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Synthesis of bioactive royleanone derivatives from *Plectranthus* spp.

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Universidade Lusófona de Humanidades e Tecnologias

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Abstract

Cancer is one of the most common causes of death worldwide. The growing incidence of cancer and the development of multidrug resistance has driven the search for novel and more effective drugs.

Plants have been used for their medicinal properties since ancient times. In developing countries, herbal preparations are still a major way of disease prevention and treatment considering their availability and cost in comparison to medicines.

Abietane diterpenoids mainly royleanones are bioactive compounds frequently found in the *Plectranthus* genus (Lamiaceae family).

P. madagascariensis (Pers.) Benth essential oil is rich in 6-7-dehydroxyroyleanone (DHR), a cytotoxic royleanone. DHR molecule possesses hydroxyl groups suitable for derivatization, which have drawn attention to the possibility of exploring its reactivity to improve its cytotoxic potential.

In this work, several hemi-synthetic reactions were performed, resourcing to reactions of carbamoylation and esterification. These reaction products were fully structurally characterized mainly by spectroscopic methods and afforded three unstable carbamoylation derivatives and three stable ester derivatives with overall good yields (86-95%).

The three stable derivatives were also submitted to biological studies to access their antimicrobial activity,general toxicity, and cytotoxicity. The biological activity of all diterpenoids found in the literature was compared to understand which chemical features are important to identify and design hit molecules.

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Acronyms and symbols

µL	Microlitres
µmol	Micromol
AcOEt	Ethyl Acetate
$CDCl_3$	Deuterated Chloroform
CH_2Cl_2; DCM	Dichloromethane
dd	Doublet of doublets
DHR	6,7-dehydroxyroyleanone
DMAP	4-dimethylaminopyridine
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
dt	Doublet of triplets
EDTA	Ethylenediaminetetraacetic acid
eq	Equivalents
h	Hours
HCl	Hydrochloric acid
Hex	Hexane
L	Liters
m	Multiplet
MBC	Minimum bactericidal concentration
MDA-MB231S	Triple-negative breast cancer cell line
MHz	MegaHertz
MIC	Minimum inhibitory concentration
mL	Milliliters
MRSA	Multi resistant Staphylococos aureus
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCI-460\R	Resistant Non-small cell lung carcinoma cell line
NCI-H460	Non-small cell lung carcinoma cell line
NMR	Nuclear Magnetic Resonance
O.E	Essential Oil
ºC	Degrees Celcius
P. madagascariensis	Plectranthus madagascariensis
PBS	Phosphate Buffer Saline
P-gp	P-glycoprotein
PPM	Parts per million

Rho 123	Rhodamine 123
S	Singlet
S.aureus	*Staphylococcus aureus*
Sept	Septet
spp.	Species
t	Triplet
TLC	Thin-layer chromatography
w\w	Weight\weight

1. Introduction

Cancer is one of the leading causes of death worldwide and chemotherapeutic drugs have been developed since the mid-XX century to try and stop the uncontrolled growth of cancer cells, however, most of the drugs currently available have a lot of side effects since they are not specific for cancer cells. (Kuchta, 2014; Torre et al., 2016) another issue related to the failure of chemotherapeutics in use is the development of multidrug resistance (Dinić et al., 2018).

Since ancient times plants have been used for their medicinal properties (Newman & Cragg, 2013). In developing countries, herbal preparations are still a major way of disease prevention and treatment considering their availability and cost in comparison to medicines (WHO, 2013). Nowadays, it was witnessed a surge of interest and acceptance of the use of natural therapies, as well as an increase in their availability, with natural remedies being sold in supermarkets and drugstores. It is estimated that 80% of the population residing in developed countries use medicinal plants as a primary source of healthcare (Ekor, 2014).

The use of medicinal plants although widespread is still a grey-area because, even though there are many herbal medicines with proven medicinal value, there still exists a vast number of untested and unmonitored natural treatments that requires studies to ascertain not only their true medical interest as well as interactions and side-effects (WHO, 2005).

1.1. Natural based drugs used in chemotherapy

Plant derived drugs have been used for cancer treatment for almost 70 years. In the 1950s Vincristine and Vinblastine (figure 1) were isolated from *Catharanthus roseus* and they act by inhibition of the microtubules assembly, this plays an important role in cancer therapeutics because microtubules are key constituents for cell proliferation, signaling, and migration (Dumontet & Jordan, 2010).

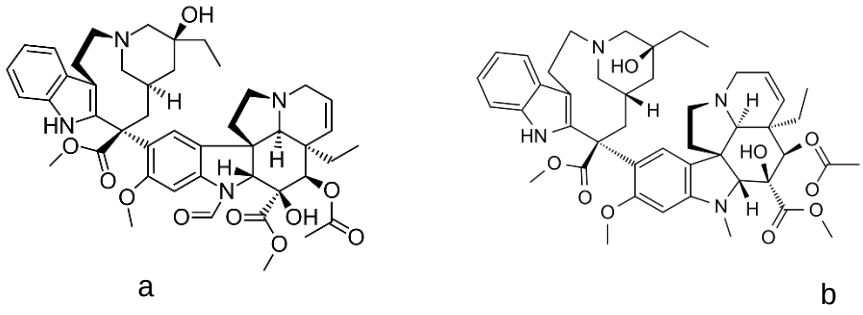


Figure 1 - a) Vincristine b) Vinblastine

Only 10 years later, Paclitaxel and Docetaxel (figure 2) were isolated from the *Taxus* genus. And they have a very similar mechanism to Vincristine and Vinblastine. (Faustino et al., 2018; Seca & Pinto, 2018). These derivatives from natural sources are used in breast cancer, non-Hodgkin's malignant lymphomas, hormone refractory and prostate cancer treatments.

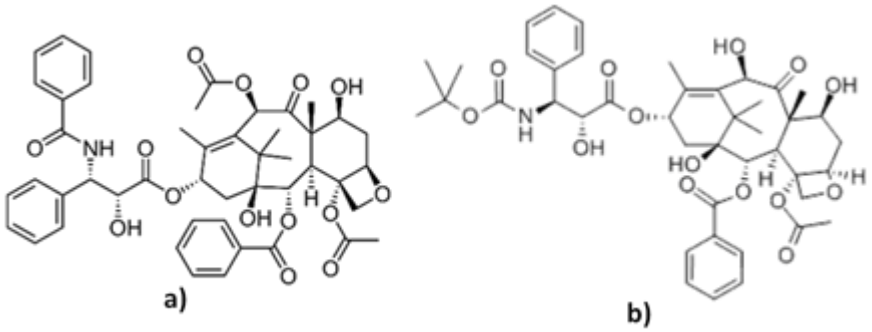


Figure 2- a) Paclitaxel / b) docetaxel

1.2. *Plectranthus* Genus

Plectranthus genus belongs to the Lamiaceae family. This family comprises a wide selection of plants, more than 350 species, with medical applications. Historically, plants from this family have been reported to be effective against several pathologies as weakness, poor circulation, skin allergies, amongst many others (Uritu et al., 2018).

Plectranthus spp. are found in the tropical and subtropical areas of Asia, Africa, Oceania, and South America (Matias, Nicolai, Fernandes, et al., 2019).

Several phytochemical studies have been conducted on *Plectranthus spp.* all over the years. These plants are a natural source of diterpene quinones, coleones, and royleanones, compounds well known for their biological activities such as antiproliferative properties (Catarina Garcia et al., 2018).

Plectranthus genus is popular for their flowers and resistance thus is used a lot as ornamental plants. In traditional medicine, this genus is used to treat infectious pathologies as well as dermatological and gastrointestinal conditions(Matias et al., 2019).

Figure 3 - *Plectranthus madagascariensis*

P. madagascariensis (Pers.) Benth. is widly known for its biological effects and consequently, for the presence of interesting compounds, namely diterpenoids, royleanones and coleones. One example is the 6,7-dehidroxyroyleanone (DHR), naturally occurrent in this plant.(Matias, Nicolai, Saraiva, et al., 2019)

In the *Plectranthus* genus, there are also some other compounds worthy to be noted, such as 7α-acetoxy-6β-dehydroroyleanone (Roy) and Parvifloron D. Parvifloron D and Roy are a diterpenoid abietanes obtained from *P.eckolinii*, *P. gradidentatus,* respectively (Ladeiras et al., 2016).

1.3. 6,7-Dehidroxyroyleanone

DHR is a tricyclic abietane with a *para*-quinone in C ring (figure 4). This compound has been isolated from several plants in the Lamiaceae family such as *Salvia* and *Plectranthus*. It is the major component of *P.madagascariensis* oil. (Catarina Garcia et al., 2018)

DHR has reported cytotoxicity since it reveals antiproliferative activity against prostate cancer cells and cytotoxic activity against mammalian cancer cells. The mode of action of this royleanone is still unknown and therefore further studies are justified (Catarina Garcia et al., 2018).

In previous studies performed on NCI-H460 and NCI-H460\R cells, it was already verified that DHR, as well as Roy, show promising cytotoxic activity in both these cell lines. This indicates that these two compounds are not P-gp substrates. It is also important to note that according to the same study, these compounds were not cytotoxic against MRC-5 (normal cell line). Regarding P-gp, when both these compounds were tested to access if they were P-gp inhibitors they showed a slight inhibition but not promising when compared with dex-verapamil which was the positive control (C Garcia et al., 2020; Catarina Garcia et al., 2018; Isca et al., 2020; Matias, Nicolai, Saraiva, et al., 2019).

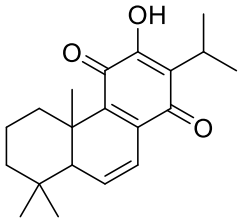


Figure 4 - Chemical structure of 6,7-dehydroxyroyleanone (DHR)

In this work, DHR was extracted and isolated from *P. madagascariensis*. This compound has an acidic hydroxylic group, suitable for derivatization, and for this reason, can be considered a drug lead for further development. Based on this, it was used to prepare new derivatives by hemi-synthesis. The DHR and obtained compounds were characterized by NMR analysis. NMR spectroscopy is utilized to identify organic compounds, through spectral analysis. It can be applied to a large variety of compounds and has a reach in the µmol range (Zhang et al., 2012).

2. Materials and Methods

2.1. General Procedures

Reagents

All solvents used in the isolation process (Hex, AcoEt) were distilled previously to use.

The RMN analyses were performed using deuterated chloroform ($CDCl_3$) as a solvent with tetramethylsilane (0,03%) (TMS) as an internal standard.

The CH_2Cl_2 used in the reactions was previously dried, under inert conditions,

The rest of the reactional reagents were used with no previous purification.

Silica

TLC was used to monitor the reactions, with commercial silica gel plates (Merck silica gel 1.07734.1000).

The extract fractionation was carried out using silica gel (Merck 7734).

The purification of reactional products was carried out using silica (1.09385.1000; 230–400 mesh; Merck KGaA 64271 Darmstadt, Germany).

Equipment

All the samples, raw material, reagents, and compounds used in this study were weighed on a Kern 770 analytical scale.

The TLC plates were visualized under UV lights (Camag, Switzerland, Catalog No.022.9230, Serial No.001216) at 254 nm.

^{1}H and ^{13}C NMR spectra were recorded at 300 MHz Bruker equipment, using deuterated $CDCl_3$ as solvent. Chemical shifts (δ) are reported in ppm referenced to the TMS peak (δ 0.00).

Plant Material

The plant material*, P. madagascariensis* (Pers.) Benth was cultivated in Parque Botânico da Tapada da Ajuda (Instituto superior Agrário, Lisbon, Portugal) from cuttings obtained from the Kirstenbosch Natural Botanical Garen (Cape Town, South Africa). Voucher specimens were deposited in Herbarium João Carvalho e Vasconcelos (ISA) with the number 841\2007. The plant material used in this study was dried and stored at room temperature, protected from light and humidity, after being collected between 2007 and 2008. The plant name has been checked with http://theplantlist.org.

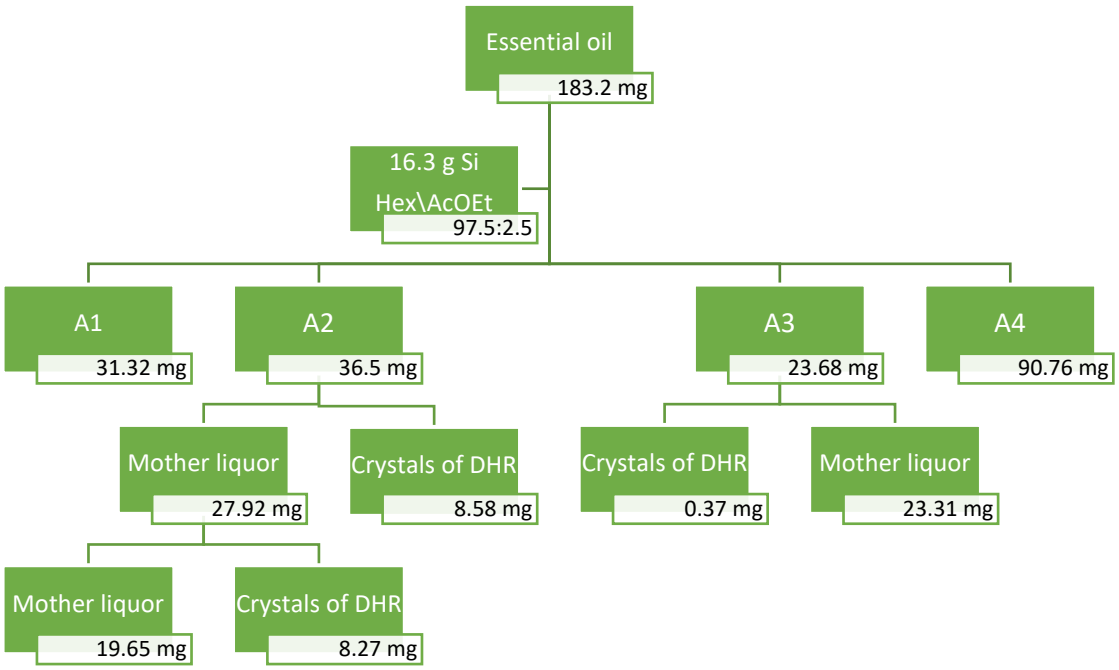
Extraction

The essential oil was extracted by hydrodistillation using hexane as an extracting solvent, in a Clevenger apparatus, according to C. Garcia et al. (C Garcia et al., 2018). The whole plant *P. madagascariensis* (371.83 g) was air-dried, grinded, and immersed in 1 L of distilled water for the hydrodistillation process, in 3 times, 30 days each. The aqueous phase was eliminated. The oil obtained was dried with sodium sulfate anhydrous and washed with hexane. The solvent was evaporated from the essential oil (under vacuum, at 40 ˚C), which yielded a residue of 0.879 g (0.2% w/w).

Isolation

The DHR was isolated from the essential oil using dry flash column chromatography. The stationary phase used was silica gel and the mobile phase was the mixture of Hex: AcOEt.

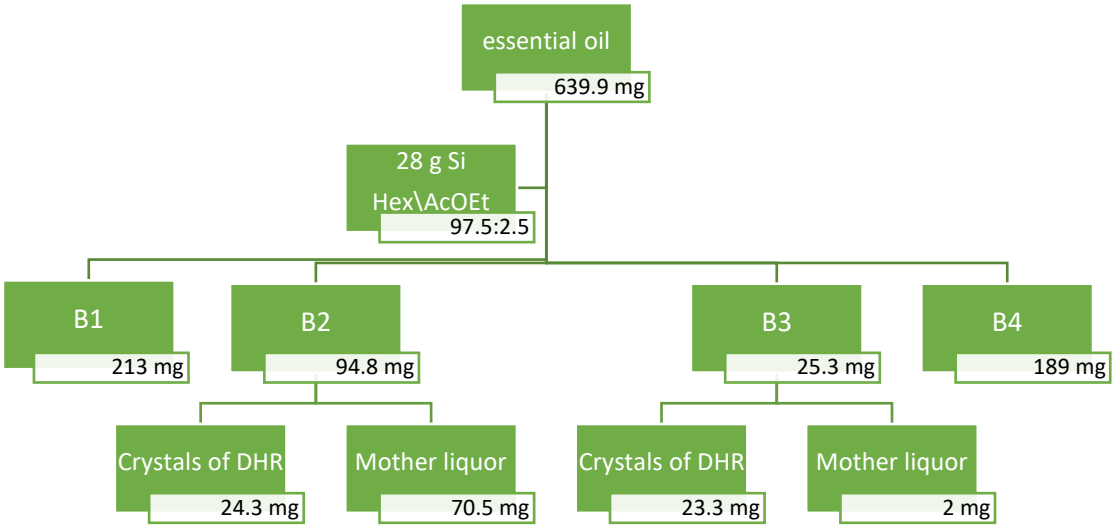
The essential oil (O.E) 1 (0,879 g) was separated in dry flash column A with silica gel (Merck 7734, 16.3 g). Several portions were collected, and then TLCs were performed. The portions that displayed similar composition were gathered in 4 fractions (A1 to A4, according to scheme 1). Fractions A2 and A3 were recrystallized from Hex to give 17.22 mg of DHR.



Scheme 1- Column chromatography A

The O.E 2 (639.9 mg) were separated in dry flash column B with silica gel (Merck 7734, 28 g). 4 fractions (B1 to B4, scheme 2) were obtained. The fractions B2 and B3 were recrystallized

from hexane to give 47.6 g of DHR. The mother liquors of the fraction B were added to the mother liquors of A for new chromatographic separation that will be made in future work.



Scheme 2- Column chromatography B

The DHR obtained from the isolation process was characterized by NMR analysis.

Orange oil, ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ 7.34 (s, 1H, OH), 6.81 (dd, 1H, H-7), 6.46 (dd, 1H, H-6), 3.16 (h, 1H, H-15), 2.88 (dt, 1H, H-1β), 2.13 (t, 1H, H-5α), 1.63-1.60 (t, 1H, H-2α), 1.52-1.50 (t, 1H, H-2β), 1.47-1.46 (m, 1H, h-1α), 1.42 (d, 1H, H-1α), 1.23 (s, 1H, H-3α), 1.22 (Me-16, overlapped signal), 1.20 (Me-17, overlapped signal), 1.03 (s, 3H, Me-20), 1.01(s, 3H, Me-18), 0,98 (s, 3H, Me-19). ^{13}C NMR ($CDCl_3$, 75 MHz, ppm): δ; 186.2 (C-14), 183.6 (C-11), 151.3 (C-12), 140.6 (C-9), 139.8 (C-6), 138.6 (C-8), 122.7 (C-13), 121.3 (C-7), 52.2 (C-5), 40.6 (C-4),39.4(C-3), 35.3 (C-1), 33.4 (C-10), 32.7 (C-19), 24.2 (C-18), 22.9 (C-15), 20.1 (C-17), 19.9 (C-16), 18.8 (C-2), 15.31 (C-20)

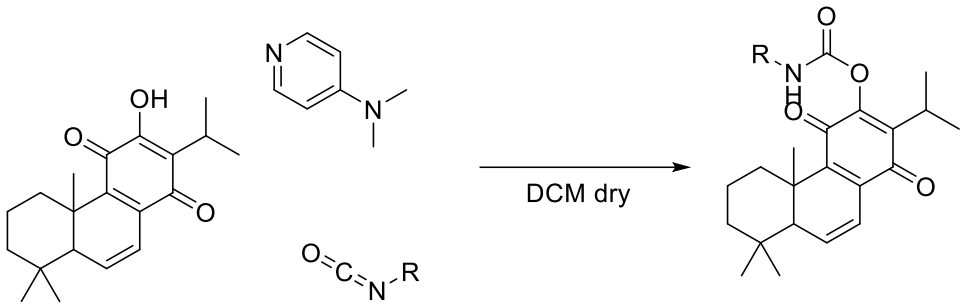
2.2. General Reaction of Carbamoylation

Using DHR as a lead compound, several reactions were preformed giving origin to 5 hemi-synthetic compounds, that were analyzed using NMR to establish their structural chemistry.

The general reaction that was performed is shown in scheme 3, and the procedure was the following:

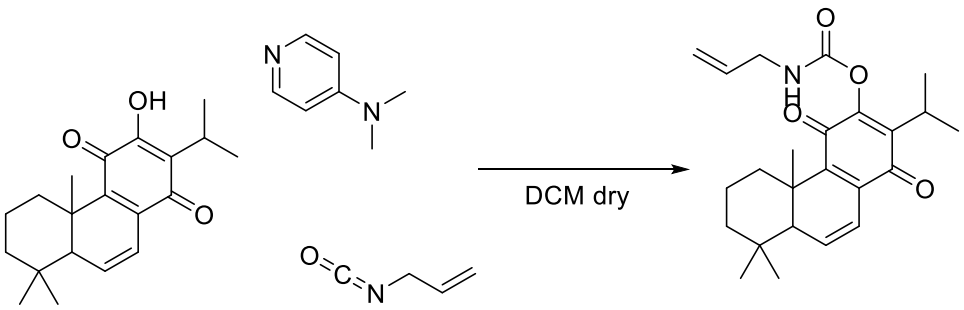
To a round bottom flask, DHR (around 10 mg, 0.032mmol) was added, along with DMAP (around 4.0 mg, 0.032mmol). Afterward, 500µL of dried CH_2Cl_2 was added under inert conditions. Isocyanate (around 0.095 mmol) was added to the reaction mixture and stirred for

48 hours at room temperature. After completion, the reaction mixture was concentrated under reduced pressure and the product was isolated by preparative TLC (Hex : AcOEt, 8:2), the product was isolated and its structural chemistry assessed by ^{1}H-NMR.



Scheme 3-General reaction of carbamoylation

Reaction 1



1

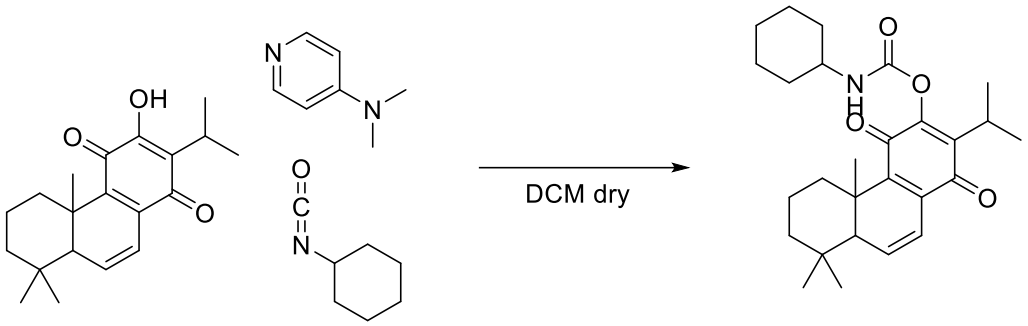
Scheme 4- Hemi-synthetic derivative 1

This reaction was performed using allyl isocyanate as a reagent and the expected product is depicted in scheme 4.

After 48 hours of reaction time, the TLC revealed that there was still DHR present.

A new reaction was performed using 20 mg of DHR and the same result was observed, therefore no characterization has been done for this product.

Reaction 2



2

Scheme 5- Hemi-synthetic derivative 2

The reaction was performed successfully using isocyanatocyclohexane as a reagent allowing the obtention of the desired product depicted in scheme 5.

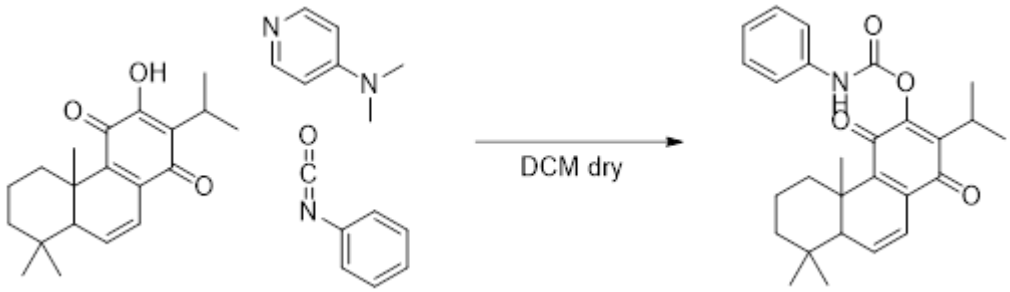
There were used 40 eq. of the reagents to 1 eq. of DHR, during 28 hours of reaction, the reaction was monitored by TLC to verify the full conversion of DHR in the product.

After completion, the reaction mixture was concentrated under reduced pressure and the solvent was immediately evaporated at low temperature, it was then stored in the freezer and a TLC was made after 24h to confirm that the degradation process was not due to the storage mechanism.

The product was separated in a flash column using Hex : AcOEt (95:5) as the eluent and silica (1.09385.1000; 230–400 mesh; Merck KGaA 64271 Darmstadt, Germany) as the stationary phase.

It was obtained a dark yelow oil, yield: 47%, ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 6.74 (dd, J= 9.8, 3.1 Hz, 1H, H-7), 6.39 (dd, J= 9.8, 3,1 Hz, 1H, H-6), 5.03 (d, J= 8.4 Hz, 1H, N-H), 3.53 (m, 1H, H-1'), 3.14 (m, 1H, H-15), 2.80 (d, J= 12,9 Hz, 1H, H-1β), 2.33-2.00 (m, 5H, H-1α, H-2α e H-2β e H-3α and H-3β, overlaped signals), 1.44-1.36 (m, 10H, H-2', H-3' and H-4', overlaped signals), 1.21 (m, 6H, Me-16 and Me-17, overlaped signals), 1.10 (s, 3H, Me-20), 1.03 (s, 3H, Me-18), 1.00 (s, 3H, Me-19); ^{13}C NMR ($CDCl_3$, 75 MHz, ppm): δ 194.55, 187.79, 183.59, 131.04, 128.95, 121.23, 109.69, 98.34, 77.48, 77.16, 76.84, 68.30, 52.24, 38.85, 33.41, 30.48, 29.85, 29.06, 23.87, 23.13, 22.95, 14.21, 11.11; MS-ESI (+): 338 [M-127+23]+, 381 [M-84+23]+, 440 [M+H]+, 462 [M+23]+.

Reaction 3



3

Scheme 6- Hemi-synthetic derivative 3

The reaction was performed successfully using isocyanatobenzene as a reagent allowing the obtention of the desired product depicted in scheme 6.

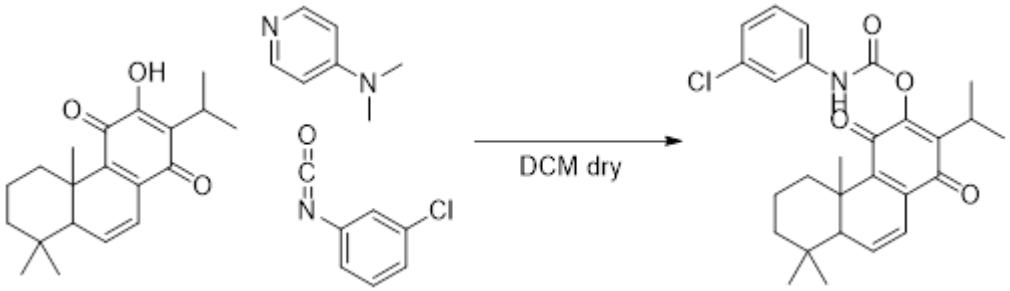
There were used 25 eq. of the reagents to 1 eq. of DHR, during 6 hours of reaction, the reaction was monitored by TLC to verify the full conversion of DHR in the product.

After completion, the reaction mixture was concentrated under reduced pressure and the product was isolated by flash column.

The flash column was made using Hex: AcOEt (95:5) as the eluent and 0,5% of triethylamine to neutralize the silica to access if the degradation process as due to the acidity of the silica reacting with the compound.

It was obtained a yelow oil, yield: 32%, ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 7.44 – 7.41 (m, 2H, H-2'), 7.34 – 7.30 (m, 3H, H-3', H-4' and H-5', overlaped signals), 7.07 – 7.01 (m, 1H, N-H), 6.74 (dd, J= 9.7, 3.1 Hz, 1H, H-7), 6.39 (dd, J= 9.7, 3.1 Hz, 1H, H-6), 3.15-3.06 (m, 1H, H-15), 2.71 (d, J = 13.9 Hz, 1H, H-1β), 2.32-1.39 (m, 5H, H-2α e H-2β e H-3α e H3-β, overlaped signals), 1.16 (d, 3H, Me-16 and Me-17), 1.11 (s, 3H, Me-20), 1.02 (s, 3H, Me-18), 0.98 (s, 3H, Me-19). ^{13}C NMR ($CDCl_3$, 75 MHz, ppm): δ; 186.2 (C-14), 153.1 (C-12), 145.? (C-9), 141 (C-Ar), 138,2 (C-8), 130.3 (C-Ar), 127.9 (C-Ar), 127.2 (C-not Ar), 123.8 (C-1'), 119.6 (C-2'), 28.8 (C-15), 23.0 (C-18), 20.2 (C-17), 18.7 (C-16), 15.1 (C-20).

Reaction 4



4

Scheme 7- Hemi-synthetic derivative 4

The reaction was performed successfully using 1-chloro-3-isocyanatobenzene as a reagent allowing the obtention of the desired product depicted in scheme 7.

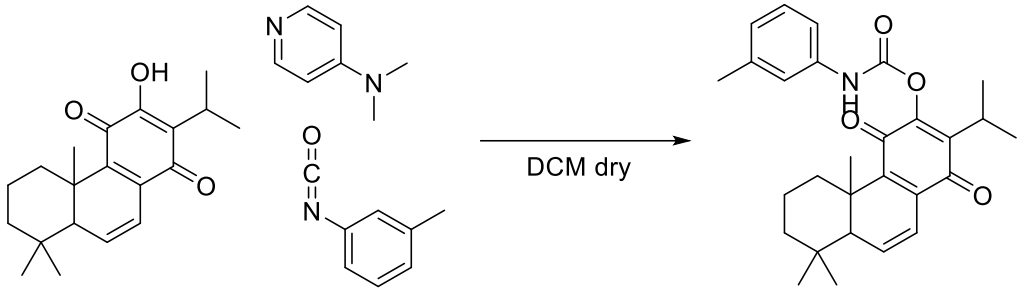
There were used 5 eq. of the reagents to 1 eq. of DHR, during 3 hours of reaction, the reaction was monitored by TLC to verify the full conversion of DHR in the product.

After completion, the reaction mixture was concentrated under reduced pressure and the product was isolated by flash column.

The flash column was made using Hex: AcOEt (95:5) as the eluent and silica as the stationary phase.

It was obtained a orange powder, yield: 54%, ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ 7.45 – 7.17 (m, 4H, H-2' and H-3', overlaped signals), 7.03 (dt, J= 7.0, 2.0 Hz, 1H, N-H), 6.75 (dd, J= 9.7, 3.1 Hz, 1H, H-7), 6.42 (dd, J= 9.7, 3.1 Hz, 1H, H-6), 3.00-2.96 (m, 1H, H-15, overlaped signals), 2.69 (d, J = 12.5 Hz, 1H, H-1β), 2.20 (s, 1H, H-5α) 1.73-1.45 (m, 4H, H-2 and H-3α, overlaped signals), 1.36 (m, 1H, H-1α), 1.25 (s, 3H, Me-16), 1.16 (d, J= 6.9 Hz, 3H, Me-17), 1.10 (s, 3H, Me-20), 1.02 (s, 3H, Me-18), 0.98 (s, 3H, Me-19). ^{13}C RMN ($CDCl_3$, 75 MHz, ppm): δ 188.20 (CO(O)NH), 186.5 (C-14), 152.5 (C-12), 146 (C-9), 144 (C-13), 139.0 (C-4'), 138.6 (C-6), 137.3 (C-8), 134.8 (C-1'), 124.2 (C-3'), 117.7 (C-2'), 51.8 (C-5), 40.6 (C-4), 39.8 (C-10), 34.7 (C-1), 33.6 (C-3), 32.7 (C-19), 28.9 (C-15), 22.9 (C-18), 21.5(C-17), 18.7(C-16), 17.5 (C-2), 15.1 (C-20).

Reaction 5



5

Scheme 8- Hemi-synthetic derivative 5

This reaction was performed using *m*-tolylisocyanate as a reagent and the expected product is depicted in scheme 8.

There were used 15 eq. of the reagents to 1 eq. of DHR, during 4 hours of reaction, the reaction was monitored by TLC to verify the full conversion of DHR in the product.

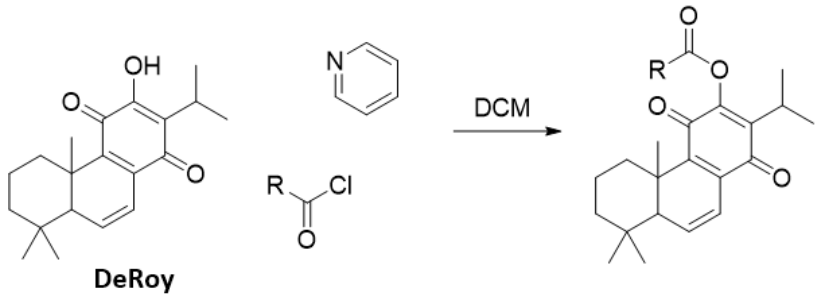
In this reaction it was not possible to obtain the pure product since there were several subproducts formed, they were then separated but it was still not possible to isolate the pure product, for that reason the NMR analysis was not performed.

2.3. General Reaction of Esterification

Using DHR as the starting material, several esterification reactions were made originating 5 hemi-synthetic compounds that were analyzed by NMR to characterize its structure.

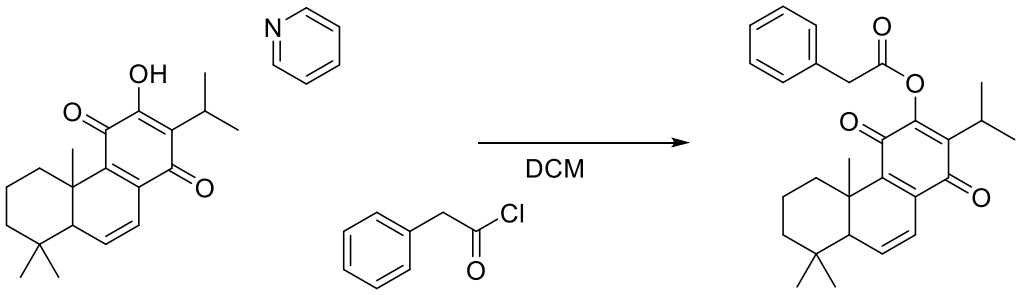
The general reaction performed is shown in scheme 9, and the procedure was the following:

To a round bottom flask, DHR (around 20 mg) was added, along with DCM (around 2 ml). Pyridine (15.43 µL) was added to the reaction mixture and stirred for 48 hours at room temperature. After completion, the reaction mixture was concentrated under reduced pressure and the product was isolated by preparative TLC (Hex: AcOEt, 8:2), the product was isolated and its structural characterization was assessed by ^{1}H-NMR.



Scheme 9- The main reaction of esterification

Reaction 6



6

Scheme 10- Hemi-synthetic derivative 6

This reaction was performed successfully using 2-phenylacetyl chloride as a reagent, allowing the obtention of the desired product depicted in scheme 10.

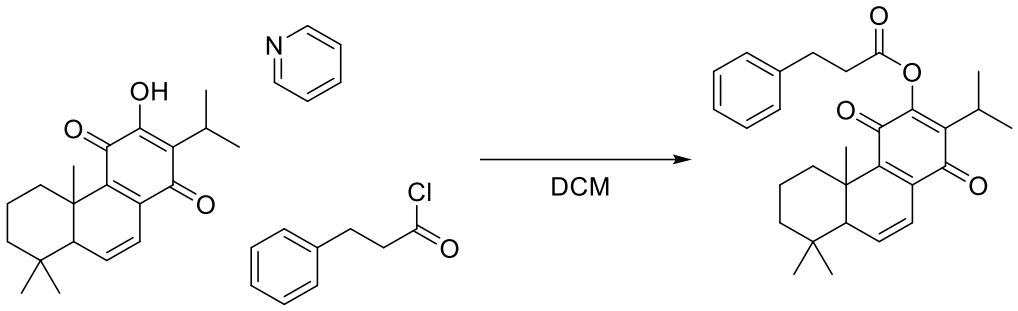
There were used 15 eq. of the reagents to 1 eq. of DHR, during 4 hours of reaction, the reaction was monitored by TLC to verify the full conversion of DHR in the product.

After completion, the reaction was stopped using 2 mL of HCl, extracted 3 times with DCM and dried with anhydrous sodium sulfate, and filtrated. Then the solvent was evaporated under low

pressure and the obtained residue was separated by preparative TLC using Hex : AcOEt (9:1) as an eluent to separate the pure product.

There was obtained a yellow oil, yield: 94%, ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 7.38 – 7.36 (m, 4H, H-2' and H-3', overlapped signals), 7.33 – 7.30 (m, 1H, H-4'), 6.74 (dd, J = 9.7, 3.1 Hz, 1H, H-7), 6.40 (dd, J = 9.7, 3.1 Hz, 1H, H-6), 3.93 (s, 2H, CH2), 2.99 (sept, J = 7.0 Hz, 1H, H-15), 2.81 (d, J = 13.4 Hz, 1H, H-1β), 2.14 (d, J = 3.2 Hz, 1H, H-5α), 1.62 – 1.58 (m, 1H, H-2α), 1.50 – 1.49 (m, 1H, H-2β), 1.46 – 1.44 (m, 1H, H-3β), 1.39 (d, J = 4.3 Hz, 1H, H-1α), 1.07 (s, 1H, H-3α), 1.05 (s, 3H, Me-16), 1.05 (s, 3H, Me-17), 1.03 (s, 3H, Me-20), 1.00 (s, 3H, Me-18), 0.98 (s, 3H, Me-19). ^{13}C ($CDCl_3$, 75 MHz, ppm): δ 185.8 (C-14), 180.3 (C-11), 168.9 (C(O)O), 149.5 (C-12), 144.0 (C-9), 138.6 (C-6), 137.7 (C-8), 136.7 (C-13), 132.6 (C-2'), 129.6 (C-1'), 128.7 (C-3'), 127.4 (C-4´), 120.1 (C-7), 51.8 (C-5), 40.8 (CH2C(O)O), 40.5 (C-4), 39.6 (C-3), 34.8 (C-1), 33.3 (C-10), 32.6 (C-19), 24.8 (C-18), 22.8 (C-15), 20.1 (C-17), 20.0 (C-16), 18.6 (C-2), 15.0 (C-20).

Reaction 7



7

Scheme 11- Hemi-synthetic derivative 7

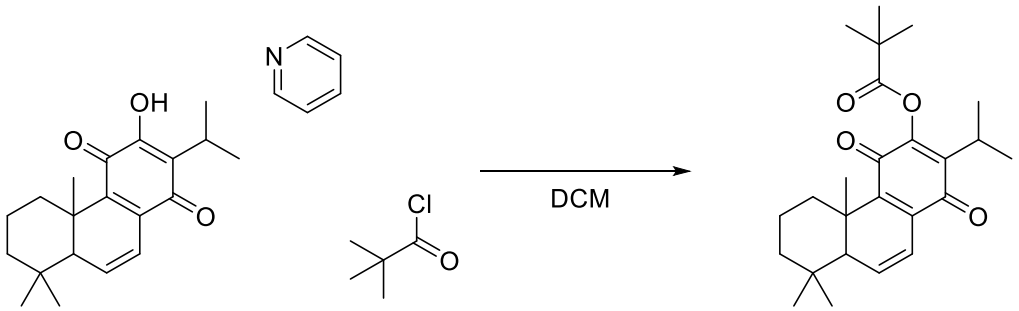
This reaction was performed successfully using trimethylacetyl chloride as a reagent, allowing the obtention of the desired product depicted in scheme 11.

There were used 6 eq. of the trimethylacetyl chloride to 1 eq. of DHR, during 4 hours of reaction, the reaction was monitored by TLC to verify the full conversion of DHR in the product. After completion, the reaction was stopped using 2 mL of HCl, extracted 3 times with DCM and dried with anhydrous sodium sulfate, and filtrated. Then the solvent was evaporated under low pressure and the obtained residue was separated by preparative TLC using Hex : AcOEt (9:1) as an eluent to separate the pure product.

It was obtained a yellow oil, yield: 95%, ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 7.32 – 7.26 (m, 3H, H-Ar), 7.24 (m, 2H, H-Ar), 6.75 (dd, J = 9.7, 3.1 Hz, 1H, H-7), 6.41 (dd, J = 9.7, 3.1 Hz, 1H, H-6), 3.07 (d, J = 6.9 Hz, 2H, $CH_2CH_2C(O)O$), 3.01 – 2.89 (m, 3H, H-15 and $CH_2CH_2C(O)O$, overlapped signals), 2.81 (d, J = 13.4 Hz, 1H, H-1β), 2.15 (t, J = 3.2 Hz, 1H, H-5α), 1.61 (d, J

= 4.8 Hz, 1H, H-2α), 1.51 – 1.47 (m, 1H, H-2β), 1.47 – 1.43 (m, 1H, H-3β), 1.40 (d, J = 4.3 Hz, 1H, H-1α), 1.14 (s, 1H, H-3α), 1.12 (d, J = 1.6 Hz, 3H, Me-16, overlapped signal), 1.10 (s, 3H, Me-17), 1.04 (s, 3H, Me-20), 1.01 (s, 3H, Me-18), 0.98 (s, 3H, Me-19).

Reaction 8



8

Scheme 12- Hemi-synthetic derivative 8

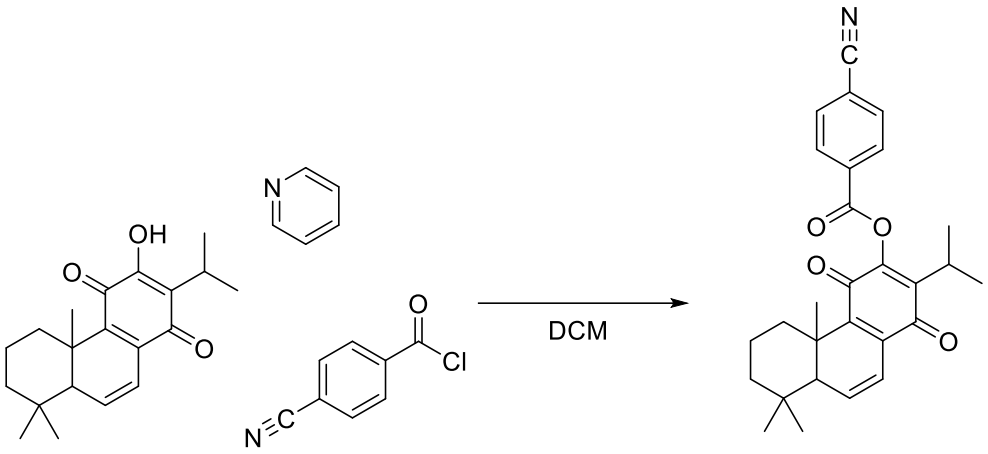
The product depicted in scheme 12 was successfully obtained through a reaction using pivaloyl chloride as a reagent.

There were used 30 eq. of the reagents to 1 eq. of DHR, during 21 hours of reaction, the reaction was monitored by TLC to verify the full conversion of DHR in the product.

After completion, the reaction was stopped using 2 mL of HCl, extracted 3 times with DCM and dried with anhydrous sodium sulfate, and filtrated. Then the solvent was evaporated under low pressure and the obtained residue was separated by preparative TLC using Hex : AcOEt (9:1) as an eluent to separate the pure product.

It was obtained a yellow oil, yield: 86%, ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ 6.75(dd, J = 9.7;3.1 Hz, 1H, H-7), 6.39 (dd, J = 9,7;3.1 Hz, 1H, H-6), 3.10 (h, J = 7,0 Hz, 1H, H-15), 2.79 (d, J = 13.4 Hz, 1H, H-1β), 2.15 (t, J = 3.2 Hz, 1H, H-5α), 1.60-1.58 (m, 1H, H-2α), 1.50-1.49 (m, 1H, H-2β), 1.46-1.43 (m, 1H, H-3β), 1.41 (d, J = 2.2 Hz, 1H, H-1α), 1.21 (s, 1H, H-3α), 1.19 (d, 3H, Me-16, J=2.1 Hz), 1.17 (s, 3H, Me-17), 1.03 (s, 3H, Me-20), 1.00 (s, 3H, Me-20), 0.97 (s, 3H, Me-18).

Reaction 9

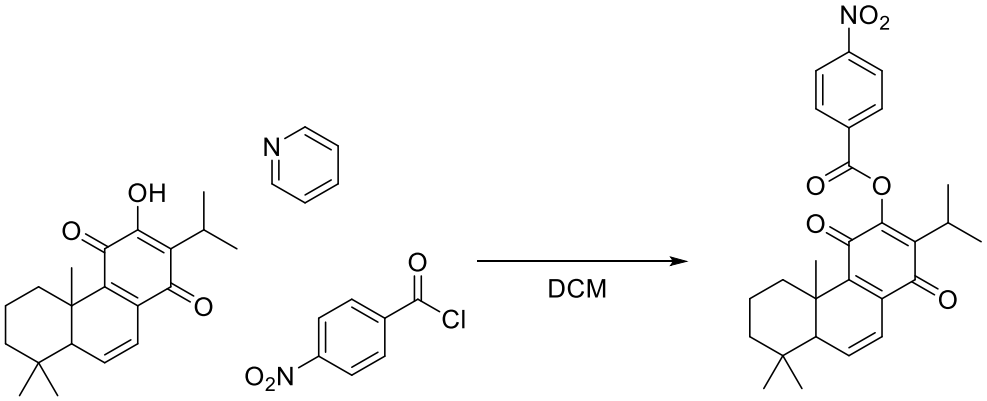


Scheme 13- Hemi-synthetic derivate 9

This reaction was performed using 4-cyanobenzoyl chloride as a reagent, and the expected product is depicted in scheme 13.

There were used 30 eq. of the 4-cyanobenzoyl chloride to 1 eq. of DHR, during 20 hours of reaction, the reaction was monitored by TLC. When observing the TLC it was possible to ascertain that no reaction had occurred even after using 30 eq for 5 days, under reflux. Thus, no further isolation or NMR characterization was performed.

Reaction 10



Scheme 14- Hemi-synthetic derivate 10

This reaction was performed using nitrobenzoyl chloride as a reagent, and the expected product is depicted in scheme 14.

There were used 33 eq. of the reagents to 1 eq. of DHR, during 20 hours of reaction, the reaction was monitored by TLC to verify the full conversion of DHR in the product.

When observing the TLC it was possible to ascertain that no reaction had occurred even after using 33 eq. of reagents during 20h of reaction, since there was no final product. Thus, no further isolation or NMR characterization was performed.

2.4. Determination of minimum inhibitory concentration

The determination of minimum inhibitory concentration (MIC) was performed using a 96 wells microplate, 100 µL of Muller-Hinton liquid was distributed in each well. In the first well of each row, 100 µL of each sample (Roy, DHR derivatives, 6, 7, and 8, the negative and positive control) were added at a concentration of 1 mg/mL in DMSO. Serial dilutions were made to 1:2 proportion between each row of wells. Finally, 10 µL of bacterial suspension of 0.5 McFarland density were added to each well. Plates were incubated for 24h at 37˚ C to allow bacterial growth, then the absorbances were read using an absorbance microplate reader (Thermo Scientific Multiskan FC, Loughborough, UK) set to 620 nm. All the assays were done in triplicate for each microorganism.

2.5. Determination of minimum bactericidal Concentration

The Minimum Bactericidal Concentration (MBC) assay was done following the conclusion of the MIC assay. In this method is taken a swab sample from the first well showed bacterial growth and seeded into a Muller-Hinton agar plate. This procedure is done also in the three wells immediately before the one where growth was observed. The plates were then incubated for 24h at 37˚ C. After the incubation period the plates were observed and the lowest concentration where there was no bacterial growth was taken as the MBC.

2.6. Preliminary Evaluation of General Toxicity on *Artemia salina* Model

To evaluate the preliminary toxicity of the derivatives, a lethality test on *artemia salina* was carried out. The general toxicity was tested adapted from the method of Zhang et al. 2012.
Brine shrimp cysts (obtained from JBL GmbH and Co. KG, D-67141 Neuhofen Germany) were hatched in artificial seawater with a concentration of 35 g/L. A container with 2 chambers connected using a handmade communicating vessel was used to hatch the brine shrimp. An air pump inserted in the container ensured a regular supply of airflow saturating the artificial seawater to promote successful hatching. The cysts were incubated for 48h at 25˚ C. The derivatives were tested at a concentration of 100 ppm. To do so ten to fifteen nauplii were transferred into 24-well plates containing the artificial seawater and 100 µL of the derivative

making the final volume of the well 1mL. The number of dead nauplii was determined after 24 h exposure thus calculating the percentage of dead nauplii.

2.7. DPPH assay

Using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) as a reagent the radical scavenging activity of the compounds was determined. A solution of DPPH in methanol (0.1 mM) was prepared. 4 mL of this solution were mixed with 1 mL of solutions containing the sample of the compound to be tested dissolved in methanol. The samples were then incubated for 30 minutes in a dark room. The scavenging capacity was then read spectrophotometrically (Hitachi U-1500, Tokyo, Japan) at 517 nm. The lower the absorbance obtained, the higher was the scavenging radical activity.

The MTT assay as well as the Rhodamine 123 assay were performed by external parties and therefore are described in annex 1.

3. **Results and discussion**

As described previously, the extraction of the O.E was performed via hydrodistillation in a Clevenger apparatus, which is the optimized method to obtain DHR with high yields according to Garcia et al. 2018 (C Garcia et al., 2018).

The plant material was immersed in water, and heated, which allows the separation of the volatile oils that are collected in hexane post-condensation.

After the extraction was completed the O.E obtained was quantified, and the yield was calculated.

The plant material was extracted in three times. The first and the second extractions were carried out mainly with *P. madagascariensis* aerial parts. On the other hand, the third extraction was carried out mainly with roots. The weight of plant material, O.E obtained, and respective yield of extraction are shown in table 1.

Table 1 - Extraction of essential oil from *P. madagascariensis*

Extraction	**Plant material (g)**	**Essencial oil (mg)**	**Essential oil extration yield**
1	80	183.2	0.22%
2	234	639.9	0.27%
3	57,83	56.9	0.1%
Final	371,83	879.1	0.2%

The extraction of the roots afforded the smallest yield when compared with the leaves and steam (extraction 1 and 2). this is in accordance with Mallavarapu et al. 2005. That reports that the aerial parts of the plants from this family possesses a higher content of essential oil (Mallavarapu et al., 2005).

This will lead to a final yield of 0.2% wich is considered low when compared with the one obtained by Catarina Garcia in a previous study (20.4%) where only leaves were used for the extraction process.(Catarina Garcia et al., 2018)

The extraction of the essential oil was monitored by TLC. This can be allowed to confirm the presence of DHR in the essential oil by comparison with the DHR standard.

TLC was also used to study which mixture of solvents would be the most effective as eluents to be used in the isolation process. The most effective eluent found was a mixture of Hex: AcOEt in a 97.5:2.5 proportion, and thus, was the eluent chosen for the isolation process.

For the isolation process, a dry flash chromatography was performed since the variation in pressure makes this method faster and more efficient. (Shusterman et al., 1997)

The obtained E.O was isolated in two different columns (Columns A and B). In column A were isolated 183.2 mg of E.O. Column A originated 4 distinct fractions (A1 to A4), the obtained mass is depicted in table 2. It was possible to obtain pure DHR from fractions A2 and A3 by recrystallization from Hex.

Table 2 – Flash chromatography A

	Weight (mg)	**Hex:AcOEt**	**Hex:AcOEt**	**MeOH: AcOEt**
A1	31.32	97.5:2.5		
A2	36.5		95:5	
A3	23.68		95:5	
A4	90.76			90:10
Final	182.2			

In the column B were isolated 639.9 mg of E.O. Similar results were observed on column B, namely, four fractions (B1 to B4, table 3), and the pure DHR was obtained from the middle fractions B2 and B3

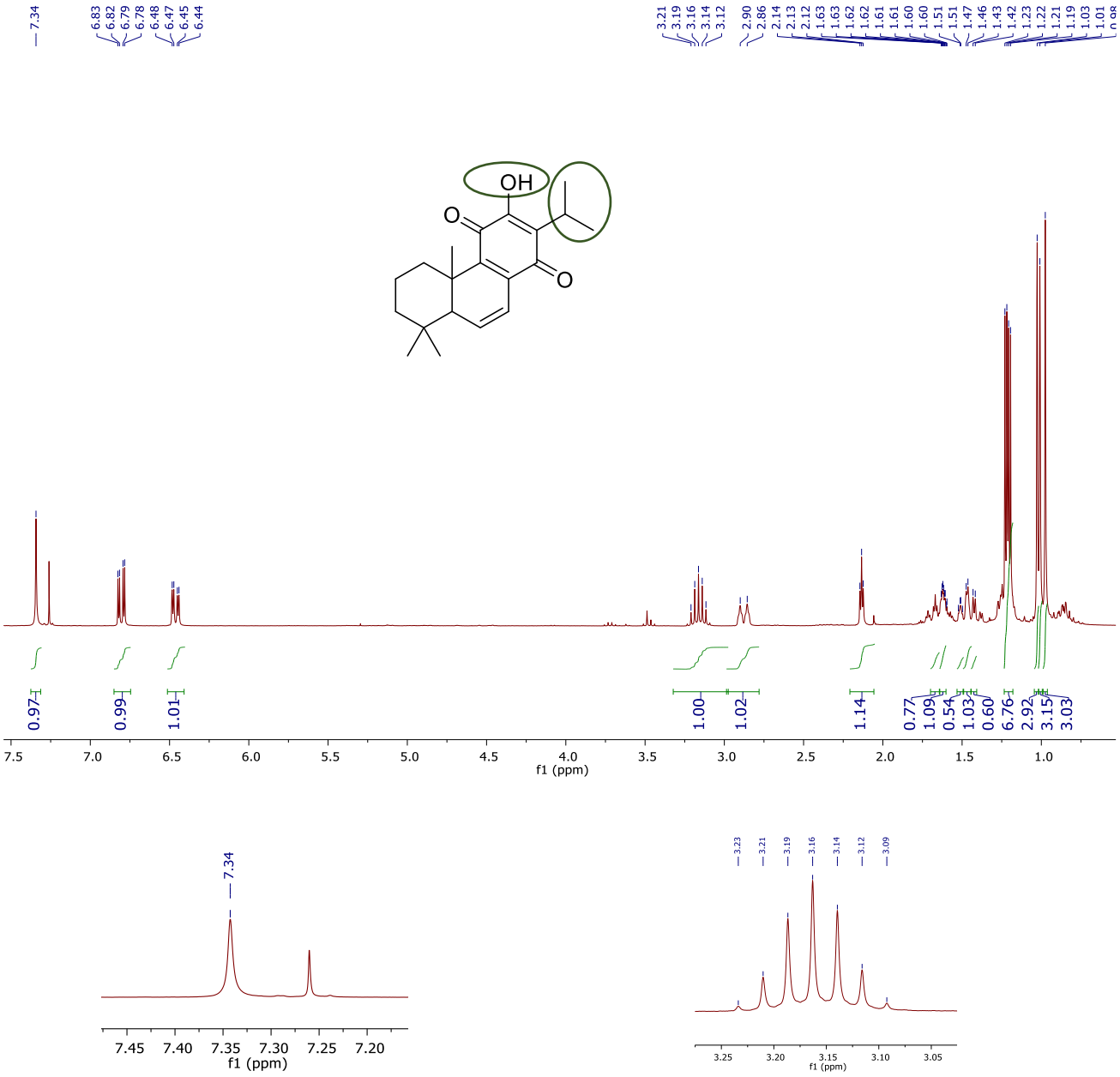
Table 3 – Flash chromatography B

	Weight (mg)	**Hex: AcOEt**
B1	213	97.5:2.5
B2	94.8	97.5:2.5
B3	25.3	97.5:2.5
B4	189	97.5:2.5
Final	522.1	

The two columns (A and B) afford 64,82 mg of pure DHR that was further used to prepare new derivatives. Nevertheless, the remain fractions A1 to A3 and B1 to B3 still have DHR to be isolated. Thus, these fractions were stored for new chromatographic separation that will be made in future work. DHR is formed by orange crystals with a molecular weight of 314.43 g/mol. DHR structure was confirmed by NMR analysis and the signals observed are according to C. Garcia et al.(Catarina Garcia et al., 2018)

^{1}H-NMR spectrum of DHR (figure 6) revealed some characteristical signs, for example, the peak OH-12 (δH-7.34 ppm) confirmed the existence of a hydroxyl group. Is this OH- group that will be replaced with other functional groups, in the reactional study.

Another very important peak in the DHR ^{1}H NMR spectrum is the signal corresponding to the H-15α (δH-3.16 ppm) since it produces a very distinctive septet (figure 6).



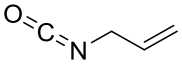
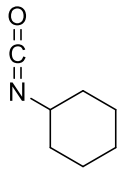
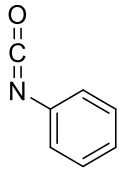
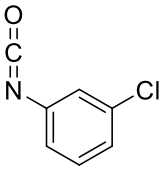
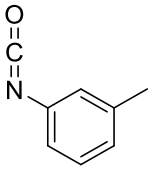
peak OH-12(δH-7.34 ppm)

peak H-15α (δH-3.16 ppm)

Figure 5 - DHR ^{1}H-NMR spectra. Extension of the signals corresponding to OH-12 (δH-7.34 ppm) and H-15α (δH-3.16 ppm)

Some reactions were performed to obtain new hemi-synthetic derivatives. In table 4 are presented the reactional conditions of the reactions performed. The amount of DHR obtained from the isolation process was not enough to perform all the reactions, thus some DHR used in the hemi-synthesis was kindly given by Professor Patricia Rijo from Universidade Lusófona de Humanidades e Tecnologias.

Table 4 - Reactional conditions for carbamoylation reactions

Isocianate	Volume isocyanate (µl)	Weight DHR (mg)	Weight DMAP (mg)	Weight product (mg)	Yield (%)	Reaction time (h)	Obs.
	8.5	10.3	3.98	n.a	n.a	48	Reaction not complete
	23	11.0	4.1	6.60	47.21	28	Complete Reaction
	39	20.2	8.6	8.7	31.55	6	Complete Reaction
	39	20.1	8.9	16.2	54.62	3	Complete Reaction
	40	21.6	9.1	n.a	n.a	4	Complete Reaction countless subproduts

Note: Although in the procedure the reaction time is set to be 48h, the reactions were being monitored through TLC, therefore when it was possible to observe that the reaction was over, the process would be terminated.

Reaction 1

In this reaction, the product depicted in figure 6 was the final product that should have been obtained. However, this was not the case since the reaction was not complete and at the end of the 48h, there was still DHR present.

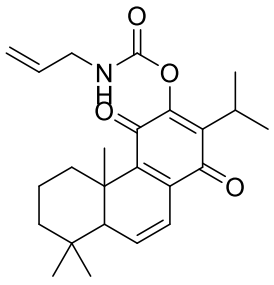


Figure 6 – Expected product of reaction 1

Reaction 2

This reaction allowed the obtention of a pure product in 47.21% of yield. The structure of the obtained product and the ^{1}H-NMR spectrum are shown in Figure 7.

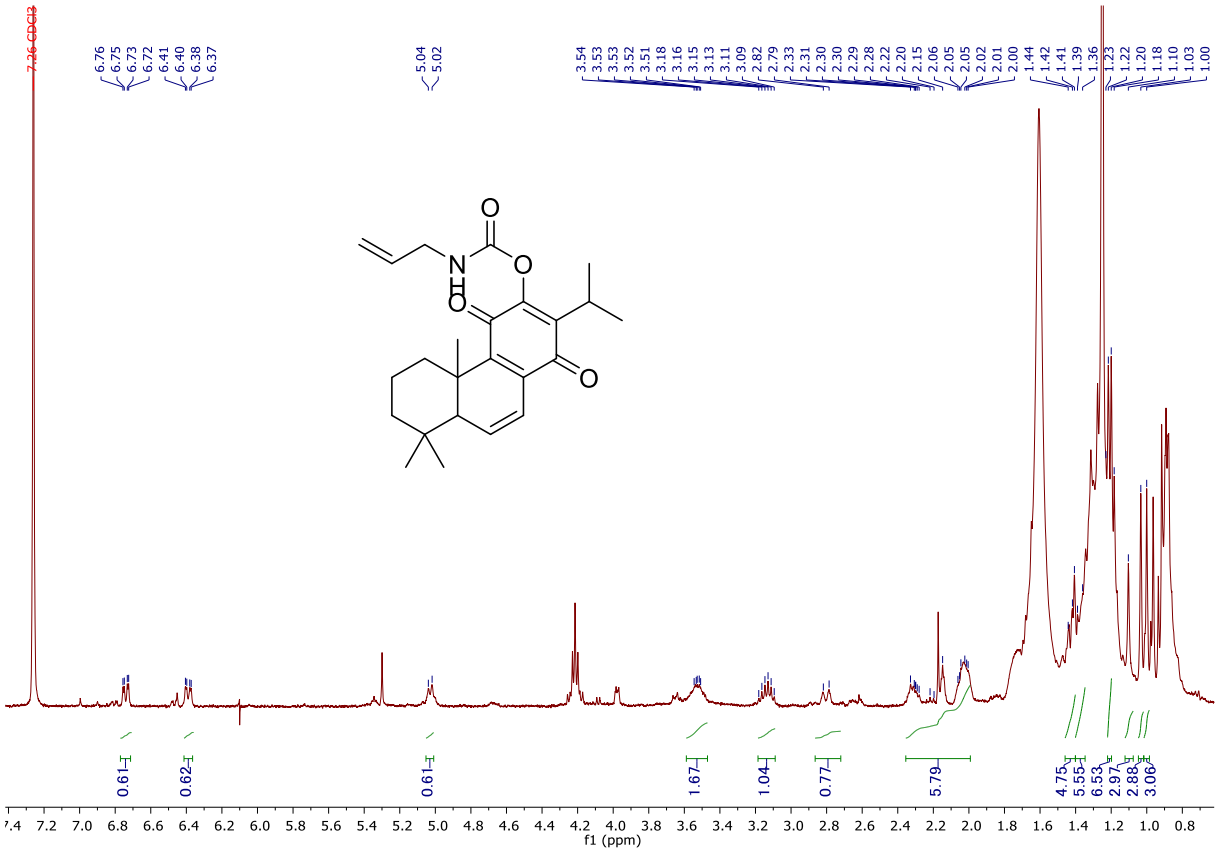


Figure 7 – ^{1}H-NMR spectrum of compound 2

Reaction 3

In this reaction, the structure of the obtained product is depicted in Figure 8 as well as the NMR spectrum. The yield of this reaction was 31.55%.

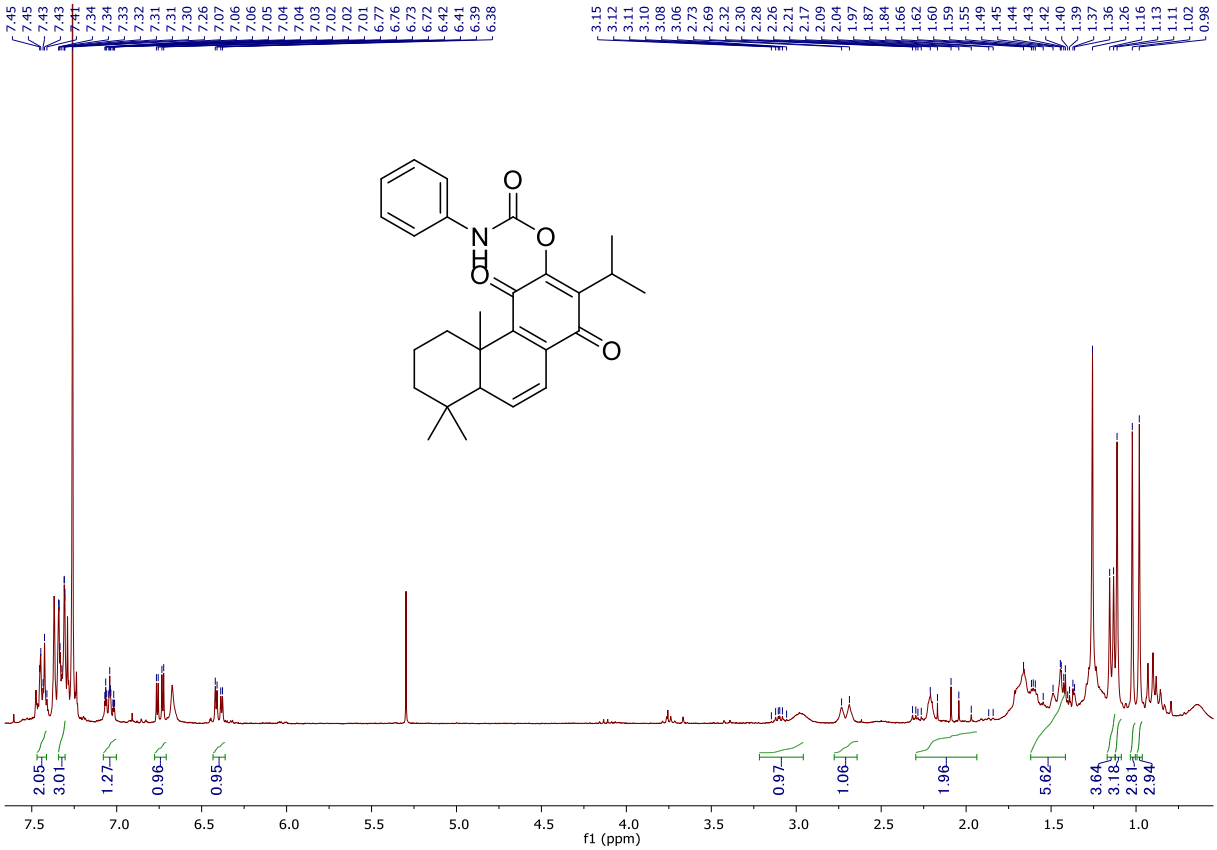


Figure 8 – ^{1}H-NMR spectrum of compound 3

Reaction 4

In this reaction, the structure of the obtained product is depicted in Figure 9 as well as the NMR spectrum. The yield of this reaction was 54.62%.

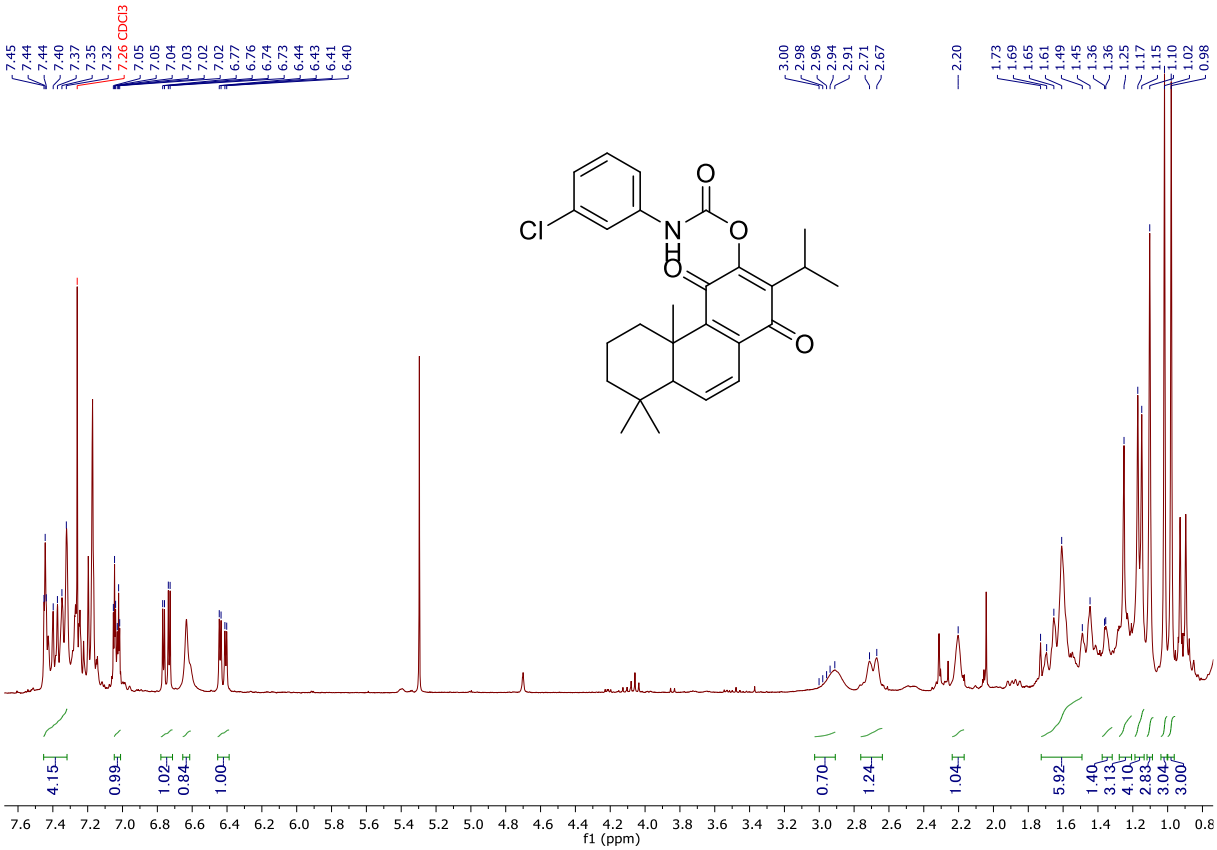


Figure 9 - ^{1}H-NMR spectrum of compound 4

Reaction 5

In this reaction, the product depicted in figure 10 was the final product that should have been obtained. However, this was not the case since the reaction produced countless subproducts and it was not possible to isolate the wanted compound, thus, no NMR analysis was performed.

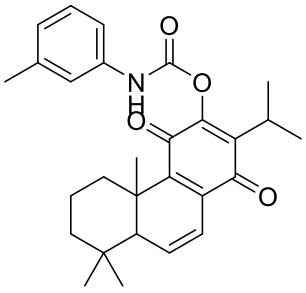


Figure 10 - Structure of expected compound 5

After all the carbamoylation reactions were performed as well as the NMR analyses, it was possible to observe that the products of these reactions were not stable and presented degradation. The comparison of product 3 and the pure DHR compound ^{1}H-NMR spectrums is presented in figure 11.

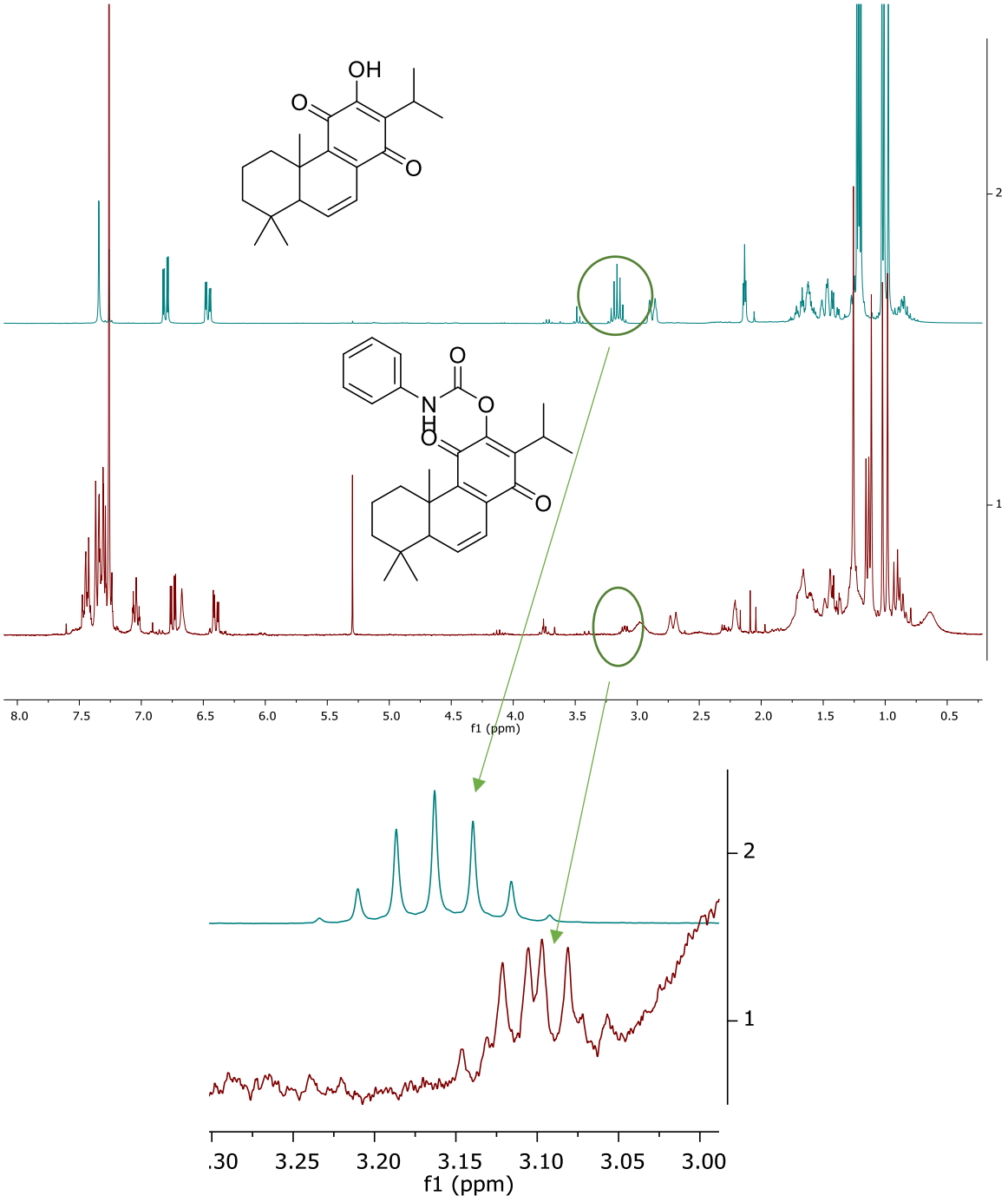
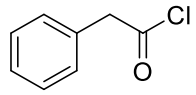
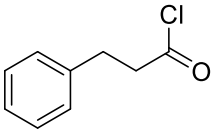
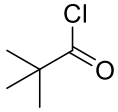
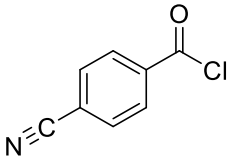
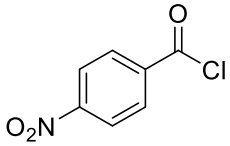


Figure 11 – Comparison of peaks of DHR (Blue) and Product 3 (Red)

After comparisation of the H-15 signal from DHR and product 3 (figure 11) it was possible to understand that this type of derivatives dysplayed some stability issues. thus, the

carbamoylation products of the DHR were considerated unviable and, therefore, another route was chosen. Recencently, Garcia et al. (2020) reported the same instability problem with other type of derivatives prepared from DHR and Roy. On the other hand, in the same study, the authors documented the hemi-synthesis of four stable products, where the most promising ones resulted from esterification reactions (C Garcia et al., 2020). Therefore, it was decided to continue this work exploring the esterification of DHR.Thus, several esterification reactions were performed, and the reactional conditions used can be consulted in Table 5.

Table 5 - Reactional conditions for esterification reactions

Reagent	Weight acyl chloride (mg)	Weight DHR (mg)	Weight product (mg)	Yield (%)	time (h)	Obs.
	29.94	20.3	26.3	94	4	Complete Reaction
	64.35	20.4	27	95	4	Complete Reaction
	262.30	22.8	24.9	86	21	Complete Reaction
	472.13	19.8	n.a	n.a	20	Reaction did not occur
	317.54	21	n.a	n.a	20h	Reaction did not occur

Note: Although in the procedure the reaction time is set to be 48h, the reactions were being monitored through TLC, therefore when it was possible to observe that the reaction was over, the process would be terminated.

Reaction 6

In this reaction, the structure of the obtained product is depicted in Figure 12 as well as the NMR spectrum. The yield of this reaction was 94%.

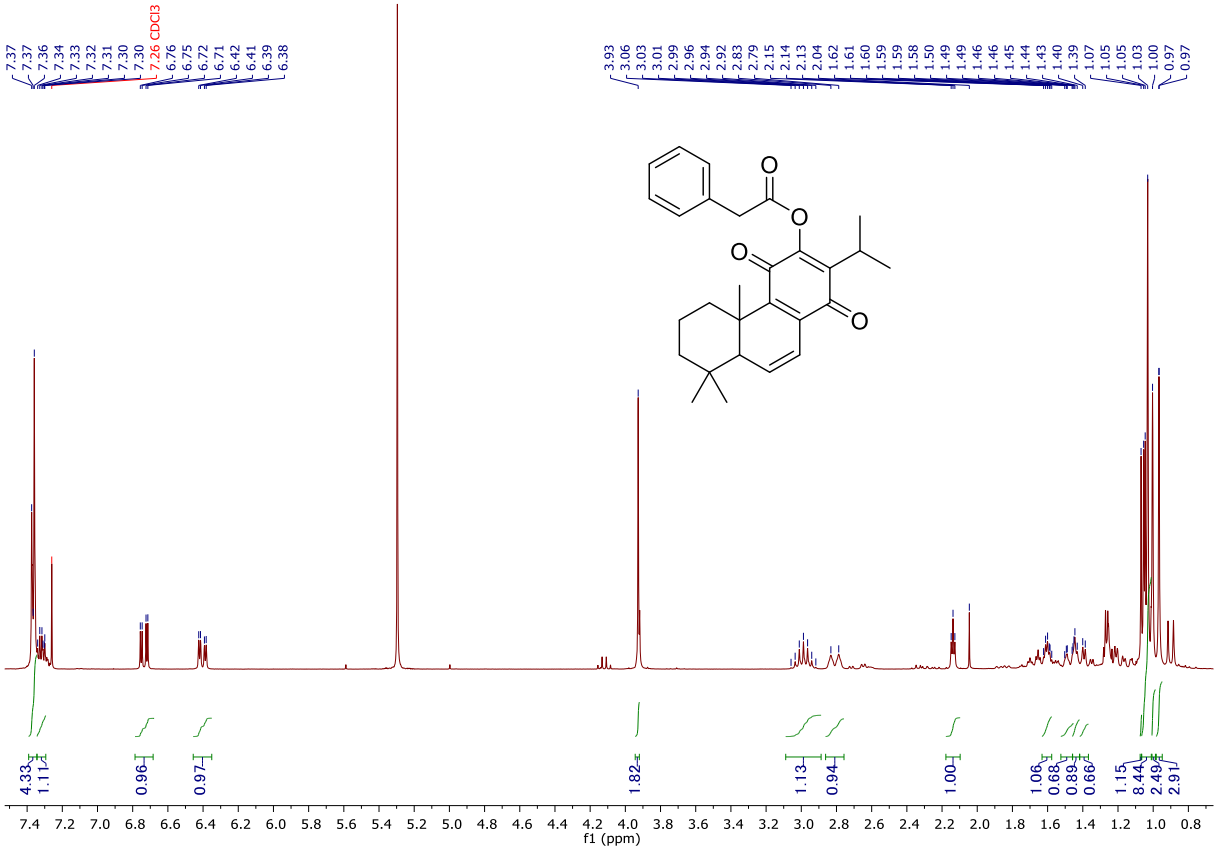


Figure 12 - ^{1}H-NMR spectrum of compound 6

Reaction 7

In this reaction, the structure of the obtained product is depicted in Figure 13 as well as the NMR spectrum. The yield of this reaction was 95%.

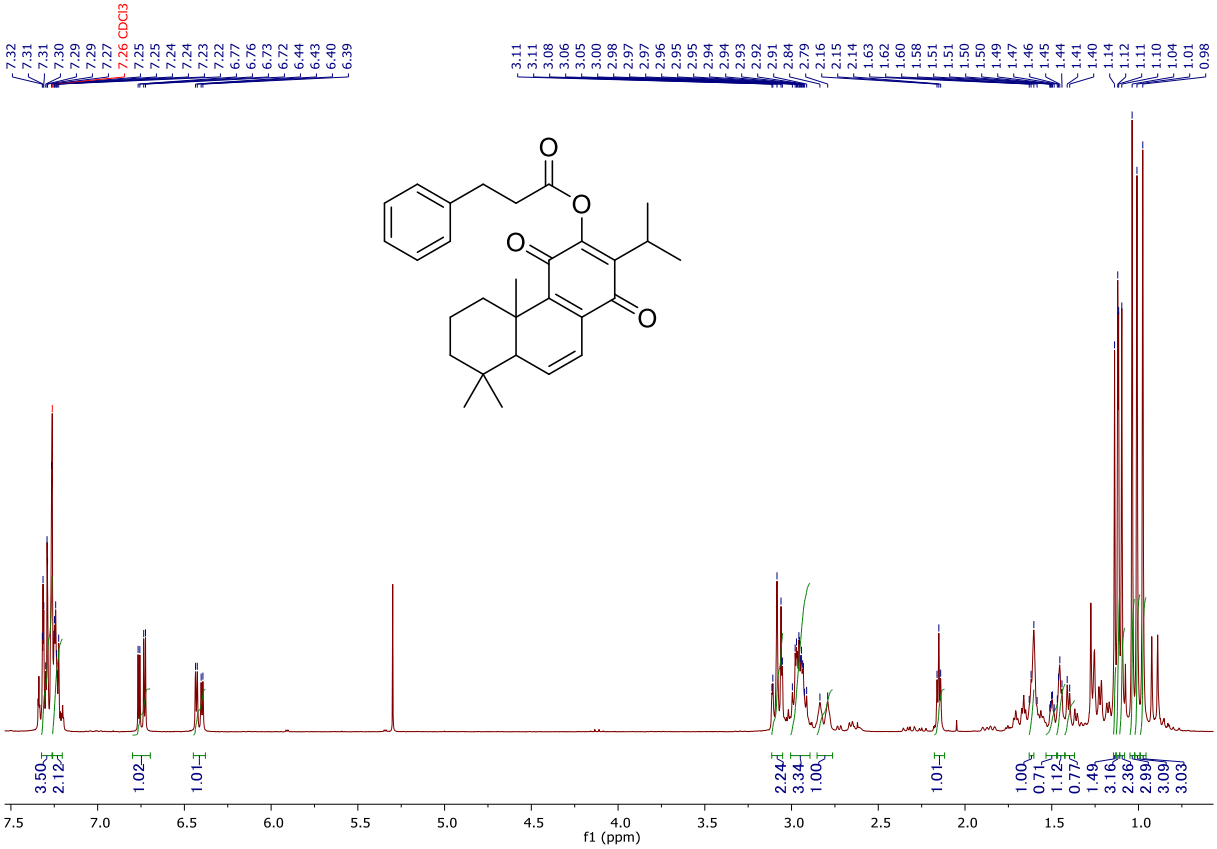


Figure 13 - ^{1}H-NMR spectrum of compound 7

Reaction 8

In this reaction, the structure of the obtained product is depicted in Figure 14 as well as the NMR spectrum. The yield of this reaction was 86%.

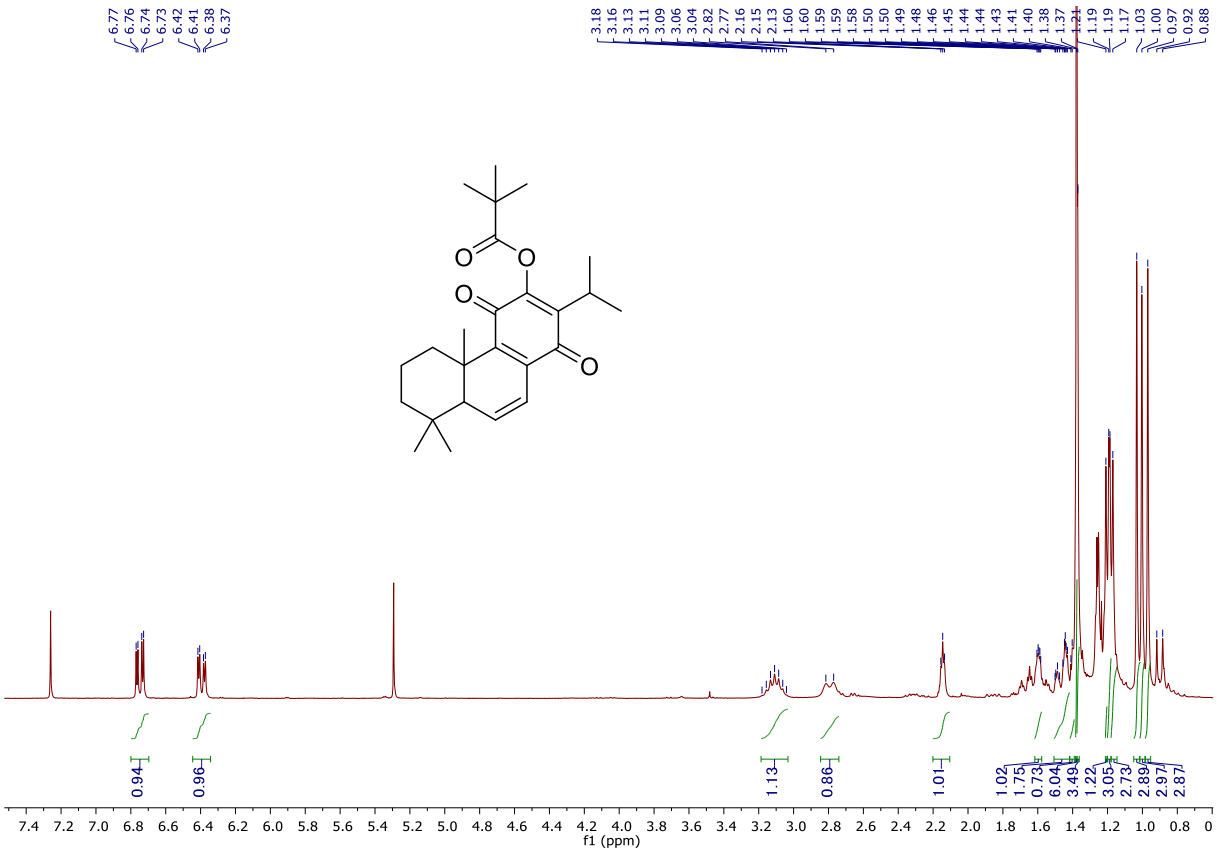


Figure 14 - ^{1}H-NMR spectrum of compound 8

Reaction 9

In this reaction, the product depicted in figure 15 was the final product that should have been obtained. However, this was not the case since, the reaction did not generate any product, thus no NMR analysis was performed.

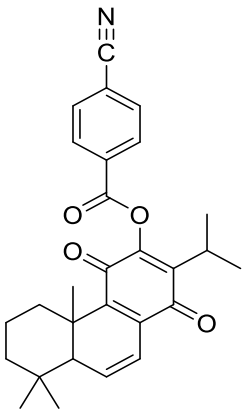


Figure 15 - The expected product of reaction 9

Reaction 10

In this reaction, the product depicted in figure 16 was the final product that should have been obtained. However, this was not the case since, the reaction did not generate any product, thus no NMR analysis was performed.

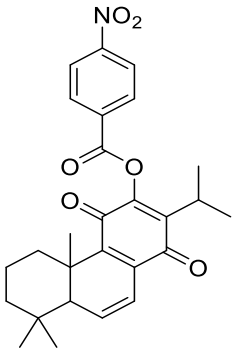


Figure 16 - The expected product of reaction 10

After all the esterification reactions were performed as well as the NMR assays, it was possible to observe that the products of these reactions presented no degradation, therefore, were stable.

As an example of the obtained results as well as their interpretation, a comparison of product 6 and the lead compound is shown below in figure 16.

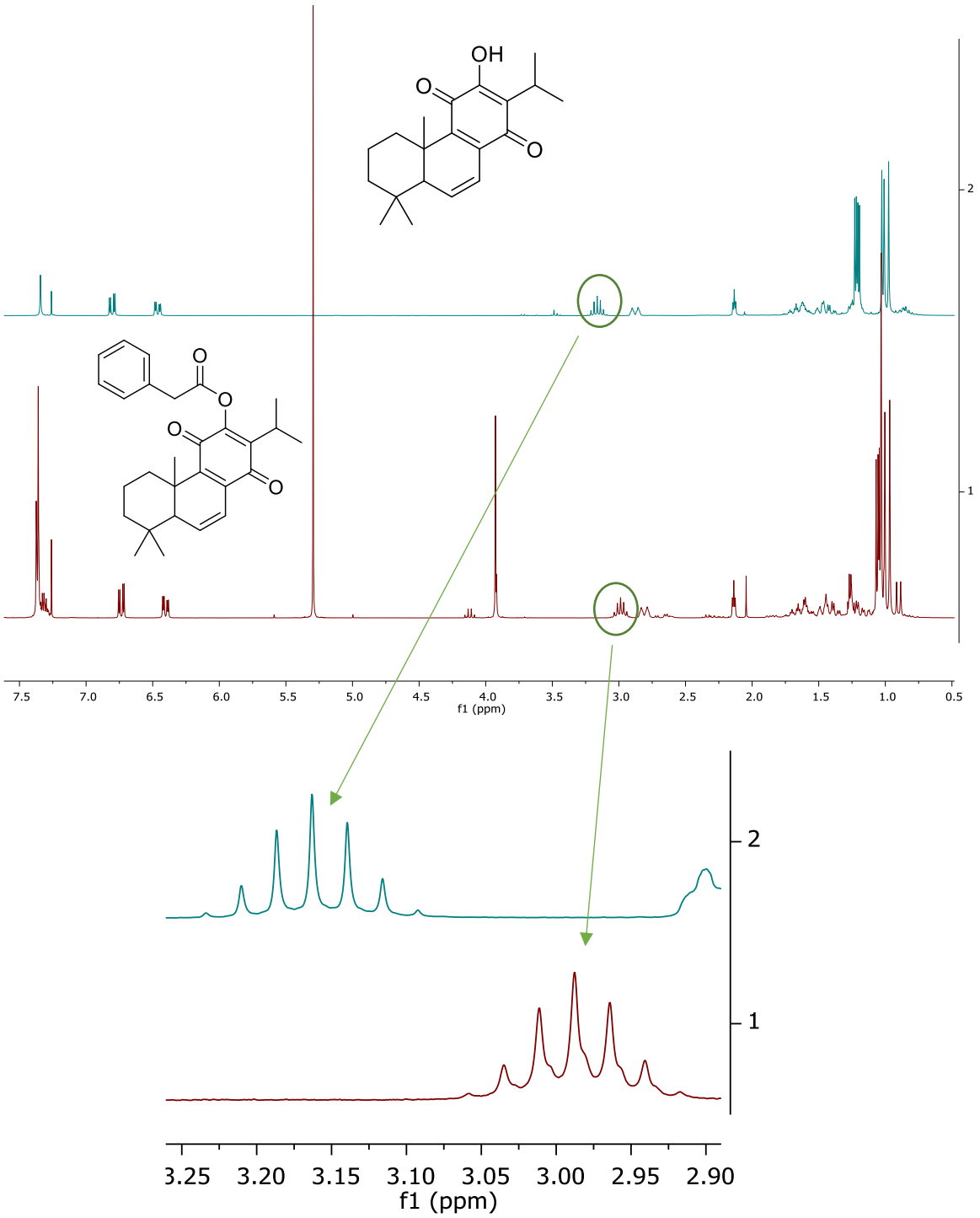


Figure 17 - Spectral comparison between DHR (blue) and compound 6 (red)

According to figure 17 the formation of the derivative from DHR is confirmed, in H-15 a very defined septet is seen which leads us to conclude that a stable product was formed, this is in agreement with reports made by Garcia and Isca regarding other esters (C Garcia et al., 2020) Only the derivatives considered stable through the NMR analysis were selected for further studies.

3.1. Antimicrobial evaluation

The antimicrobial activity of the derivatives 6 to 8 was evaluated. The MIC and MBC were assessed. additionaly, the natur compounds Roy, Parvifloron D, and DHR were also tested. The results are shown below in table 6.

Table 6 - Antimicrobial Activity of the derivatives 6 to 8

Compounds	***S.aureus***		***MRSA***	
	MIC	**MBC**	**MIC**	**MBC**
Roy	3.91	31.25	3.91	31.25
Parvifloron D	125	125	62.5	nt
DHR	3.91	31.25	3.91	31.25
6	3.91	31.25	3.91	31.25
7	3.91	31.25	3.91	31.25
8	3.91	31.25	3.91	31.25
+	1.95	nt	0.976	nt
-	125	nt	125	nt

+ = vancomycin - = DMSO nt = not tested

In this assay it is possible to access that the derivatives show antimicrobial activity against *S.aureus* and MRSA, portraying identical results as Roy and DHR. It is also possible to identify that Parvifloron D does not present a significant antimicrobial activity. In the same way, the derivatives show MBC results identical to the starting material (DHR). Considering that the derivatives were obtained using DHR as the starting material, we can conclude that the hemi-synthetic process did not increase the antimicrobial activity of the compounds.

3.2. Evaluation of General Toxicity on *Artemia salina* Model

A lethality test was carried out to evaluate the preliminary toxicity of the derivatives 6 to 8 (figure 18).

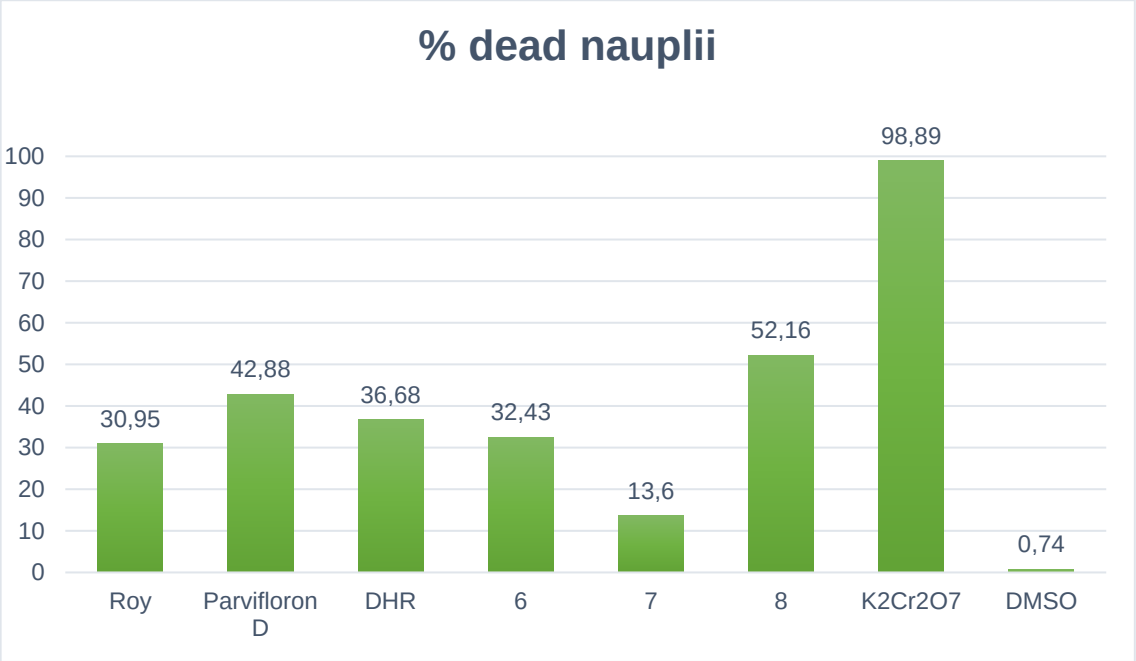


Figure 18 – Evaluation of General Toxicity on *Artemia salina* Model

Observing the results obtained it is possible to determine that when comparing with the starting material, the hemi-synthetic reactions performed increased general toxicity in derivative 8, but decreased toxicity in 6 and 7. This can be an indicator that the *terc*-butyl group added to the molecule may be responsible for the increase of the general toxicity observed.

3.3. Antioxidant activity

Antioxidant activity was evaluated through DPPH assay. The IC50 (mM) of the natural compounds, DeRoy, Roy, and Parvifloron D, and the derivatives 6 to 8 are presented in figure 19.

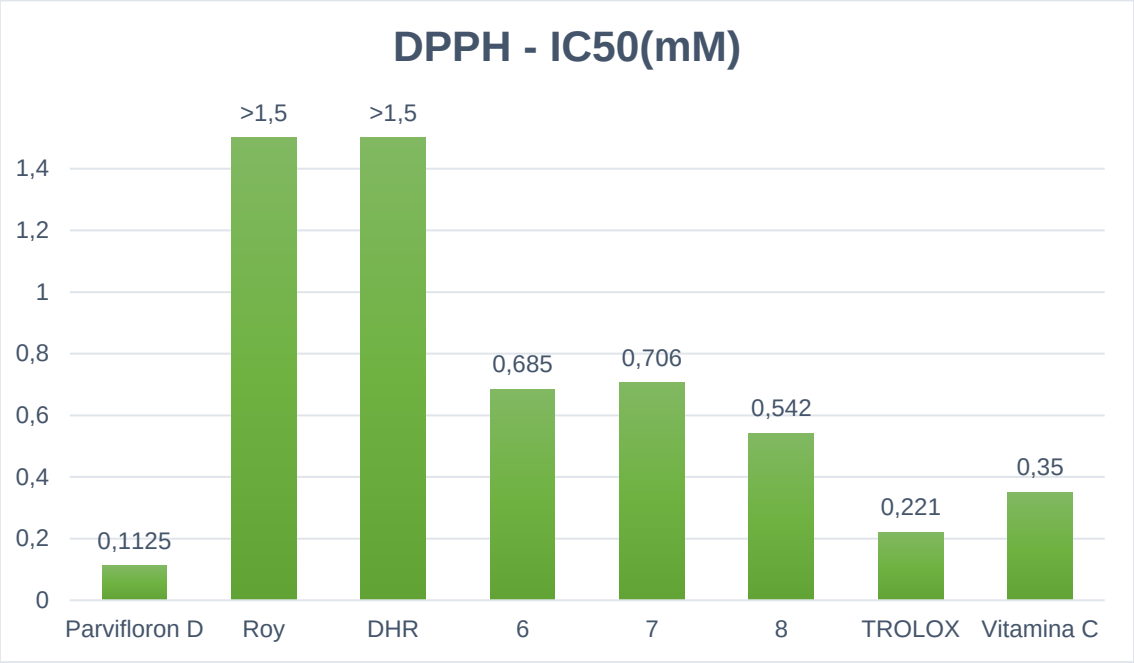


Figure 19 - Antioxidant activity evaluated through DPPH: - IC_{50}(mM)

Comparing with the positive controls (Trolox and ascorbic acid) most of the compounds show less antioxidant activity, Parvifloron D however is an exception, portraying a higher antioxidant activity when compared with the control samples.

It is also possible to observe that all the derivatives present a higher antioxidant activity when compared with the starting material making it possible to conclude that the hemi-synthesis reactions improved the antioxidant activities of the molecules.

3.4. Cytotoxic activity

The cytotoxic activity of the natural compounds and derivatives were evaluated in the MDA-MB-231S triple-negative cancer cell line. The results are presented in figure 20.

Figure 20 - IC_{50} (µM) observed in MDA-MB-231S cancer cell lines

Both Roy and DHR show a moderate activity against the MDA-MB-231S cell line, but the derivatives do not show promising results in this cell line, it is, however, important to note that being this a triple-negative cell line, it is a cell line that presents resistance to most of the compounds.

3.5. Evaluation of P-gp inhibition potential.

Rhodamine 123 Flow cytometry assay was performed to test the ability of P-gp inhibition of the compounds, and the results are shown in figure 21.

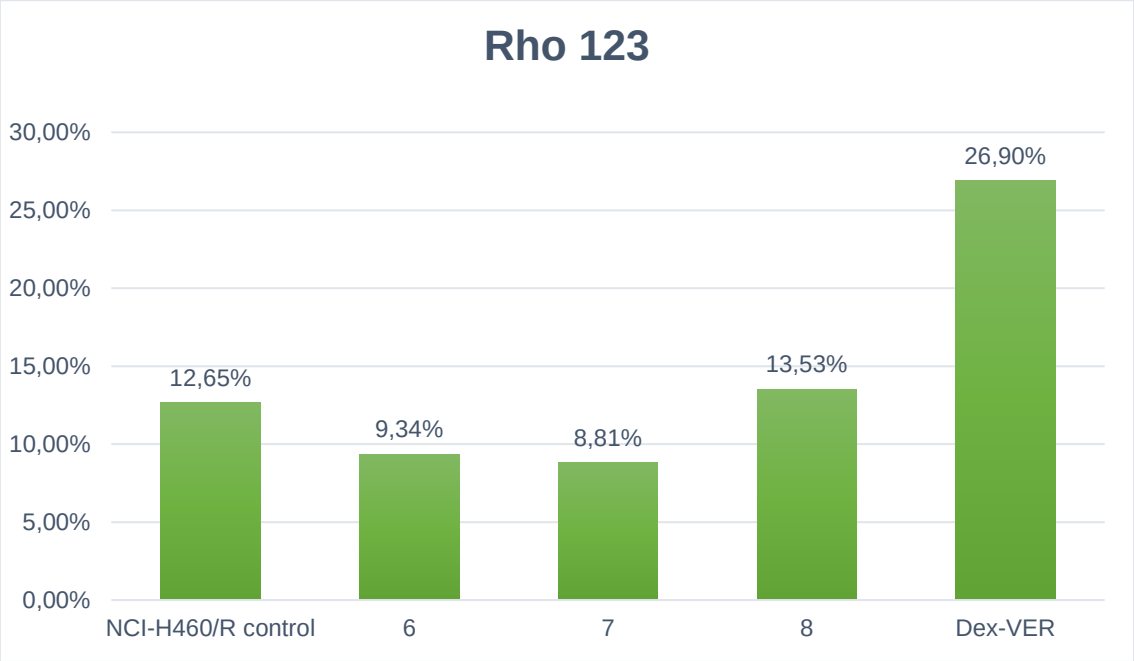


Figure 21 - Evaluation of P-GP inhibition potential through Rho123 assay

When observing the graph, it is possible to understand that these compounds are not active when compared with the positive control, however when compared with other similar compounds published by Vera Isca in 2020 Frontiers, it is noted that other DHR derivatives do not show activity in this cell line overexpressed with P-gp. In this paper it is possible to note that there are two very similar compounds, 6,7-dehydro-12-O-benzoylroyleanone and 7α-acetoxy-6β-hydroxy-12-*O*-benzoylroyleanone obtained from different natural products where the latter displays a promising result for P-gp inhibition which suggest that substituents in position 6 (-OH) and 7 (-OAc) can be a contributor for P-gp inhibition, this hypothesis, however, needs further studies to be proven (C Garcia et al., 2020; Isca et al., 2020).

Conclusion

This project aimed to prepare and characterize new derivatives from the reported cytotoxic DHR.

In total ten hemi-synthetic reactions were carried out, five of these reactions were carbamoylation and the other five were esterification reactions. Six of the reactions were completed, one resulted in degradation (carbamoylation) and three did not occur (two esterifications and one carbamoylation). Considering the stability of the six obtained compounds only three stable esterification products were sucessfully obtained.

In this work, it is possible to conclude that carbamoylation reactions originate unstable compounds. On the other hand, esterification reactions produce stable compounds, with overall good yieldings (86-95%). Furthermore, other reactions of esterification should be selected to produce additional derivatives in future work.

Using only the derivatives that were considered stable in the NMR analysis, assays were performed to evaluate the antimicrobial activity. The hemi-synthesis reactions did not improve the antimicrobial activity due to the values of minimum inhibitory concentration and minimum bactericidal concentration which were not different from the starting material. It is important to note that antimicrobial activities were mostly tested against Gram + bacteria, therefore in further studies, other strains should be tested, such as Gram – bacteria, yeasts, and other Gram + bacteria.

The *Artemia salina* model, showed that the derivatization process was responsible for an increased toxicity in derivative 8. However, in derivatives 6 and 7 there was a decrease of toxicity when compared with the starting material. This can be an indicator that the hydroxyl group present in DHR may have an important role in the toxicity of this molecule.

In the DPPH assay to access the antioxidant a activity of the compounds, it was possible to conclude that the derivatives obtained dysplayed increased antioxidant activity when compared with the starting material.

In the MTT assay the compounds did not show enough activity against the cell line where they were tested, it is however important to note that this cell line is a triple-negative breast cancer cell line, which is the most aggressive, therefore further studies against other less aggressive cell lines must be made.

In the Rhodamine 123 assay, the compounds did not show promissing P-gp inhibition activity. Nevertheless, the results suggest that some moieties in key locations (C-6 and C-7) can be important for P-gp inhibition. Therefore further studies must be conducted, such as using other royleanones as starting materials for the derivatization process and conducting the assays

performed in this work. In future studies, we aim to test these derivatives against other cancer cell lines, including breast cancer cell lines less agressive. Additionally, other hemi-synthetic pathways should be explored and also using Roy as starting material to have a large library of derivatives. Finally, some structure-activity relationship studies should be performed.

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Annex 1

Cells and Cell culture

The non-small cell lung carcinoma cell line (NCI-H460) was purchased from American Type Culture Collection, Rockville, MD.

Resistant Non-small cell lung carcinoma cell line (NCI-H460\R) cells were selected from the original cell line and cultured in a medium containing 100 nM doxorubicin, according to Pesic et al., 2006 (Pesic et al., 2006). At 72h intervals, the cells were subcultured sung 0.25% trypsin\EDTA and seeded with 8.000 cells\cm3 and 16.000 cells\cm3 densities for NCI-460 and NCI-460\R respectively (C Garcia et al., 2020).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with a triple-negative breast cancer cell line (MDA-MB231S)

The cellular line MDA-MB231S was cultivated in DMEM L050-500 medium, supplemented with 10% of Fetal Bovine Serum, L-glutamine, and Penicillin and Streptomycin at 37˚ and 5% CO_2. 1000 cells for well were seeded for the realization of the MTT assay in a 96 well plate and 24h later the compounds were added.

The compounds and derivatives were dissolved in DMSO (shrlarlau; SU1531000) at a concentration of 2 mg/mL for the extracts and a concentration of 10 mM for the derivatives.

The compounds were diluted in the following concentrations 80 µg\mL, 40 µg\mL, 20 µg\mL, 10 µg\mL, and 2 µg\mL.

The derivatives were diluted in the following concentrations 10 µM, 3 µM, 1 µM, 0,3 µM and 0,1 µM. all the assays were done in triplicate.

After 48h, the MTT was added and incubated for 2h. At the end of the 2h, the medium was taken out and the crystals that formed were diluted in 200 µL of DMSO. Finally, absorbance was read at 595 nm.

Rhodamine 123 Flow cytometry assay

The accumulation of Rhodamine 123 (Rho 123) was analyzed through flow cytometry since Rho 123 possesses the capability of emitting fluorescence. The intensity of the fluorescence is proportional to the accumulation of Rho 123. NCI-H460 and NCI-H460\R cells were grown to 80% confluence in 75 cm^3 flasks, trypsinized, and then resuspended in 10 mL centrifuge tubes in a medium that contained Rho123. The cells were treated with Dex-Verapamil (used as positive control) and with the compounds and derivatives and incubated at 37˚ C in 5% CO_2 for 30 minutes. Then the cells were centrifuged, washed with Phosphate-buffered saline (PBS), and placed in cold PBS. The analysis was performed by CyFlow Space Partec flow-cytometer (Sysmex Partes GmbH, Germany) and the fluorescence of the Rho123 was assessed on the FL1 channel. Each sample was assayed for a minimum of 20.000 events and the results were analyzed using Summit Dako Software (C Garcia et al., 2020).