade devido à indução de radicais livres[18]. A coleona U também está descrita como um inibidor seletivo da proteína quinase C delta[19]11,12,14-tetrahydroxy-abieta-5,8,11,13-tetraene-7--one (coleon U. Este mecanismo de ação poderá ser útil na modelação de vias moleculares que se encontram alteradas em alguns tipos de cancro.

Conclusion

This study showed that the extract containing the 7α,6β-dihydroxyroyleanone as its main compound was successfully encapsulated into lipid structures. Phytosomes with reduced and uniform dimension with smaller and more uniform size was archived by the use of acetone as reaction medium over two hours and with the addition of a molar proportion of 2.5% of cholesterol. The selection of any drug delivery system must always be careful and dependent on the therapeutic target. This study suggests that phytosomes could be considered excellent natural bioactive products delivery systems with interest for the cosmetic and pharmaceutic industries.

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Conflict of interests

The authors declare that there are no financial and personal relationships that could be viewed as presenting a potential conflict of interests

Conclusão

Este estudo demonstrou que o extracto contendo como composto principal a 7α,6β-dihidroxiroileanona foi encapsulado com sucesso em estruturas lipídicas. Foram obtidos fitossomas de reduzida dimensão e uniforme após utilização de acetona como meio reacional, durante 2h e com adição de uma proporção molar de 2,5% de colesterol. A selecção de qualquer sistema de veiculação de fármacos deverá ser sempre cuidadosa e dependente do fim terapêutico. Este estudo sugere que os fitossomas poderão ser aqui considerados excelentes sistemas de veiculação de compostos naturais bioactivos com interesse nas indústrias cosmética e farmacêutica.

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Conflitos de interesses

Os autores declaram não existir qualquer relação pessoal ou financeira que possa ser entendida como representando um potencial conflito de interesses.

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