UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA

STRUCTURAL CHARACTERIZATION AND BIOLOGICAL ACTIVITIES OF TERPENIC AND PHENOLIC COMPOUNDS ISOLATED FROM *EUPHORBIA LAGASCAE* AND *EUPHORBIA TUCKEYANA*

Noélia Maria da Silva Dias Duarte

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(QUÍMICA FARMACÊUTICA)

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2008

The studies presented in this thesis were carried out at Centro de Estudos de Ciências Farmacêuticas (CECF), now integrated in the Medicinal Chemistry Group of the Institute for Medicines and Pharmaceutical Sciences (*i*Med.UL), Faculdade de Farmácia da Universidade de Lisboa, under the supervision of Professor Maria José U. Ferreira.

Dedicated to

Filipe

and to our daughters

Helena and Gabriela

"Try looking at tomorrow, not yesterday
And all the things you left behind
All those tender words you did not say
The gentle touch you couldn't find
In these days of nameless faces
There's no one truth, but only pieces
My life is all I have to give
Dare to live, until the very last
Dare to live, forget about the past
Dare to live,
Giving something of yourself to others
Even when it seems there is nothing more left to give..."

Andrea Bocelli

"Dare to live", In "Vivere", 2007

ABSTRACT

The main goal of this study was to search for new bioactive compounds, mainly effective modulators of P-glycoprotein in resistant cancer cells, from two species of the Euphorbiaceae family. In this way, the methanolic extracts of *Euphorbia lagascae* and *Euphorbia tuckeyana* were studied and several terpenic and phenolic compounds were isolated by chromatographic techniques. The chemical structures were deduced from their physical and spectroscopic data (IR, MS, 1D and 2D NMR experiments).

From *Euphorbia lagascae* (aerial parts and seeds), several macrocyclic diterpenes with the lathyrane skeleton containing the rare C_5,C_6-epoxy function were isolated, five of them are new compounds that were named latilagascenes A, B, D, E and F. Latilagascene B was acylated using various reagents, affording four new derivatives (latilagascenes C, G, H, and I). Two new jatropholane-type diterpenes, named lagaspholones A and B, and characterized by the rare 5:6:7:3 fused ring system, were also isolated. This is the first reported occurrence of this type of compounds from *Euphorbia* species. A possible biogenetic pathway for these compounds is proposed and would reinforce the importance of casbene-derived diterpenes as chemotaxonomic biomarkers for the Euphorbiaceae family. Besides the new compounds, diterpenes with the tigliane, atisane and kaurane skeletons, several pentacyclic taraxastane and oleanane-type triterpenes, stigmastane and ergostane-type steroids, a *nor*-sesquiterpene and several phenolic compounds, with diverse chemical structures, were also isolated and identified. From *Euphorbia tuckeyana*, three new jatrophane-type diterpenes, having a different and unique acylation pattern were isolated and characterized. Moreover, diterpenes with the tigliane and *ent*-abietane structures, two flavonoids and a neolignan were also isolated and identified.

Several biological activity studies were performed, with some of the metabolites described above. In particular, it should be emphasized that the best results were obtained with the macrocyclic lathyrane and jatrophane diterpenes as modulators of multidrug resistance in human *MDR1* gene-transfected mouse lymphoma cells. The majority of these compounds showed to be very strong inhibitors of the efflux-pump activity of P-glycoprotein, increasing therefore, the drug retention in resistant cancer cells. Moreover, the studied lathyrane diterpenes may be considered a homogenous set of compounds, allowing considerations of structure-activity relationship, particularly highlighting the influence of the ring A and the acylation pattern in the inhibition of Pgp, and showing, as well, that other parameters may be important in the interaction with the protein, such as lipophilicity and the presence of functional groups, which can be involved in H-bond formation. Some of these macrocyclic diterpenes were assayed, *in vitro*, for their antiproliferative effects in combination with epirubicine, and all of them showed to synergistically enhance the effect of the antitumour drug. These results reinforce the importance of macrocyclic lathyrane and jatrophane diterpenes as effective lead compounds for the reversal of multidrug resistance.

The inhibition of Multidrug Resistance Associated Protein 1 (MRP1) transport activity in human erythrocytes was carried out. Some compounds were also evaluated as apoptosis inducers, being the more significant results obtained for the stilbene piceatannol and its tetramethylated derivative.

Some of the isolated compounds were also investigated for their antiproliferative activity in sensitive and resistant cancer cell lines derived from three human gastrointestinal carcinomas. Particularly, latilagascenes D and C were found to be highly effective against one drug resistant subline derived from gastric carcinoma. Moreover, other biological assays were performed, such as, the evaluation of the anti-leishmania activity of a stilbene, and the antimycobacterial activity of an ergostane steroid.

Keywords: Euphorbiaceae, *Euphorbia lagascae*, *Euphorbia tuckeyana*, diterpenes, lathyrane, jatrophane, jatropholane, triterpenes, steroids, stilbenes, multidrug resistance, P-glycoprotein.

RESUMO

Este estudo teve como principal objectivo, o isolamento de compostos bioactivos, principalmente com actividade moduladora da multirresistência aos fármacos anticancerígenos (Multidrug Resistance - MDR), em células tumorais. Nesse sentido, procedeu-se ao estudo fitoquímico de duas espécies do género *Euphorbia*: *Euphorbia lagascae* e *Euphorbia tuckeyana* (Euphorbiaceae).

Os compostos foram isolados utilizando técnicas cromatográficas (cromatografia em coluna, cromatografia preparativa em camada fina e cromatografia líquida de alta resolução). As estruturas químicas dos compostos isolados foram deduzidas com base nas suas características físicas e dados espectroscópicos (IV, MS e RMN unidimensional e bidimensional: ^{1}H, ^{13}C, DEPT, COSY, HMQC, HMBC e NOESY).

A partir do extracto metanólico das sementes de *Euphorbia lagascae* foram isolados e identificados: um estilbeno (piceatanol), duas cumarinas e dois diterpenos com o esqueleto do tigliano. O piceatanol, isolado em grandes quantidades, foi acetilado e metilado, originando cinco derivados.

Do extracto metanólico de *Euphorbia lagascae* (partes aéreas), foram isolados e caracterizados vários diterpenos macrocíclicos, com o esqueleto do latirano, com a particularidade de possuírem a rara função epóxido entre C-5 e C-6 e a maioria deles estarem oxidados em C-16. Cinco dos diterpenos isolados são compostos novos que foram denominados latilagascenos A, B, D, E e F. O latilagasceno B, isolado em maiores quantidades, foi acilado utilizando vários anidridos/cloretos de ácido, o que deu origem a quatro novos derivados (latilagascenos C, G, H e I), que diferem no padrão de acilação do anel A. Foram isolados dois novos diterpenos tetracíclicos com o esqueleto do jatrofolano, denominados lagasfolonas A e B. O isolamento deste tipo de compostos é raro e até à data só tinham sido obtidos a partir de espécies do género *Jatropha* (jatrofolonas A e B). É apresentada uma discussão sobre a possível via biogenética destes compostos. Assim, a partir do rearranjo de diterpenos bicíclicos do tipo latirano, as lagasfolonas A e B poderão ser considerados intermediários no processo biossintético das jatrofolonas. O isolamento de diterpenos com o esqueleto do jatrofolano, a partir de espécies do género *Euphorbia* vem, deste modo, reforçar a importância dos diterpenos derivados do casbano, como marcadores quimiotaxonómicos da família Euphorbiaceae. Foram também isolados e identificados: dois diterpenos com o esqueleto do atisano e kaurano, um triterpeno tetracíclico e vários triterpenos pentacíclicos, entre os quais se incluem o simiarenol, o lupeol e outros com o esqueleto do oleanano e taraxastano, vários esteróides com o esqueleto do stigmastano e ergostano, um *nor*-sesquiterpeno e compostos fenólicos, com estruturas químicas variadas. Foi também isolado e identificado um alcalóide (3-indolcarbaldeído).

A partir do extracto metanólico de *Euphobia tuckeyana* (partes aéreas), foram isolados e caracterizados três diterpenos macrocíclicos novos, com o esqueleto do jatrofano, denominados tuqueanol A, tuqueanol B e eufotuqueanol. Foram igualmente isolados e identificados dois diterpenos com o esqueleto do tigliano, várias lactonas *ent*-abietânicas (helioscopinolidos A, B, D, e E), dois flavanóides (naringenina e aromadendrina) e um neolinhano. Os compostos obtidos em maiores quantidades foram acetilados ou metilados, originando os respectivos derivados.

Foram realizados vários estudos para avaliar a actividade biológica dos compostos isolados. Estudou-se a inibição da actividade transportadora da glicoproteína-P (Pgp) em células de linfoma de rato transfectadas com o gene humano *MDR1*. Neste estudo, analisou-se por citometria de fluxo, a acumulação intracelular da rodamina-123, um substrato fluorescente, análogo da epirrubicina. Foram avaliados os diterpenos macrocíclicos com o esqueleto do latirano e do jatrofano, assim como os estilbenos, flavanóides e outros compostos fenólicos. São de salientar os resultados obtidos com os diterpenos macrocíclicos. Com efeito, os latiranos e jatrofanos testados mostraram ser potentes inibidores da

actividade da glicoproteína-P, aumentando deste modo, a retenção intracelular da rodamina-123 nas células resistentes. Foram também avaliados *in vitro*, os efeitos antiproliferativos de alguns dos diterpenos macrocíclicos em combinação com a epirrubicina ou a doxorrubicina. Todos eles apresentaram um efeito sinergístico sobre a actividade do fármaco antitumoral.

Os estudos da actividade moduladora da glicoproteína-P permitiram retirar algumas conclusões sobre a relação estrutura-actividade dos diterpenos com o esqueleto do latirano. São de salientar, além de alguns aspectos gerais como a lipofilia e a presença de grupos funcionais com capacidade para estabelecer ligações de hidrogénio, alguns aspectos estruturais, tais como, a importância de um grupo hidroxilo livre em C-3 e o padrão de acilação do anel A, nomeadamente a presença de dois anéis aromáticos. Estes resultados corroboram a importância dos diterpenos macrocíclicos (jatrofanos e latiranos) como moléculas padrão no estudo da reversão da multirresistência associada à Pgp.

Para além da inibição da glicoproteína-P, foi também estudada a inibição da actividade transportadora da MRP1 (Multidrug Resistance Associated Protein 1). Este ensaio, efectuado em eritrócitos humanos, baseou-se no estudo do efluxo do 2',7'-bis-(3-carboxipropil)-5-(e-6)-carboxifluoresceina (BCECF), um substracto fluorescente da MRP1. O estilbeno piceatanol e os flavanóides naringenina e aromadendrina mostraram possuir actividade inibitória.

Procedeu-se à avaliação da capacidade de alguns compostos diterpénicos e fenólicos, induzirem a apoptose em células de linfoma de rato transfectadas com o gene humano *MDR1*. Para tal recorreu-se a um ensaio por citometria de fluxo, utilizando marcadores celulares como a anexina-V e o iodeto de propídio, de modo a avaliar as alterações da superfície da membrana celular. Neste ensaio, os resultados mais significativos foram obtidos com o estilbeno piceatanol.

A actividade antiproliferativa de alguns dos compostos isolados foi avaliada contra três linhas celulares, derivadas de três carcinomas humanos: gástrico (EPG85-257), pancreático (EPP85-181) e cólon (HT-29). Foram estudadas as linhas celulares sensíveis aos fármacos antitumorais e duas variantes multirresistentes: as linhas celulares associadas à sobre-expressão da glicoproteína-P (multirresistência clássica, células EPG85-257RDB, EPP85-181RDB e HT-29RDB) e as linhas celulares associadas à alteração da expressão da topoisomerase II (multirresistência atípica, células EPG85-257RNOV, EPP85-181RNOV e HT-29RNOV). São de salientar os resultados obtidos com o latilagasceno D, que mostrou um índice de resistência de 0.03, tendo uma forte actividade antiproliferativa contra a linha celular resistente EPG85-257RDB derivada do carcinoma gástrico. Contra a linha celular resistente EPG85-257RNOV, derivada do carcinoma pancreático, o composto mais activo foi a naringenina (índice de resistência de 0.06). Para todos os compostos, as linhas celulares derivadas do carcinoma do cólon (HT-29), mostraram ser mais resistentes do que as outras linhas celulares estudadas (EPG85-257 e EPP85-181).

Foram igualmente efectuados outros ensaios de avaliação da actividade biológica, tais como a avaliação da actividade anti-leishmania do piceatanol e o estudo da actividade antimicobacteriana do peróxido de ergosterol.

Palavras-chave: Euphorbiaceae, *Euphorbia lagascae*, *Euphorbia tuckeyana*, diterpenos, latirano, jatrofolano, jatrofano, triterpenos, esteróides, estilbenos, multirresistência, glicoproteína-P.

ACKNOWLEDGMENTS

I would like to express my profound gratitude to Professor Maria José U. Ferreira, my supervisor, for her permanent support, scientific guidance and enthusiasm. Thanks for her careful revision of all my work and for our fruitful but sometimes hard discussions, which have allowed my scientific and personal growth. I also would like to thank her friendship.

I would like to thank Professor Matilde Castro, Professor Camila Batoréu and all the others Professors and Colleagues from the Analytical Chemistry Group at Faculdade de Farmácia da Universidade de Lisboa, who have allowed my leave of absence.

I am very grateful to Professor Joseph Molnár from University of Szeged, Hungary, for his willing collaboration in the Multidrug Resistance assays and most of all for providing so many important considerations and advices in order to improve the biological studies presented in this thesis.

I would like to thank Professor Leonard Amaral from Instituto de Higiene e Medicina Tropical de Lisboa, Professor Krystina Michalak from Wroclaw Medical University, Poland, Professor Hermann Lage from Institute of Pathology, Germany, Professor Andras Varga from Humbolt University, Germany and Professor Oliver Kayser from GRIPS, Netherlands, and all their teamwork for the collaboration in various biological activity studies.

I also wish to thank Professors Matilde Castro, Helder Mota-Filipe and Maria Eduardo Figueira from Faculdade de Farmácia da Universidade de Lisboa, for their collaboration in the anti-inflammatory and anti-oxidant activity studies.

I would like to thank Professor Pedro Abreu from Universidade Nova de Lisboa and Professor José Ascenso from Instituto Superior Técnico, for their help in getting some NMR and MS spectra, respectively.

I would like to thank Cátia Ramalhete, Maria João Catalão, Andreia Gonçalves and Maria Carlos Lopes for their collaboration in the phytochemical study of *E. lagascae* and *E. tuckeyana*.

I would like to thank Engenheira Teresa Vasconcelos from Instituto Superior de Agronomia de Lisboa, for the identification of plant material.

I wish to express my thanks to Helena Brito and Francisco Carvalho for their technical support in the laboratory. I also wish to thank Helena for her willingness to help me whenever I asked.

I would like to thank Dra. Virgínia Carvalho and Dra. Isabel Joglar from Faculdade de Farmácia da Universidade de Lisboa, for their support with the GC and HPLC techniques, respectively.

I also would like to thank Professor Rui Moreira as Coordinator of Centro de Estudos de Ciências Farmacêuticas / Medicinal Chemistry Group, where this work was carried out.

My sincere thanks to my friends and colleagues, particularly Ana Margarida Madureira, Ana Bela Santana, Ana Paula Francisco and Emilia Valente for the great time I have had in our group. I have enjoyed their friendship and their emotional support in the bad and good moments. I also would like to thank my friends Cristina and Antonio Alfaia for their availability to sometimes babysitting my daughter Helena.

I deeply appreciate and thank the welcome and encouragement I have received during this past year from my colleagues Maria Luísa Mateus and Maria Rosário Bronze.

My graduate studies would not have been the same without the scientific conversations, friendship and good spirit of Professor José Nascimento, who always has been confident in me and in my work.

Finally, I wish to express my gratitude to my family for the support they provided me, and in particular, I am profoundly indebt with my husband and best friend, Filipe, without whose love, encouragement, assistance and patience, I would not have finished this long task.

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ABBREVIATIONS AND SYMBOLS

Ac	Acetyl
AcOH	Acetic Acid
ADP	Adenosine Diphosphate
Ang	Angeloyl
ATP	Adenosine Triphosphate
ax	Axial
BCRP	Breast Cancer Resistance Protein
Bu	Butanoyl
Bz	Benzyl
c	Concentration
^{13}C NMR	^{13}C Nuclear Magnetic Resonance
calcd	Calculated
$CDCl_3$	Deuterated Chloroform
CH_2Cl_2	Dichloromethane
Cin	Cinnamoyl
cm	Centimetre
CNS	Central Nervous System
COSY	Correlation Spectroscopy
COX	Cycloxygenase
d	Doublet
dd	Double doublet
DEPT	Distortionless Enhancement by Polarization Transfer
dm	Decimetre
DMAPP	Dimehylallyl Diphosphate
DMSO	Dimethylsulphoxide
DNA	DesoxirriboNucleic Acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl radical
DXP	Deoxyxylulose Phosphate Pathway
E.	*Euphorbia*
EIMS	Electronic Impact Mass Spectrometry
eq	Equatorial
et al	and others
Et_2O	Ethyl ether
EtOAc	Ethyl acetate

eV	Electron volt
FABMS	Fast Atom Bombardment Mass Spectrometry
FID	Flame Ionization Detector
FAR	Fluorescence Activity Ratio
FITC	Fluorescein isothiocyanate
FL	Fluorescence Activity
FPP	Farnesyl Diphosphate
FSC	Forward Scatter
g	Gram
GC	Gas Liquid Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
GGPP	Geranyl Geranyl Diphosphate
GPP	Geranyl Diphosphate
h	Hour
^{1}H NMR	^{1}H Nuclear Magnetic Resonance
HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple-Quantum Correlation
HPLC	High Performance Liquid Chromatography
HR-ESIMS	High Resolution Electrospray Mass Spectrometry
HR-LSIMS	High Resolution Liquid Secondary Ion Mass Spectrometry
HSV	Herpes Simplex Virus
Hydrp	Hydroperoxyl
Hz	Hertz
iBu	Isobutanoyl
IC_{50}	Sample concentration causing 50% inhibition
iNOs	Nitric Oxide Synthase
IPP	Isopentenyl Diphosphate
IR	Infrared
J	Coupling Constant
$^{2}J_{C\text{-}H}$	C-H coupling through two bonds (geminal coupling)
$^{3}J_{C\text{-}H}$	C-H coupling through three bonds (vicinal coupling)
L	Litre
log P	Octanol/water partition coeficient
LRP	Lung Resistance Protein
m	Multiplet
m.p.	Melting Point

m/z	Ratio of mass to charge
max	Maximum
MDR	Multidrug Resistance
Me	Methyl
Me_2CO	Acetone
MeBu	2-Methylbutanoyl
MeCN	Acetonitrile
MeOH	Methanol
MEP	Methylerythritol Phosphate pathway
mg	Milligram
MHz	Megahertz
min	Minute
mL	Millilitre
mM	Millimolar
mm	Millimetre
MR	Molecular Refractivity
mRNA	Messenger Ribonucleic Acid
MRP	Multidrug Resistance Associated Protein
MVA	Mevalonoic Acid
MW	Molecular Weight
n.d.	Not Described or Not Determined
NADH	Nicotinamide Adenine Nucleotide
NBD	Nucleotide Binding Domain
NGF	Nerve Growth Factor
Nic	Nicotinoyl
nM	Nanomolar
nm	Nanometre
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
n^o	Number
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Enhancement Spectroscopy
NPP	Neryl diphosphate
PAR	Parental cells
PG	Prostaglandins
Pgp	P-glycoprotein (P stands for permeability)
PKC	Protein Kinase C

ppm	Parts per million
QSAR	Quantitative Structure Activity Relationship
Rel. int.	Relative intensity
Rel. Ach	Relative to cholesterol acetate
R_f	Retention fraction
RNA	Ribonucleic Acid
R_t	Retention time
s	Singlet
SAR	Structure Activity Relationship
sh	Shoulder
SSC	Side Scatter
t	Triplet
Tig	Tigloyl
TLC	Thin Layer Chromatography
TMD	Transmembrane Domain
TMS	Tetramethylsilane
TNF	Tumor Necrosis Factor
Topo	Topoisomerase
UV	Ultraviolet
WHO	World Health Organization
δ	Chemical shift
δ_C	Carbon chemical shif
$\Delta^{x,y}$	Insaturated bond between carbons X and Y
λ_{max}	Maximum Wave Lenght
$[\alpha]_D^{20}$	Specific rotation
µg	Microgram
µL	Microlitre
µm	Micrometre
µM	Micromolar
ν_{max}	Maximum Wave Number
µmol	Micromole
$[M]^+$	Molecular ion