CHAPTER VI

Conclusions
The main goal of this study was to search for bioactive compounds, mainly effective modulators of P-glycoprotein in resistant cancer cells. In this way, the methanolic extracts of *Euphorbia lagascae* and *Euphorbia tuckeyana* (Euphorbiaceae) 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, HRMS, $^1$H and $^{13}$C NMR and also 2D NMR - COSY, HMQC, HMBC and NOESY experiments).

From *Euphorbia lagascae* (aerial parts), 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 (36), B (42), D (18), E (30) and F (19). Jolkinol B (21), a known lathyrane diterpene was also isolated.

![Diagram of latilagascenes A, B, D and E](image)

Latilagascenes A, B, D and E have the peculiarity of being oxidized at C-16, a feature that was found for the first time in lathyrane diterpenes. Latilagascene F has a new acylation pattern, besides the C$_5$,C$_6$-epoxy function. Latilagascene B, isolated in larger quantities, was
acylated using various reagents yielding four new lathyrane derivatives that were named latilagascenes C (43), G (44), H (25) and I (46).

Two new jatropholane-type diterpenes, named lagaspholone A (28) and lagaspholone B (34) were also isolated and identified. Jatropholane diterpenes, characterized by the unusual 5:6:7:3 fused ring system, have been rarely isolated and only in their oxidized form (jatropholones A and B). This is the first reported occurrence of this type of compounds from Euphorbia species.

A biogenetic pathway for lagaspholones A and B as biosynthetic intermediates of jatropholones was proposed. Jatropholones have been isolated from three species of Jatropha genus (Euphorbiaceae). Lagaspholones might be considered intermediates in the biosynthetic process leading to jatropholones by the rearrangement of lathyrane-type diterpenes. The isolation of lagaspholone A and B from Euphorbia species reinforces the importance of casbene-derived diterpenes as useful chemiotaxonomic biomarkers for Euphorbiaceae family.
Besides the new compounds, two atisane and kaurane-type diterpenes were isolated, in large amounts. This polycyclic diterpenes are not exclusively found in Euphorbiaceae but are common to other plant families. Acetylation reactions were carried out to obtain their acetylated derivatives. In addition, several pentacyclic triterpenes, particularly with the taraxastane and oleanane-type scaffolds, a tetracyclic triterpene with the cycloartane skeleton, stigmastane and ergostane-type steroids, a nor-sesquiterpene [(+)-dehydrovomifoliol] and several phenolic compounds, with diverse chemical structures, were also isolated and identified. To the best of our knowledge, (+)-dehydrovomifoliol, dehydrodiconiferyl diacetate, 4-hydroxy-3,5-dimethoxybenzaldehyde and (±) cleomiscosin A were isolated for the first time from Euphorbia species.

The study of Euphorbia lagascae defatted seeds, has afforded two known diterpenes with the tigliane scaffold and three phenolic compounds: two coumarins and a stilbene (piceatannol, 47). The latter compound, which was obtained in larger quantities, was acetylated and methylated affording four stilbene derivatives.

From Euphorbia tuckeyana, three new jatrophane-type diterpenes named tuckeyanol A and B, and euphotuckeyanol, which have a different and unique acylation pattern, were isolated and characterized.

\begin{align*}
63: & R = iBu \\
64: & R = MeBu \\
65: &
\end{align*}
In $\Delta^{6(17)},\Delta^{11}$ jatrophane-type diterpenes, the macrocyclic ring can adopt two predominant conformations (exo and endo-type) which are dependent on the spatial orientation of the exomethylene group with respect to the mean plane of the macrocycle. Analysing the coupling constant pattern and specific NOESY correlations of the isolated jatrophanes, it was concluded that tuckeyanol A and B adopt preferentially the exo-type conformation, where the exomethylene group is parallel to the mean plane of the molecule. In contrast, euphotuckeyanol had a strong preference to remain in the endo-type conformation, due to the perpendicular orientation of the exomethylene group with respect to the mean plane of the macrocycle.

Moreover, diterpenes with the tigliane and ent-abietane skeletons, two flavonoids (naringenin and aromadendrin) and a neolignan were also isolated and identified. Some compounds, isolated in larger quantities were also acetylated or methylated.

Several biological activity studies were performed with some of the metabolites described. 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 ability of those compounds to modulate the transport activity of Pgp was studied by flow cytometry. Reversion of MDR was performed using a standard functional assay with rhodamine-123 as a fluorescent substrate analogue of epirubicine and verapamil as positive control. 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. 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. Therefore, it may be concluded that these lathyrane and jatrophane diterpenes interact with the Pgp function directly and not only by changing the physicochemical properties of the membranes of resistant cells. The present data lead to conclude that ring A, with a free hydroxyl group at C-3, and the presence of two aromatic rings in the diterpene molecule, plays a significant role in the modulation of MDR. As general structural requirements, lipophilicity and the ability to establish H-bonds seem to play an important role.
Piceatannol (47) and its acetylated and methylated derivatives were also tested on the reversal of MDR. From the results obtained, it was concluded that the structurally related stilbenes differ significantly in their interaction with Pgp. In fact, trans-3,5,3′,4′-tetramethoxystilbene (49) strongly inhibited Pgp, in a dose-dependent manner, while piceatannol and the other derivatives (48, 50 and 51) were found to be ineffective in the MDR reversal assay. Once more, these results corroborate the importance of the existence of electron donors functional groups (e.g. methoxy groups), which can be involved in favourable electron-charge transfer type of interactions, between the modulator and its binding site on Pgp. Moreover, in combination with doxorubicine, trans-3,5,3′,4′-tetramethoxystilbene (49) showed and additive antiproliferative effect, on the human MDR1 gene-transfected mouse lymphoma cells.

Some of the isolated compounds were also evaluated as apoptosis inducers, on multidrug resistant (MDR1) L5718 mouse lymphoma cell line, by flow cytometry, using the FITC-annexin-V/propidium iodide assay. The more significant results were obtained for the stilbene piceatannol.

A search for inhibitors of Multidrug Resistance Associated Protein 1 (MRP1) transport activity in human erythrocytes was carried out, among some of the studied terpenic and phenolic compounds. The functional assay was based on measuring the efflux of a fluorescent MRP1 substrate (BCECF) from human erythrocytes. Piceatannol (47) and the flavonoids naringenin (58) and aromadendrin (61) were found to be MRP1 inhibitors.

Some of the isolated compounds were also investigated for their antiproliferative activity in several human cancer cell lines which were derived from three different tumour entities: gastric (EPG85-257), pancreatic (EPP85-181) and colon carcinomas (HT-29). Two different multidrug resistant variants of these cells were also investigated: cell lines with classical MDR phenotype (associated with the over expression of Pgp, EPG85-257RDB, EPP85-181RDB and HT-29RDB) and cell lines with atypical MDR phenotype (associated with altered topoisomerase II expression, EPG85-257RNOV, EPP85-181RNOV and HT-29RNOV).

The results obtained for latilagascene D should be emphasized. This compound was found to be highly effective against the drug resistant subline EPG85-257RDB (associated with the classical MDR phenotype) derived from gastric carcinoma. Concerning the drug resistant subline EPG85-257RNOV (associated with altered topoisomerase II expression), the
most effective compound was naringenin (58). With regard to the activity against the colon carcinoma cells (HT-29) it should be noted that, for all the compounds studied, this cell line appeared markedly more resistant than the other two gastrointestinal EPG85-257 and EPP85-181 cell lines.

Moreover, other biological assays were performed, such as, the evaluation of the anti-anti-leishmania activity of a stilbene, and the antimycobacterial activity of an ergostane steroid.

Traditionally used in folk medicine to treat cancers, tumours and warts, *Euphorbia* species have been the source of several bioactive compounds. *Euphorbia* plants synthesize a wide range of structurally unique macrocyclic diterpene polyesters, which have revealed to be promising multidrug resistance reversal agents in cancer cells. Therefore, this work represents a contribution, not only to the phytochemical study of *Euphorbia lagascae* and *Euphorbia tuckeyana*, but above all, corroborates the importance of the research of this genus, particularly in the discovery of effective anti-tumour lead compounds.