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Unravelling new ethnopharmacological roles of *Plectranthus* species: biological activity screening

Joana Eulália da Cruz Marçalo de Andrade

Dissertação de Mestrado

MESTRADO EM CIÊNCIAS BIOFARMACÊUTICAS

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Scientific production

Papers

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Poster Communications

National

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International

Faustino C., **Marçalo J.**, Nicolai M., Rodrigues L.M., Reis C., Rijo P. *In vitro* efficacy assessment of extracts and compounds from *Plectranthus* species for skin delivery. International Society for Biophysics and Imaging of the Skin World Congress, Lisboa, Portugal, May 31st – June 3rd, 2016.

Marçalo J., Frias D., Nicolai M., Rodrigues L.M., Reis C., Faustino C., Rijo P. Antioxidant, antiacetylcholinesterase, antityrosinase, antielastase and anticollagenase activity of extracts and compounds from *Plectranthus* species. 6th International Congress of Aromatic and Medicinal Plants (CIPAM), Coimbra, Portugal, May 29th – June 1st, 2016.

Marçalo J., Custódio L., Rodrigues M.J., Nicolai M., Rodrigues L.M., Reis C., Faustino C., Rijo P. Exploring the biological activity potential of *Plectranthus* spp. diterpene derivates. Conference: 6th European Workshop in Drug Synthesis, Siena, Italy, May 15th – 19th, 2016.

Stanković T., Dinić J., Podolski-Renić A., Garcia C., **Andrade J**., Simões M.F., Rijo P., Pešić M. Diterpenoids isolated from *Plectranthus* spp. are selective towards human non-small cell lung carcinoma cells with P-glycoprotein overexpression. 2nd COST meeting. Centro de Investigaciones Biológicas (CIB-CSIC), Madrid, Spain, April 4th – 5th, 2016.

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Abbreviations

1D	One dimension
2D	Two dimensions
AA	Arachidonic acid
Abs	Absorbance
Roy	7α-acetoxy-6β-hydroxyroyleanone
Ach	Acetylcholine
AChE	Acetylcholinesterase
AChI	Acetylcholine iodide
AChR	Acetylcholine receptors
Ala	Alanine
ANOVA	Analysis of variance
AP-1	Activator protein-1
BHA	Butylated hydroxyanisole
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CBIOS	Research Center for Biosciences & Health Technologies
CCMAR	Center of Marine Sciences
$CD14^+$	Cluster of Differentiation 14 monocytes
CFU	Colony-forming units
ChC	Collagenase
COSY	Homonuclear correlation spectroscopy
COX	Cyclooxygenase
Cyclo-abietene	(13*S*,15*S*)-6β,7α,12α,19-tetrahydroxy-13β,16-cyclo-8-abietene-11,14-dione
DeHRoy	6,7-dehydroroyleanone
DiHRoy	6β,7α-dihydroxyroyleanone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
EC	Enzyme Commission
ECM	Extracellular matrix
EGCG	Epigallocatechin gallate
Ela	Elastase

ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
***et al*.**	*et alii* / *et aliae*
ETAN	Ethambutol
FAD	Flavin adenine dinucleotide
FALGPA	*N*-(3-[2-furyl]acryloyl)-Leu-Gly-Pro-Ala
FBS	Fetal bovine serum
FMN	Flavin mononucleotide
Forskolins	1,6-di-*O*-acetylforskolin:1,6-di-*O*-acetyl-9-deoxyforskolin
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
gHMBC	Gradient selected phase-sensitive - Heteronuclear multiple bond correlation
gHSQC	Gradient selected phase-sensitive - Heteronuclear single quantum correlation
Glu	Glutamine
Halimane	11*R**-acetoxy-halima-5,13*E*-dien-15-oic acid
Halimane Bu	(11*R**,13*E*)-15-butyryloxyhalima-5,13-dien-11-ol
Halimane Diol	(11*R**,13*E*)-halima-5,13-diene-11,15-diol
Halimane Ester	(11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic methyl ester
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
HSD	Honest significant difference
IC_{50}	Drug concentration causing 50% inhibition
IFN-γ	Interferon-γ
IL	Interleukin
INH	Isoniazid
iNOS	Inducible nitric oxide synthase
IR	Infrared
KOJ	Kojic acid
LC3	Microtubule-associated protein 1A/1B-light chain 3
LDH	Lactate dehydrogenase
L-DOPA	L-3,4-dihydroxyphenylalanine
L-NAME	ω-Nitro-L-arginine methyl ester hydrochloride
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDR	Multiple drug resistance

MIC	Minimum Inhibitory Concentration
MMP	Metalloproteinase
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
Mtb	*Mycobacterium tuberculosis*
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NED	*N*-(1-naphthyl)ethylenediamine
NF-κB	Nuclear factor-κB
NMR	Nuclear magnetic resonance
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOESY	Nuclear overhauser spectroscopy
NOS	Nitric oxide synthase
NSAID	Nonsteroidal anti-inflammatory drug
NT	Not tested
p62	Nucleoporin p62
Parvifloron D	11-hydroxy-2α-(4-hydroxybenzoyloxy)-abieta 5,7,9(11),13-tetraen-12-one
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PE	*Plectranthus ecklonii* Benth.
PG	*Plectranthus grandidentatus* Gürke
PGE_2	Prostaglandin E_2
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PM	*Plectranthus madagascariensis* (Pers.) Benth
PN	*Plectranthus neochilus* Schltr.
PO	*Plectranthus ornatus* Codd.
PP	*Plectranthus porcatus* van Jaarsv. & P.J.D. Winter
PPP	*Plectranthus prostratus* Gürke
PS	*Plectranthus saccatus* Benth.
ROS	Reactive oxygen species
rpm	Revolutions per minute

RPMI	Roswell Park Memorial Institute
SANA	*N*-succinyl-$(Ala)_3$-*p*-nitroanilide
SAR	Structure-activity relationships
SCF	Supercritical fluid
SD	Standard deviation
SIRS	Systemic inflammatory response syndrome
Smad	Mothers against decapentaplegic homolog
SOD	Superoxide dismutase
SQSTM1	Sequestosome 1/p62
STS	Staurosporine
TB	Tuberculosis
TDR	Totally-drug resistance
TGF-β	Transforming growth factor-β
TMPD	*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine
TNF-α	Tumor necrosis factor-α
TOCSY	Total correlation spectroscopy
TPP	Tuberculosinyl diphosphate
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
Tyr	Tyrosinase
U	Enzyme unit
URS	Ursolic acid
UV	Ultraviolet
v/v	Volume/volume
WHO	World Health Organization
XDR	Extensively-drug resistant

Abstract

This dissertation focused on the biological activity screening of several *Plectranthus* spp. plants, aiming to unravell novel ethnopharmacological roles and further research, of several extracts (methanol, ethyl acetate, acetone and water) and isolated compounds. Previous evidences on interesting biological activities of *Plectranthus* spp. constituents, directed the study for antioxidant, anti-skin ageing, anti-inflammatory, and anti-mycobacterial activities.

Antioxidant results revealed an increased activity of the methanol extracts (20-76%), due to the presence of polyphenols widely known as antioxidants. Moreover, *P. grandidentatus* (62.3 ± 0.43%) and *P. ecklonii* (55.5 ± 1.66%) registered a high scavenging activity in the ethyl acetate extract, in comparison with quercetin (89.0 ± 2.5%), most likely due to the presence of abietane diterpenes.

Acetylcholinesterase (AChE) was studied *in vitro* to evaluate the enzymatic inhibition, due to recent discoveries on non-neuronal cholinergic system in the skin. This assay showed that the *Plectranthus* spp. organic extracts did not significantly inhibited AChE.

Concerning the tyrosinase inhibition assay, it was observed a high inhibition for the *P. ecklonii* methanol (65.9 ± 3.42%), *P. grandidentatus* acetone (67.9 ± 3.55%), and *P. saccatus* acetone (56.5 ± 5.68%) organic extracts. On the other hand, the aqueous extract of *P. porcatus* was the only one that showed enzymatic inhibition (65.0 ± 8.67%). From the tested isolated compounds, abietane diterpenes, mainly present in the organic extracts of *P. grandidentatus, P. madagascariensis*, and *P. ecklonii* were highly active against tyrosinase, in more than 46% and up to 75%, especially compared to kojic acid (92.9 ± 7.28%).

In the collagenase assay, all tested extracts and compounds showed a high enzymatic inhibition which was in the range of 28-76%. The higher inhibition results were obtained from *P. neochilus* methanol extract (76.4 ± 2.09%), *P. ecklonii* aqueous extract inhibited (75.59 ± 6.5%) and rosmarinic acid (44.78 ± 4.53%), in comparison with epigallocatechin gallate (93.1 ± 5.27%).

In contrast to the results of the previous enzymatic assays, the anti-elastase assay revealed that in general the extracts did not decrease elastase activity (30-42%). Nonetheless, the isolated compounds were tested, and they were able to highly inhibit elastase activity. Particularly, the oleanolic:ursolic acids mixture (1:4) with 63.4 ± 2.56% and Parvifloron D with 52.8 ± 3.76%, were the most efficient in elastase inhibition specially compared with ursolic acid used as positive control (69.9 ± 3.65%).

The anti-inflammatory assay was performed by the quantification of NO production using the Griess reaction. The non-cytotoxic isolated compounds revealed to be unable to reduce NO production, after LPS stimulated inflammation (ranging from 16-23 μM), in

comparison with the normal quantities of NO production within the cells (17.7 ± 0.67 µM), and with the positive control L-NAME that decreased NO until reaching 3.9 ± 0.24 µM.

Finally, the preliminary results of *Mycobacterium tuberculosis* H37Rv growth assay, revealed low CFU/mL (dilution 10^{-3}) especially regarding one halimane diterpene compound (2.1×10^5 CFU/mL), similar to the positive controls isoniazid (1.2×10^5 CFU/mL), and ethambutol (2.0×10^5 CFU/mL), suggesting a potential alternative for further anti-tubercular studies.

Overall, according to the references of this study, this was the first report on Plectranthus spp., concerning the assays of skin-related enzymatic inhibition in vitro, anti-inflammatory assay, and *Mycobacterium tuberculosis* H37Rv growth, with a preliminary scientific validation upon known ethnopharmacological uses.

KEYWORDS: *Plectranthus* spp.; Anti-inflammatory; Antioxidant; Skin enzymatic inhibition; *Mycobacterium tuberculosis*.

Resumo

As plantas do género *Plectranthus* L'Héritier (família *Lamiaceae*) são selecionadas em várias investigações científicas devido aos vários usos etnofarmacológicos pelas populações indígenas, e facilidade de crescimento em zonas temperadas. Estas plantas são compostas por cerca de 350 espécies reconhecidas pelos seus óleos essenciais e compostos terpénicos.

Nesta dissertação pretendeu-se desenvolver e complementar o *screening* de várias atividades biológicas, a partir de extratos e compostos isolados de sete espécies de *Plectranthus* (*P. grandidentatus* Gürke, *P. ecklonii* Benth., *P. ornatus* Codd., *P. madagascariensis* (Pers.) Benth., *P. porcatus* van Jaarsv. & P.J.D.Winter, *P. neochilus* Schltr., e *P. prostratus* Gürke). Estes estudos biológicos envolveram a pesquisa de atividades antioxidante, anti-envelhecimento da pele, anti-inflamatória e anti-micobacteriana.

Foram obtidos vinte e oito extratos (sete aquosos e vinte e um orgânicos, nomeadamente de acetona, metanol e acetato de etilo) de todas as plantas em estudo, obtendo-se os resíduos secos (mg de extrato/g de planta).

Nos resultados preliminares da atividade antioxidante executada pelo método da recaptação do radical DPPH, verificou-se que os extratos de metanol detiveram os valores mais elevados de recaptação de radicais livres (20-76%), e, portanto, de maior atividade antioxidante. Concluiu-se que esta atividade poderá estar relacionada com as elevadas quantidades em polifenóis nos extratos metanólicos, amplamente citados como antioxidantes. Adicionalmente, foi também registada uma elevada atividade antioxidante para os extratos de acetato de etilo de *P. ecklonii* (55.5 ± 1.66%) e *P. grandidentatus* (62.3 ± 0.43%), muito provavelmente devido à presença de diterpenos abietânicos. Na verdade, estes foram os resultados que demonstraram maior atividade antioxidante comparável ao controlo positivo usado, quercetina (89.0 ± 2.5%).

A acetilcolinesterase (AChE, EC 3.1.17), conhecida pela sua ação na terminação do impulso nervoso pela hidrólise da acetilcolina, produzindo colina, foi estudada *in vitro* usando o método de Ellman, de modo a avaliar a inibição enzimática pelos extratos e/ou compostos isolados. Este ensaio foi realizado com base nos dados recentes da literatura, evidenciando um sistema colinérgico não-neuronal pela presença de acetilcolina na pele (humana). Com base nos resultados obtidos, foi possível concluir que nenhum dos vinte e um extratos orgânicos estudados inibiram a AChE no ensaio enzimático *in vitro*.

Relativamente aos ensaios *in vitro* de inibição enzimática relacionada com a pele, o ensaio de inibição da tirosinase (EC 1.14.18.1), uma monooxigenase de cobre que catalisa a síntese de melanina pela produção de L-DOPA a partir de L-tirosina, revelou que os extratos orgânicos de *P. ecklonii* metanol (65.9 ± 3.42%), *P. grandidentatus* acetona (67.9 ± 3.55%), e *P. saccatus* acetona (56.5 ± 5.68%), diminuíram significativamente a atividade enzimática da tirosinase.

Por outro lado, o extrato aquoso de *P. porcatus* foi o único a exibir inibição enzimática da tirosinase (65.0 ± 8.67%). De modo a confirmar a atividade registada para os extratos, foram testados os compostos isolados. De facto, os compostos abietânicos (maioritariamente presentes nos extratos orgânicos de *P. grandidentatus*, *P. madagascariensis*, e *P. ecklonii*) demonstraram forte atividade contra a tirosinase, em mais de 46% e até 75%, especialmente em comparação com o ácido kójico (92.9 ± 7.28%).

Relativamente ao ensaio enzimático de inibição da colagenase (EC 3.4.24.3, metaloproteinase envolvida na clivagem do colagénio), todas as amostras inibiram, de um modo geral, a atividade enzimática (28-76%). Na verdade, o extrato metanólico de *P. neochilus* inibiu a colagenase em 76.4 ± 2.09%, bem como o extrato aquoso de *P. ecklonii* (75.6 ± 6.5%) e o composto ácido rosmarínico em 44.78 ± 4.53%. Estes resultados preliminares sugerem que a presença de compostos polifenólicos e diterpenos abietânicos nos extratos de *Plectranthus*, têm uma elevada eficiência inibitória da colagenase semelhante à epigallocatequina galhato (93.1 ± 5.27%).

Em contraste com o que foi obtido nos ensaios enzimáticos da tirosinase e da colagenase, no ensaio enzimático de inibição da elastase (EC 3.4.21.36, protease de serina envolvida na quebra da elastina) não foi observada a diminuição da atividade enzimática após tratamento com os vinte e oito extratos de *Plectranthus* (30-42% de inibição enzimática). No entanto, os compostos isolados testados demonstraram uma elevada inibição. Particularmente, esta ação foi observada para a mistura de ácidos oleanólico:ursólico 1:4 (63.4 ± 2.56%) e para a Parviflorona D (52.8 ± 3.76%), em comparação com o ácido ursólico usado como controlo positivo (69.9 ± 3.65%).

De um modo geral, os extratos e/ou compostos isolados das sete espécies de *Plectranthus*, revelaram uma elevada capacidade, como redutores da híper pigmentação da pele (provocada pela atividade excessiva da tirosinase), e na manutenção da integridade dérmica, através da manutenção de colagénio e elastina, pela inibição da colagenase e da elastase. Assim, em conjunto com a atividade antioxidante contra a produção de ROS, e na inibição de enzimas relacionadas com a integridade dérmica e pigmentação, será possível o desenvolvimento de uma aplicação cosmética em ensaios futuros.

Por outro lado, a capacidade anti-inflamatória dos compostos foi avaliada através da inibição da produção de NO, usando a reação de Griess. Assim, após a avaliação da citotoxicidade dos compostos testados (em várias concentrações) através do ensaio MTT, foi medida a inflamação em células de macrófagos RAW 264.7. Após estimulação da inflamação celular com LPS, e tratamento com os compostos não tóxicos, este ensaio revelou que os compostos testados não foram capazes de reduzir a produção de NO (16-23 μM), em comparação com a quantidade de NO produzida naturalmente pelas células (17.7 ± 0.67 μM) e com o L-NAME, usado como controlo positivo (3.9 ± 0.2 μM). Uma vez que a inflamação

não é apenas proporcional à produção de NO, mas também de outros mediadores inflamatórios, será necessário expandir a investigação desta atividade biológica. De modo a compreender, se de facto, os compostos isolados de *Plectranthus* são anti-inflamatórios por outros mecanismos, como por exemplo, por inibição enzimática da ciclooxigenase-2 (COX-2).

Finalmente, foi avaliado o crescimento de *Mycobacterium tuberculosis* (Mtb) H37Rv após a infeção de macrófagos, derivados de células mononucleares do sangue periférico (PBMC). Numa primeira abordagem, o crescimento bacteriano foi monitorizado pela contagem de unidades formadoras de colónias (UFC), nas primeiras horas de infeção, e 13 dias após infeção e tratamento com os compostos selecionados. Após a normalização dos valores de UFC/mL, os resultados revelaram uma diminuição substancial de colónias de Mtb (na diluição 10^{-3}), apenas após o tratamento com um composto halimano obtido por hemi-síntese (2.1×10^5 UFC/mL).

Este resultado foi muito semelhante ao dos controlos positivos aplicados, isoniazida (1.2×10^5 UFC/mL) e etambutol (2.0×10^5 UFC/mL), sugerindo a possível aplicabilidade deste tipo de compostos em estudos futuros de terapia anti-tuberculose. Dado o resultado deste halimano na notável diminuição de colónias de Mtb, será relevante prosseguir na possível avaliação do mecanismo de ação que conduziu à inibição do crescimento bacteriano. Para tal, poder-se-ão aplicar técnicas de Western blot e imunofluorescência usando marcadores de autofagia, e avaliar a biogénese do fagolisossoma pelos macrófagos infetados.

No geral, de acordo com as referências consultadas, são aqui reportados os primeiros estudos em ensaios de inibição de enzimas relacionadas com a pele, na atividade anti-inflamatória por via do NO, e na atividade anti-tuberculose de extratos e compostos isolados de *Plectranthus*. Os resultados obtidos nesta dissertação fornecem uma validação científica preliminar, sobre os usos amplamente conhecidos e reportados, por estas plantas medicinais.

Na verdade, são necessários mais estudos na pesquisa de novos alvos terapêuticos mais específicos e com menos efeitos adversos, a partir das atividades etnofarmacológicas de *Plectranthus* reportadas até agora. Assim como validações científicas no uso de produtos à base de plantas usados no tratamento, cura e prevenção de doenças.

Uma vez que os produtos naturais representam uma fonte única de compostos protótipo no desenvolvimento de novos fármacos, os metabolitos secundários isolados de *Plectranthus* revelam neste estudo, o seu potencial no *design* de novos medicamentos.

PALAVRAS-CHAVE: *Plectranthus spp.; Anti-inflamatório; Antioxidante; Inibição enzimas da pele; Mycobacterium tuberculosis.*

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I. INTRODUCTION AND OBJECTIVES

I.1 Overview of natural products from medicinal plants

Natural products from medicinal plants have been used by indigenous populations for thousands of years, as traditional medicines, remedies, potions, and oils for the treatment of many ailments (Rijo *et al*., 2014a; Dias *et al*., 2012). For their vast ethnomedicinal and ethnopharmacological applications which inspired current research in drug discovery, natural products provide new and important leads against various pharmacological targets (Butler, 2004; Dias *et al*., 2012). In addition, it is well known that many of the drugs in the market have been developed from natural products (Rijo *et al*., 2013a; Pereira *et al*., 2015; Balunas and Kinghorn, 2005).

The 20th century was known for the intensive search for new compounds using chemical synthesis from most of multinational pharmaceutical companies. Natural products operations were dismissed or scaled down, despite the significant number of natural product-derived drugs, that reached phase III clinical trials (Butler, 2004; Dias *et al*., 2012; Mishra and Tiwari, 2011). However, the chemical synthesis approach did not fulfill the high expectations concerning the discovery of new lead alternatives, thus there has been a renewed interest on natural products, regarding their unique structural diversity (Butler, 2004; Cragg *et al*., 2008; Dias *et al*., 2012; Mishra and Tiwari, 2011; Rey-Ladino *et al*., 2011).

The most ancient records of natural products go back to the “Ebers Papyrus” dated 2900 B.C., the “Chinese Planta Medica” from 1100 B.C., and “Ayurveda Pharmacopoeia” from India more than 5000 years ago (Cragg and Newman, 2013; Samuelsson and Bohlin, 2010). The first was an Egyptian pharmaceutical document recording over 700 plants many still used today to alleviate cough, inflammation, and colds. These plants were to be used as gargles, infusions, ointments, and even pills. The second, documented over 100,000 prescriptions, plants practice, raw material, and most are still used today in Chinese Traditional Medicine (Cragg *et al*., 2008; Dias *et al*., 2012).

According to the World Health Organization (WHO), by 2012 about 80% of people still depended on traditional medicines as primary health care, and 80% of the 122 plant-based drugs were being used according to their ethnopharmacological use (Cragg and Newman, 2013; Dias *et al*., 2012). Medicines considered basic and essential by WHO are about 250, 11% of which are exclusively of vegetable origin. The vast majority of synthetic drugs originate from natural precursors, and over 60% of antitumor and anti-infective drugs are based on plants natural metabolites (Bandaranayake, 2006; Robbers et al., 1996; Rout et al., 2009).

Two well-known examples of drug discovery from natural products are acetylsalicylic acid (aspirin), obtained from salicin, a natural product isolated from *Salix alba* L. (Dias *et al*., 2012; Mishra and Tiwari, 2011), and morphine isolated from *Papaver somniferum* L. (Cragg and Newman, 2013; Cragg *et al*., 2008; Dias *et al*., 2012). Thus, plants can have an important

role on pharmacological research and drug development, not only when the bioactive compounds are directly used as therapeutic agents, but also when they are used as raw material for drug synthesis, or as a base model for new biologically active compounds (Mendonça-Filho, 2006; Swain, 1972).

However, validating and using plants as a phytopharmaceutical requires careful and exhaustive applied research, to set this resource at the same level of conventional pharmaceutical products (Batanouny *et al*., 1999). In order to keep up with the drug discovery market, the research on natural products needs to improve screening, isolation, and chemical characterization (Butler, 2004; Dias *et al*., 2012).

Since less than 10% of the world's biodiversity has been evaluated, many potential natural lead compounds await discovery of their biological activity (Dias *et al*., 2012). Thus, it is imperative to improve medicinal plants study, and learn from the traditional health practitioners that bear the knowledge of many generations of trial and error.

I.2 *Plectranthus* plants L' Héritier genus (*Lamiaceae*)

Plants from the *Plectranthus* L' Hérit. genus belong to the *Lamiaceae* family, and are also known as spur flowers (Rice *et al*., 2011). The *Lamiaceae* is well known for its biologically active essential oils, common to many family members, its ornamental and culinary herbs such as basil, lavender, mint, rosemary, sage, and thyme (Naghibi *et al*., 2005; Wagstaff *et al*., 1998).

Being easy to grow, there are about 350 species of *Plectranthus* widely distributed across the warm and tropical areas of Africa, Asia and Oceania. These plants were probably brought to the Mediterranean areas due to the 16th century Portuguese maritime discoveries (Rijo *et al*., 2014a; Rosa *et al*., 2015).

The genus *Plectranthus* was first described in 1788 by Charles L' Héritier a keen French botanist who named these plants after the Latin "*plecton"* meaning spur, and "*Anthos"* for flower (Rijo *et al*., 2013b). This particular genus is widely known for their biologically active essential oils regarding its aromatic nature (Rice *et al*., 2011), and so it is commonly used in traditional medicine (see Table 1). In fact stems, leaves, roots, and tubers are used to treat around thirteen categories of ailments as described in Economic Botany Data Collection Standard (Cook, 1995).

Some traditional application include uses as vermicides, antiseptics, purgatives, pain relief, and nausea (Rijo *et al*., 2005), making the *Plectranthus* plants the most recurrent medicinal plants cited in literature (Gaspar-Marques *et al*., 2006; Lukhoba *et al*., 2006). Generally, *Plectranthus* species are rich in essential oils having more than 0.5% of volatile oil on a dry weight basis, composed mainly of mono- and sesquiterpenes (Abdel-Mogib *et al*.,

2002). Also these plants are notorious as household (culinary, fly repellent, aromatic) ornament and for horticulture (gardens, bonsai, rockeries), since they are very aromatic, produce stunning flowers, and are plague resistant (Lukhoba *et al*., 2006; Rice *et al*., 2011).

Table 1 Traditional medicinal uses of the studied *Plectranthus* spp. plants

***Plectranthus* spp.**	**Ethnobotanical uses**	**Reference**
***P. madagascariensis* (Pers.) Benth.**	Cough; colds; asthma; scabies; small wounds; skin ailments; infection	(Lukhoba *et al*., 2006)
***P. grandidentatus* Gürke**	Blood circulation; infection	(Lukhoba *et al*., 2006)
***P. neochilus* Schltr.**	Hepatic insufficiency; dyspepsia; anti-helmintic; infection	(Gabriel *et al*., 2016; Lukhoba *et al*., 2006)
***P. ecklonii* Benth.**	Nausea; meningitis; tuberculosis; antifungal; headache; allergic rhinitis; skin ailments; gastrointestinal infections; malaria	(Rice *et al*., 2011)
***P. porcatus* van Jaarsv. & P.J.D.Winter**	Infections	(Lukhoba *et al*., 2006)
***P. prostratus* Gürke**	Genito-urinary system problems	(Lukhoba *et al*., 2006)
***P. ornatus* Codd.**	Digestive tract (stomach and liver complications); diuretic; pain; fever; inflammations; infections	(Lukhoba *et al*., 2006)
P. saccatus Benth.	Infections; fungicide; insect anti-feedant	(Rijo, 2011; Wellsow *et al*., 2006)

In Africa and Asia, *Plectranthus* are mostly used for digestive, pain, skin, and infections complaints whereas Caribbean populations exploited them for epilepsy symptoms relief (Lukhoba *et al*., 2006).

Plectranthus plants are very difficult to catalogue because of the ambiguous morphology criteria, resulting in copious taxonomic setback. Due to these issues, one *Plectranthus* plant can be recorded with many names thus preventing suitable research on ethnobotanical uses (Lukhoba *et al*., 2006). Helpful information about the medicinal uses of most *Plectranthus* plants can be found in online databases such as NAPRALERT, CAB-direct, SEPASAL and Kew Library Catalogue.

The worldwide interest in the use of medicinal plants has been growing, with its beneficial effects being rediscovered for the development of new drugs. Therefore, new therapeutic agents with specific targets and less adverse side effects, supports the need to extend the studies on *Plectranthus* plants.

I.2.1 Isolated compounds from *Plectranthus* plants

Several phytochemical studies on *Plectranthus* plants have described a great number of isolated compounds, and Figure 1 depicts the link between *Plectranthus* species and isolated compounds, with chemical structures and compounds number reported in this thesis.

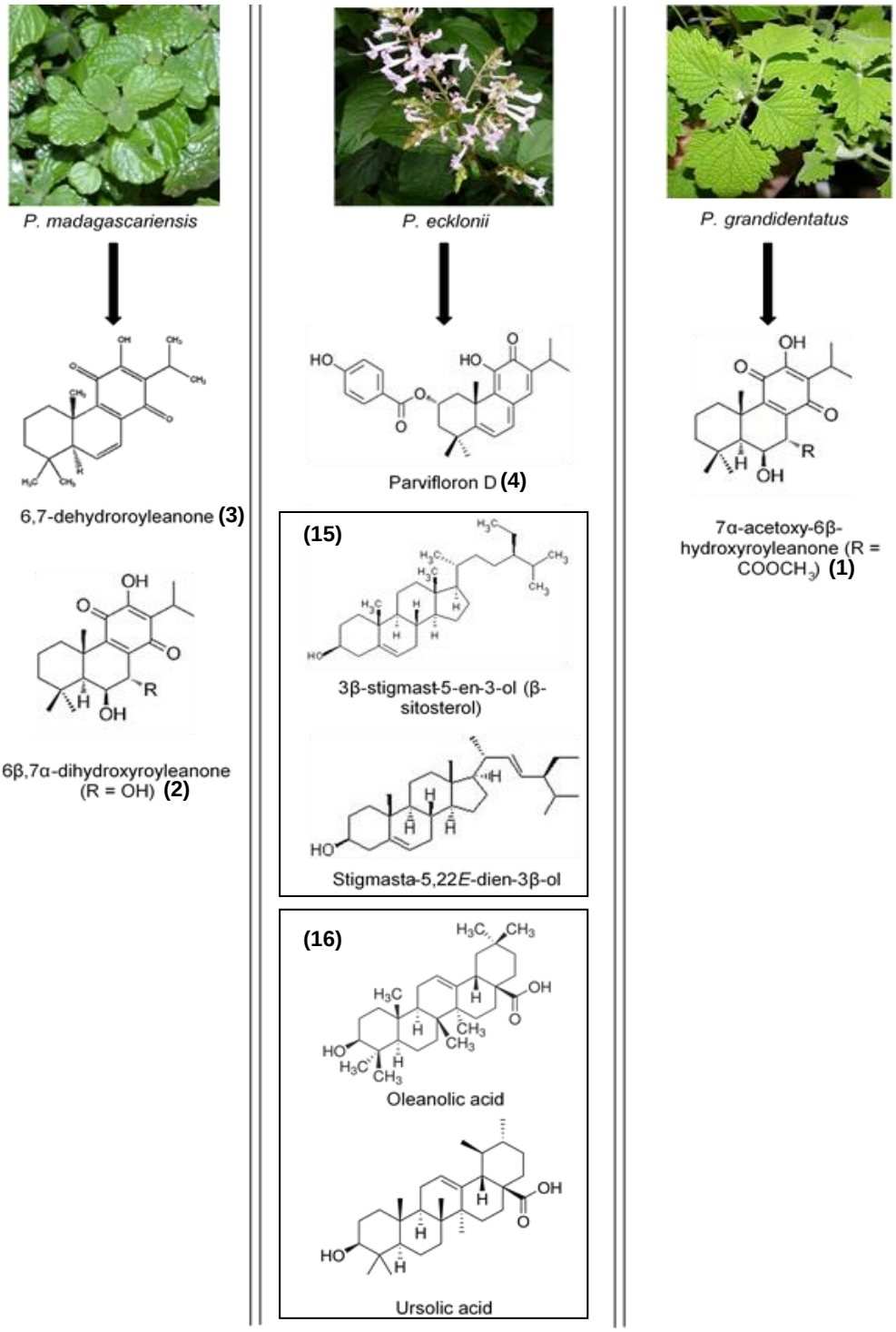
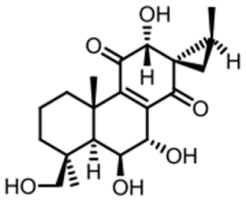


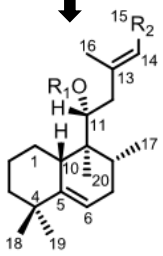
Figure 1 Studied *Plectranthus* plants species and related isolated compounds with internal identification numbers. Compounds **(15)** were studied in a 1:1 mixture and **(16)** in a 1:4 mixture. Plants pictures were obtained from (Van Jaarsveld E., 2006).

P. porcatus



(13*S*,15*S*)-6β,7α,12α,19-tetrahydroxy-13β,16-cyclo-8-abietene-11,14-dione **(6)**

P. ornatus

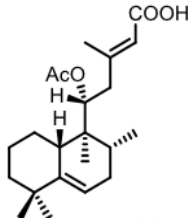


R1=Ac; R2= COOH
(11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid **(5)**

R1= OH; R2= $CH_2OCOCH_2CH_2CH_3$
(11*R**,13*E*)-15-butyryloxyhalima-5,13-dien-11-ol **(9)**

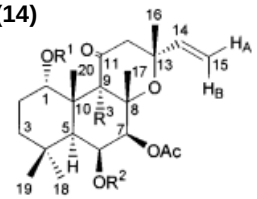
R1=OH; R2= CH_2OH
(11*R**,13*E*)-halima-5,13-diene-11,15-diol **(10)**

R1=Ac; R2=COOMe
(11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic methyl ester **(8)**



1α,6β-diacetoxy-8α,13*R**-epoxy-14-labden-11-one **(7)**

(14)

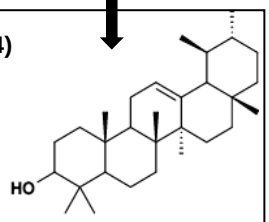


R1=R2=Ac; R3=OH
1,6-di-*O*-acetylforskolin

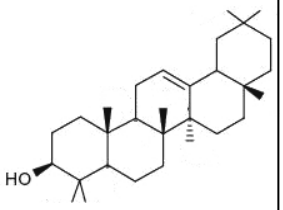
R1=R2=Ac; R3=H
1,6-di-*O*-acetyl-9-deoxyforskolin

P. neochilus

(14)

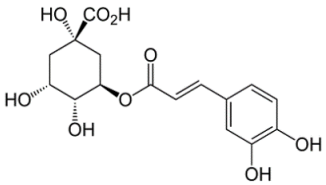


α-amyrin



β-amyrin

P. saccatus



Chlorogenic acid **(11)**

Figure 1 (Cont.) Studied *Plectranthus* plants species and related isolated compounds with internal identification numbers. Compounds **(14)** were studied in a 1:1 mixture and **(14)** in a 3:1 mixture. Plants pictures were obtained from (Van Jaarsveld E., 2006).

The main compounds found in *Plectranthus* plants are abietane, phyllocladane, kaurane, clerodane and labdane diterpenoids (Figure 1), together with long-chain alkylphenols, aristolane sesquiterpenes, ursane, oleane, and lupine triterpenois, flavonoid, and phenolic compounds (Naghibi *et al*., 2005; Rijo *et al*., 2005).

Terpenes are usually present in essential oils and resins, which include over 10,000 compounds and are divided into mono-, di-, tri- and sesquiterpenes, depending on the number of carbon atoms and isoprene (C_5H_8) groups (Doughari, 2012; Lovkova *et al*., 2001). *Lamiaceae* is one of the families where diterpenes have been found, and they can be divided in 91 skeletons, although the majority belong to the abietane skeleton (Rijo, 2011).

Isolation of diterpenes usually requires organic extracts together with maceration, ultrasound, or super critical fluid extraction methods (Rijo et al., 2012). For higher isolation efficacy, there should be an adequate selection of solvents with different polarities, according to the desired compounds (usually with greater scientific relevance like phenols, terpenes and flavonoids).

The most common organic solvents have intermediate polarity such as acetone, dichloromethane and ethyl acetate, or high polarity like methanol (Rijo et al., 2005, 2014a, 2014b; Waksmundzka-Hajnos and Sherma, 2011).

The often used maceration technique is long-lasting, environmentally unfriendly, hazardous and with some associated human toxicity. However, other extraction methodologies can overcome these problems and simultaneously extract the decomposable highly oxygenated terpenes contained in some *Plectranthus* plants (Marques *et al*., 2002).

For natural products extraction and isolation, supercritical fluid extraction (SCF), especially employing supercritical CO_2, has become a widespread choice. Cutting-edge technologies allow precise regulation of changes in temperature and pressure, and thus manipulation of the solvating property of the SCF. These settings enhance the quality of extraction of a wide range of natural products (Bernardo-Gil *et al*., 2011).

Structural identification of the metabolites is based on physicochemical and spectroscopic data, typically UV, IR, ^{1}H and, ^{13}C NMR spectra. Usually NMR can be further completed with 2D COSY, TOCSY, gHSQC, gHMBC, and 1D NOESY spectra, mass spectra, and elemental analysis (Maria Fátima Simões, 2010; Rijo *et al*., 2005).

I.2.1.1 Diterpenes

Diterpenes have a wide structural variability due to their singular roles in plant growth development, and in the resistance to environmental stress (Rijo *et al*., 2013b). These compounds have a hydrocarbon skeleton, mostly cyclic, and highly oxygenated with hydroxyl, carbonyl, and carboxylic groups in an aliphatic or aromatic framework (Rijo *et al*., 2014b; Maria Fátima Simões, 2010; Coutinho *et al*., 2009; Abdel-Mogib *et al*., 2002). It is the cyclization

method of the carbonated skeleton allied with the oxygenated groups that gives diterpenes the wide range of pharmacological abilities (Rijo *et al*., 2013b). Some of the most common diterpene skeletons can be found in Figure 2.

Due to their high boiling point, diterpenes are not considered as essential oils, but rather as a resin, the remaining material after steam distillation (Wang *et al*., 2005).

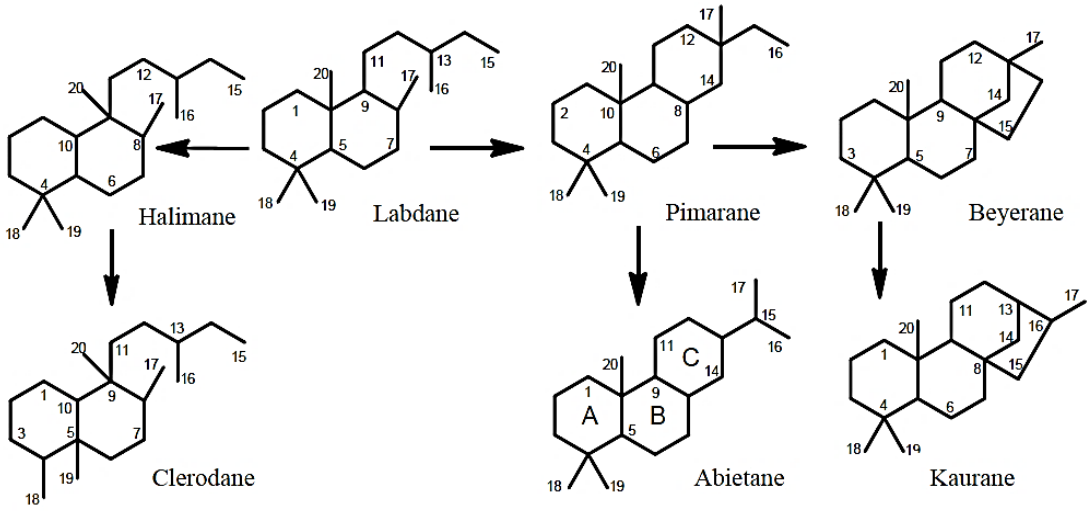


Figure 2 Pathway of the halimane, labdane, pimarane, beyerane, clerodane, abietane and kaurane diterpenoid skeletons, widely isolated from *Plectranthus* species, showing the numeration of the hydrocarbon skeleton. Adapted from (Rijo *et al*., 2013b).

I.2.1.1.1 Abietane diterpenes

Abietane diterpenes (mainly isolated from *P. madagascariensis*, *P. grandidentatus* and *P. ecklonii*, are the skeleton with the highest occurrence and most widespread in *Lamiaceae*.

Indeed the royleanone structure of abietane diterpenes, as well as its derivatives, are already extensively known for their antimicrobial, cytotoxic, antiproliferative, and antifungal activities (Rijo et al., 2013a, 2013b; Pereira et al., 2015; Rijo et al., 2014b; Burmistrova et al., 2015, 2013; Rijo et al., 2012).

Two of most the promising metabolites are Parvifloron D and 7α-acetoxy-6β-hydroxyroyleanone. Because of the oxidized abietane and phenol groups, these compounds show a wide range of biological activities (Figure 3). Biological activity has been improved by their derivatization into more bioactive compounds such as 6β,7α-dihydroxyroyleanone or ester derivatives (Rijo, 2011).

Parvifloron D, isolated from *P. ecklonii* is widely known for its toxicity, but has also been reported with high antioxidant power at *in vitro* assays, suggesting an interesting efficacy in converting free radicals into more stable products. However, these do not take into account the compound bioavailability or toxicity at *in vivo* systems (Rosa *et al*., 2015).

The quinone moiety present in abietane diterpenes compounds has been associated with antimycobacterial, antimicrobial, and antitumoral activities (Gaspar-Marques *et al*., 2006; Rijo, 2011; Rijo *et al*., 2010, 2014b). Often found in nature, quinones represent important features at stabilizing free radicals in many biological systems suffering from oxidative stress, protein inactivation through irreversible reactions enhancing antimicrobial activity, and intermediate melanin synthesis pathway in human skin (Rijo, 2011; Riley, 1997; Shadyro *et al*., 2002).

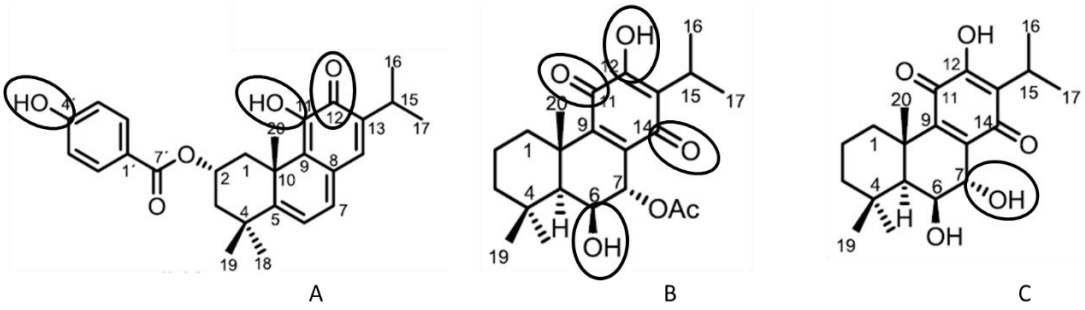


Figure 3 Structural representation of three abietane diterpenes, Parvifloron D (A), 7α-acetoxy-6β-hydroxyroyleanone (B), and 6β,7α-dihydroxyroyleanone (C). Here are also depicted some of the most important structural characteristics granting a wide biological activity. Adapted from (Rijo, 2011).

Structure activity relationships (SAR) analyses have shown that due to their amphipathic character, these diterpenes can damage bacteria cytoplasmatic membrane. Also, they can increase lipophilicity and/or promote hydrogen-bonding of the hydrophilic moiety (Rijo *et al*., 2014b). The cytotoxic and antiproliferative abilities against human tumor cells are probably due to their alkylating properties (Burmistrova *et al*., 2013, 2015).

Since royleanones can be present in many traditional remedies, more *in vitro* and *in vivo* studies should be performed, to understand their medicinal value consistent with the ethnopharmacological uses.

I.2.1.1.2 Labdane diterpenes

By 2005 the isolation of new diterpenes concerning the labdane skeleton was reported (Rijo *et al*., 2005). These diterpenes had a forskolin-like backbone (isolated from *P. ornatus* at ethyl acetate extract, see Figure 1), one of the most interesting compounds originally obtained from *Plectranthus barbatus* Andr. (also known as *Coleus forskohlii* Briq.) (Rijo *et al*., 2005, 2013b).

Forskolin compounds are specially notorious for several biological activities, regarding their cardiotonic, platelet aggregation inhibition, and anti-inflammatory properties (Klatz and Goldman, 2003; Rijo *et al*., 2005). Also, forskolin (Figure 4) has a catecholamine-like action by increasing intracellular cyclic adenosine monophosphate (cAMP), through the activation of adenylate cyclase. Previous studies suggest that this activation occurs in synergy with a wide

range of hormones and neurotransmitters (Bone and Mills, 2013). This interesting bioactivity leads to vasodilatory properties, and improved fat loss in double-blind clinical trials (Bone and Mills, 2013; Hayashida *et al*., 2001; Pizzorno and Murray, 2013).

Regarding the presence of non-volatile compounds, 1,6-di-*O*-acetylforskolin (Figure 4B) is obtained by acetylation of forskolin, whereas 1,6-di-*O*-acetyl-9-deoxyforskolin (Figure 4C) is an intermediate of forskolin, starting with 9-deoxyforskolin. Although the potential therapeutic benefit of forskolin-like compounds, there is a concern about the use of these products on drug metabolizing enzymes in the liver (Lukhoba *et al*., 2006).

Another labdane diterpene is Plectrornatin C depicted in Figure 4D, that was isolated from the acetone extract of *P. ornatus*. This compound was previously demonstrated to have moderate antifungal activity (Rijo *et al*., 2002), and cyclooxygenase-2 (COX-2) inhibition by 59% at a concentration of 0.6 mM (Rijo, 2011).

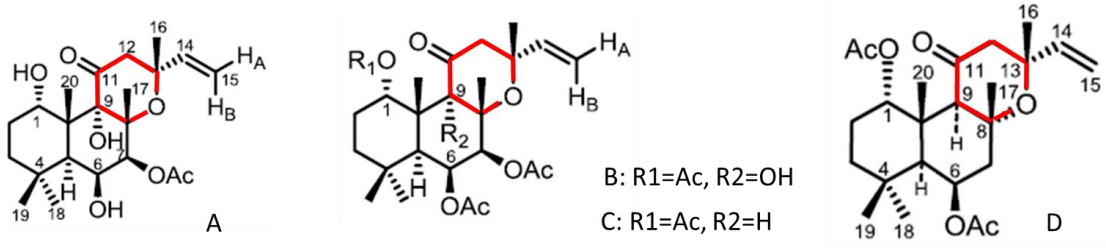


Figure 4 Resemblance of the chemical structures of some labdane diterpenes with relevance for Forskolin (A), 1,6-di-O-acetylforskolin (B), 1,6-di-O-acetyl-9-deoxyforskolin (C), and Plectrornatine C (D). Here are also depicted the flavanone ring (in red) granting the labdanes a wide biological activity. Adapted from (Rijo, 2011).

As seen in Figure 4, some labdane diterpenes (A-D) seem also to be related with flavanones. Flavanones have been a promising source for lead compounds as the focus of many researches for biologically active components. Indeed, they were reported to have significant cytotoxic activity against leukemia and lung carcinoma cells, senescence, and cardiovascular diseases (Ketabforoosh *et al*., 2014; Shi *et al*., 2010). However, they are rarely present in natural sources, which limits the flavanone biological applications, and realizing their existence in *Plectranthus* spp. plants could bring a new light into labdane pharmacological activities.

I.2.1.1.3 Halimane diterpenes

Diterpenes with a halimane skeleton are mainly isolated from the acetone extract of *P. ornatus* (see Figure 1Figure), and still have some unknown pharmacological properties. Considering halimane biosynthesis, they appear to be related to the labdanes by a series of methyl and hydride shifts (Silva *et al*., 2011).

Unfortunately, studies on halimane biological activities are scarce. In 2011 some halimane derivatives derived from the halimane diterpene A (Figure 5), namely halimane diterpenes B-D, were reported to be strong inhibitors of COX-2 in a range of 59 to 79% (Rijo, 2011).

More recently, new findings regarding tuberculosis, namely the pathogenic strain of *Mycobacterium tuberculosis*, reported the existence of tuberculosinols (Nakano *et al*., 2011), very similar in structure to halimane diterpenes. Tuberculosinols have been reported to suppress phagosome maturation, and inhibit phagocytosis by macrophage cells (Hoshino *et al*., 2011). More details will be discussed in Section I.3.4.

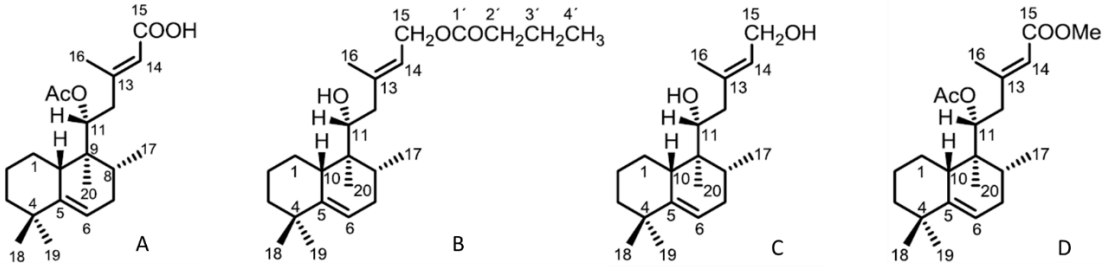


Figure 5 Chemical structures of some halimane diterpenes. (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid (A), (11*R**,13*E*)-15-butyryloxyhalima-5,13-dien-11-ol (B), (11*R**,13*E*)-halima-5,13-diene-11,15-diol (C), and (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid methyl ester (D). Compounds B-D are derivatives from A. Adapted from (Rijo, 2011).

I.2.1.2 Triterpenes and phytosterols

Triterpenes and sterols are two types of compounds that genetically derive from the same squalene precursor, whose cyclic structures comprise *trans* or *cis* cyclohexane and cyclopropane units (Wink, 2010). Distinguishing triterpenes from sterols can be hard since they share common structural characteristics, therefore one possible way is by identifying their synthetic routes (Wink, 2010). Whereas sterols can be present in the *Animalia*, triterpenes are unique of the *Plantae* kingdom (Gabay *et al*., 2010).

Pentacyclic triterpenes such as ursolic and oleanolic acids (Figure 6A and 6B), α- and β-amyrin (Figure 6C and 6D), can be isolated from *P. neochilus*, *P. ornatus*, and *P. ecklonii*, using acetone or hexane extracts (Gabriel *et al*., 2016; Rijo *et al*., 2005; Simões *et al*., 2010). Oleanolic and ursolic acids are usually isolated simultaneously and are isomers that only differ on the methyl group at C-29 as depicted in Figure 6.

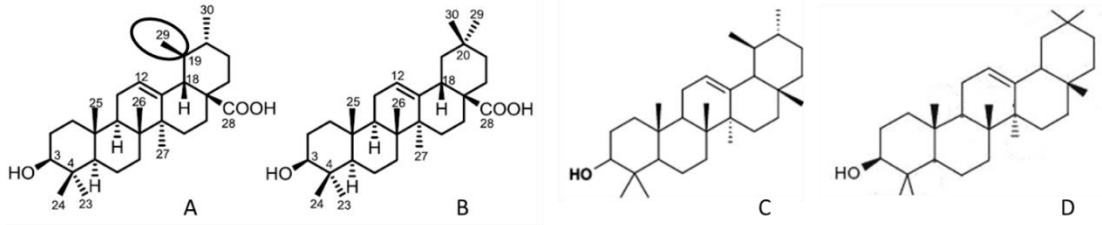


Figure 6 Chemical structure of ursolic (A) and oleanolic (B) acids and their characteristic difference at C-29, α-amyrin (C) and β-amyrin (D). Adapted from (Rijo, 2011).

The triterpenoids α- and β-amyrins are known for their antinociceptive properties, that have been demonstrated in several *in vivo* models (Gabriel *et al*., 2016; Rijo *et al*., 2005). Although the mechanisms of action are not yet fully understood, it is considered that they are independent from important endogenous systems (like opioidergic, serotoninergic and

noradrenergic), stabilizing the action of mast cell membranes involved in inflammation process (Backhouse *et al*., 2008).

Oleanolic and ursolic acids have proven efficacy as antioxidant regarding radical scavenging and superoxide decrease (Ghimeray *et al*., 2015; Hwang *et al*., 2014), antimicrobial activities (Maria Fátima Simões, 2010), anti-inflammatory, antitumor including inhibition of skin tumorigenesis (Cha *et al*., 1998), and as potent elastase and collagenase inhibitors (Lee *et al*., 2001; Thring *et al*., 2009; Tu and Tawata, 2015; Ying *et al*., 1991).

Phytosterols are notorious for their anti-inflammatory properties (Backhouse *et al*., 2008). These compounds are synthesized by the mevalonate pathway of terpenoid formation, and can be regarded as direct precursors of steroid saponins, alkaloids, pregnanes, androstanes, that share the same backbone (Wink, 2010).

Two examples of these compounds that can be isolated from *Plectranthus* plants, usually from *P. ecklonii* (Figure 1), are β-sitosterol and stigmasterol. These have been validated for anti-inflammatory and analgesic purposes (Backhouse *et al*., 2008; Ghimeray *et al*., 2015), although it was previously though that stigmasterol could not be considered as major anti-inflammatory agent (García *et al*., 1999). Nonetheless, both compounds distinctly reduce myeloperoxydase activity influenced by the neutrophil migration inhibition into inflamed tissue (De la Puerta *et al*., 2000; García *et al*., 1999; Villaseñor *et al*., 2002, 2004)

Furthermore, β-sitosterol was proven to inhibit human prostate cancer cells growth, revealing an effectiveness in apoptosis induction regarding prostaglandin production (Awad *et al*., 2005). According to the investigation held by Nashed et al. (Nashed et al., 2005), phytosterols are strongly associated with reduced plasma cholesterol concentrations and atherosclerosis lesions by 20% and 60% respectively, together with lowering of pro-inflammatory cytokines (Ghimeray et al., 2015).

I.2.1.3 Polyphenolic compounds

Besides diterpenes, *Plectranthus* plants are rich in phenolic compounds (mainly isolated from aqueous extracts, see Figure 1). Polyphenols are chemical compounds primarily responsible for the fruit coloring (Doughari, 2012). These are classified as phenolic acids, flavonoids and non-flavonoid (Doughari, 2012). In addition to their antioxidant properties, they play a very important role in the plant defenses against herbivores, pathogens and predators; therefore they have an application in the control of human agents infections (Doughari, 2012).

Polyphenols, such as rosmarinic acid ubiquous to all *Plectranthus* spp., and chlorogenic acid obtained mainly from *P. saccatus* (Rijo *et al*., 2014a), are present in aqueous fractions using decoction, infusion microwave or ultrasound extraction methods (Rijo et al., 2012, 2014a). Overall polyphenols are present in the diet and are known for their antioxidant,

antimicrobial, and anti-inflammatory properties (Ghimeray *et al*., 2015; Pereira *et al*., 2015; Sergent *et al*., 2010).

I.3 Biological activities of *Plectranthus* natural products

In line with the literature, the most common compounds in *Plectranthus* are pimarane, labdane, neoclerodane, halimane, and abietane diterpenes, and they have been extensively studied for their biological activity (Rijo *et al*., 2013b).

Previous studies on the secondary metabolites samples used in this project, were reported in partial studies regarding their medicinal use, although the pharmacological properties of some compounds remain unknown (Rijo *et al*., 2013c).

Although many compounds seem to have great potential according to their biological activities as described in Table 2, research focused on the screening and scientific validation of *Plectranthus* plants are still half-way.

The next sections will review in more detail the state of the art concerning the studied biological activities of *Plectranthus* secondary metabolites within this thesis.

Table 2 Terpene, phytosterols, and polyphenols isolated from the described *Plectranthus* spp. plants, and their main biological activities.

Plant material	Isolated compounds	Biological activities	References
P. madagascariensis **(PM)**	6,7-dehydroroyleanone	Antibacterial (Gram-positive, MRSA and VRE)	(Ascensão *et al*., 1998; Rijo *et al*., 2014b)
	6β,7α-dihydroxyroyleanone	Antibacterial (Gram-positive, MRSA and VRE)	(Kubínová *et al*., 2014; Rijo *et al*., 2014b)
P. ecklonii **(PE)**	Parvifloron D	Cytotoxic Antioxidant	(Burmistrova *et al*., 2013; Rosa *et al*., 2015; Simões *et al*., 2010)
	3β-stigmast-5-en-3-ol Stigmasta-5,22*E*-dien-3β-ol Oleanolic acid Ursolic acid	Antimicrobial; Apoptotic; Antinociceptive; Antioxidant	(Ghimeray *et al*., 2015; Simões *et al*., 2010)
P. grandidentatus **(PG)**	7α-acetoxy-6β-hydroxyroyleanone	Antibacterial (Gram-positive, MRSA and VRE); Mild cytotoxicity	(Rijo *et al*., 2014b)
P. porcatus **(PP)**	(13S,15S)-6β,7α,12α,19-tetrahydroxy-13β,16-cyclo-8-abietene-11,14-dione	Antimicrobial	(Maria Fátima Simões, 2010)

Table 2 (Cont.) Terpene, phytosterols, and polyphenols isolated from the described *Plectranthus* spp. plants, and their main biological activities.

Plant material	Isolated compounds	Biological activities	References
***P. ornatus* (PO)**	(11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid (11*R**,13*E*)-15-butyryloxyhalima-5,13-dien-11-ol (11*R**,13*E*)-halima-5,13-diene-11,15-diol (11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic methyl ester	Antimicrobial (MRSA); Antimycobacterial	(Layre *et al*., 2014; Mann and Peters, 2012; Nakano *et al*., 2011; Rijo *et al*., 2011)
	1α,6β-diacetoxy-8α,13*R**-epoxy-14-labden-11-one	Antibacterial	(Rijo *et al*., 2013a)
	1,6-di-*O*-acetylforskolin 1,6-di-*O*-acetyl-9-deoxyforskolin	Anti-inflammatory	(Rijo *et al*., 2005)
***P. neochilus* (PN)** ***P. ornatus* (PO)**	α-amyrin β-amyrin	Cytotoxic; Anti-inflammatory; Analgesic	(Backhouse *et al*., 2008; Gabriel *et al*., 2016; Rijo *et al*., 2005)
***P. saccatus* (PS)**	Chlorogenic acid	Antioxidant; Antimicrobial Anti-acetylcholinesterase; tyrosinase; collagenase; elastase; Anti-inflammatory	(Falé *et al*., 2012, 2011; Ghimeray *et al*., 2015; Pereira *et al*., 2015)
***Plectranthus* spp.**	Rosmarinic acid	Antioxidant; Antimicrobial; Anti-acetylcholinesterase; Anti-inflammatory	(Falé *et al*., 2012, 2011; Ghimeray *et al*., 2015; Pereira *et al*., 2015)

I.3.1 Antioxidant Activity

Reactive oxygen species (ROS) are mainly produced at mitochondrial oxidative phosphorylation, in cellular response to xenobiotics, cytokines, and bacterial attack. Therefore, oxidative stress refers to the imbalance of ROS over the cell ability to have an effective antioxidant response (Ray *et al*., 2012).

Although a natural physiological process in biologic systems, the consequences of oxidative stress are present in many pathologies such as cancer, inflammation, and neurodegenerative disorders, renewing the interest to discover new antioxidant molecules (Rosa *et al*., 2015). When ROS are extensively generated, such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$), redox-active metal ions like Fe^{2+} or Cu^{2+} originate harmful products from reacting rapidly with lipids, DNA and proteins and thus resulting in mutagenesis and enzymatic denaturation (Dhawan, 2014; Ray *et al*., 2012; Thring *et al*., 2009). Antioxidants are known to interfere with oxidative reactions either by reacting with

free radicals and chelating catalytic metals, or by acting as oxygen scavengers (Nsimba *et al*., 2008). However, the most used antioxidants such as butylated hydroxyanisole (BHA) or propyl gallate, are suspected to induce liver damage and carcinogenesis in animals (Nsimba *et al*., 2008). Therefore, it is widely important to discover new and eventually more powerful and less cytotoxic antioxidant agents from natural sources, such as *Plectranthus* plants.

Polyphenols are powerful ROS scavengers, therefore they work as the most powerful antioxidants (Wahab *et al*., 2014) and they can be found in many plants, including *Plectranthus* (Rijo et al., 2012; Rosa et al., 2015). These plant-derived compounds exhibit a wide range of biological effects as antioxidants, such as anti-ageing, vitamin C protection and reduction of α-tocopheryl radical (Wahab *et al*., 2014; Rijo *et al*., 2014b).

The phenol groups present in rosmarinic and chlorogenic acids, can form relatively stable phenoxyl radicals, thereby disrupting chain oxidation reactions in cellular components (Pandey and Rizvi, 2009).

Also, abietane diterpenes, Parvifloron D and 7α-acetoxy-6β-hydroxyroyleanone have been proved to have the capacity to chelate the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. Parvifloron D (IC_{50} 0.11 ± 0.018 mM) was found to be a much stronger antioxidant than 7α-acetoxy-6β-hydroxyroyleanone (IC_{50} 1.85 ± 0.071 mM), when compared to the positive control quercetin (IC_{50} 0.0075 ± 0.0010 mM) (Rijo, 2011; Rosa *et al*., 2015). Besides, Parvifloron D can protect DNA from oxidative stress by plasmid DNA cleavage assay in the presence of H_2O_2 and Fe (II) (Rosa *et al*., 2015).

Since one of the antioxidant mechanisms is the capacity to scavenge the free radicals that are formed during carcinogenesis, neurodegeneration, diabetes, inflammation, and ageing processes, an antioxidant agent can be useful as an adjuvant therapy (Falé *et al*., 2011; Ray *et al*., 2012).

Also, compounds associated with reduced levels of oxidative stress, are supposed to have skin-protective properties, through the inhibition of natural skin-related enzymes such as tyrosinase, elastase, and collagenase (Chen *et al*., 2015; Ghimeray *et al*., 2015). Further elucidation will be given throughout section I.3.2.

I.3.2 Inhibition of skin-related enzymes

The skin is one of the most important organs of the body, since it provides protection from the external environment, and from any damage caused by the UV-light, chemicals, and microorganisms (Moon *et al*., 2010; Thring *et al*., 2011).

There are many forms of skin ageing, but one the most common stress-inducing factor is exposure to UV irradiation, that disturbs the intrinsic pathways involving elasticity and pigmentation changes (Wahab *et al*., 2014; Thring *et al*., 2011; Moon *et al*., 2010). Solar

exposure causes ultimately the formation of lipid peroxides, and ROS that can overcome the endogenous cellular antioxidant system, thus induce proteinases capable of changing the matrix (Thring *et al*., 2009). Moreover, skin disorders are associated with increased oxidative stress, that enhances enzymatic activity such as tyrosinase, elastase, and collagenase (Ghimeray *et al*., 2015; Vallisuta *et al*., 2014). Therefore, there is an interest to identify natural antioxidants that can be inhibitors of these enzymes to fight against skin ageing and hyperpigmentation (Popoola *et al*., 2015; Tu and Tawata, 2015).

I.3.2.1 Enzymes in skin pigmentation and ageing – Tyrosinase, elastase, and collagenase

Tyrosinase (EC 1.14.18.1) is a bi-nuclear copper monooxygenase that catalyzes the first two stages of melanin biosynthesis. The copper ions are coordinated with the histidine residues on the active site, and are critical for catalytic activity (Chen *et al*., 2015; Popoola *et al*., 2015). In the initial step, L-tyrosine is hydroxylated to L-3,4-dihydroxyphenylalanine (also known as L-DOPA), that is further oxidized to the corresponding dopaquinone. The oxidative polymerization of several dopaquinone derivatives originates melanin (Masuda *et al*., 2009; Tu and Tawata, 2015), thus tyrosine is considered to be the rate-limiting regulator (Tu and Tawata, 2015).

Melanin is an important cellular component responsible for skin color, and has photo-protective properties against harmful effects of UV radiation in normal skin pigmentation. In contrast, abnormal pigmentation caused by formation of excessive ROS can lead to senile lentigines, feckles, and melasma (Chen *et al*., 2015; Tu and Tawata, 2015).

Many tyrosinase inhibitors such as hydroquinone, kojic acid (IC_{50} 0.03 mM (Masuda *et al*., 2009)), azelaic acid, electron-rich phenols, and arbutin (IC_{50} 83.3 mM (Masuda *et al*., 2009)), have been reported for their capability of inhibiting melanin overproduction (Chen *et al*., 2015). However, due to their adverse side effects, low formulation stability, and poor skin penetration their use is limited, hence the search for new agents from natural products. In fact, quercetin, a strong antioxidant compound, has been reported to highly inhibit tyrosinase activity (IC_{50} 0.1 mM (Masuda *et al*., 2009)).

Besides hyperpigmentation, UV irradiation can also cause changes in the extracellular matrix (ECM) by the breakdown of collagen, elastin, proteoglycans, and fibronectin, thus damaging skin collagenous tissues (Jung *et al*., 2014). It is believed that ROS can upregulate expression of several matrix metalloproteinases (MMP), and decrease the synthesis of ECM proteins such as collagen and elastin. In Figure 7 two signaling pathways are outlined according to previous studies.

Collagenase (EC 3.4.24.3) and elastase (EC 3.4.21.36) are two of the main enzymes responsible for breaking down of collagen and elastin, the major components of the connective tissue skin. Skin weight contains 70-80% of collagen to provide structural stability, and 2-4% of elastin to maintain skin elasticity (Wahab *et al*., 2014). These two ECM under the epidermis comprise fibers networks granting tensile strength of the skin the mechanical properties of the connective tissue (Moon *et al*., 2010; Thring *et al*., 2009; Vallisuta *et al*., 2014; Wahab *et al*., 2014).

Since many plants contain a wide variety of polyphenols, recent studies reported that these antioxidant compounds are able to protect skin against the damaging effects of ROS, such as photo-ageing, sagging skin, and skin-ageing (Wahab *et al*., 2014). Triterpenoids isolated from *Boswellia* spp., and resin and extracts from *Rosmarinus officinalis* have also been shown to have anti-elastase and anti-collagenase activity. Indeed, oleanolic and ursolic acids are known inhibitors of elastase (ursolic acid IC_{50} 31 µg/mL), and collagenase (oleanolic acid IC_{50} 21 µg/mL) (Baylac and Racine, 2004; Barrantes and Guinea, 2003; Thring *et al*., 2009). It is believed that triterpenes bind reversibly to elastase and collagenase catalytic sites, since there is an activity recovery upon dilution of the enzyme-inhibitor mixture. Other triterpenes belonging to α- and β-amyrins have also been assessed, and the similar pentacyclic structure, revealed the same inhibitory effect as ursolic and oleanolic acids (Ying *et al*., 1991).

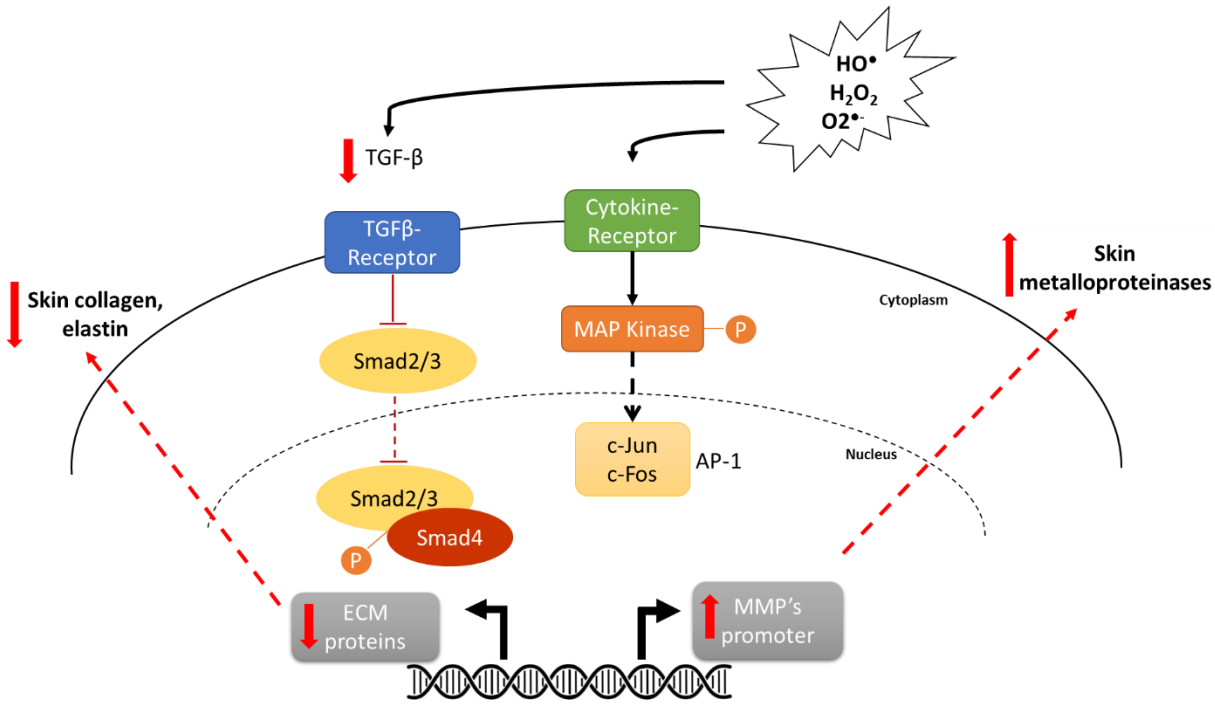


Figure 7 Signaling pathway induced by ROS causing skin damaging. ROS up-regulated mitogen-activated protein kinases (MAPK) cascades that will enhance transcriptional activity of AP-1 heterodimer comprised of c-Jun and c-Fos, thus increasing metalloproteinases (MMP) expression. In addition, TGF-β/Smad signaling pathway is down-regulated by the over accumulation of ROS, decreasing the synthesis of major ECM proteins such as collagen and/or elastin.

The biochemistry of collagenase has been reported to the catalytic domain composed by an active zinc (Zn^{2+}) atom linked to three aminoacid side chains, with a fourth coordination site occupied by a water-binding atom (Nsimba *et al*., 2008; Thring *et al*., 2009).

Elastase is a member of the serine protease enzyme family that breaks down elastin. Damage to the skin results in reduced skin elasticity and the loss of linearity of dermal elastic fibers, thus inducing wrinkling and sagging. Therefore, certain elastase inhibitors have been utilized for dermatological preparations to reduce the wrinkling and aging of skin (Vallisuta *et al*., 2014).

Many phenolic compounds, such as catechin and epigallocatechin gallate (EGCG) have been reported to be not only metal chelators, thus inhibiting collagenase by making Zn^{2+} unavailable (Jung *et al*., 2014; Thring *et al*., 2009), but also the hydroxyl and/or benzene group of polyphenols can form hydrogen bonds or hydrophobic interaction with elastase functional groups (Wahab *et al*., 2014). Most compounds contain carboxylic, hydroxamic, or sulfonamide groups that coordinate the zinc ion in the active site of the MMP using two main binding modes: hydrogen bonding and electrostatic interactions (Nsimba *et al*., 2008).

The bioactive inhibitors in these enzymes can act by inducing conformational changes, metal-chelation, non-covalent bonding, or by reducing radicals from UV stimulation (Masuda *et al*., 2009; Wahab *et al*., 2014).

Concerning *Plectranthus* antioxidant potential, the search for non-cytotoxic compounds and skin permeable for new anti-ageing, anti-sagging, and anti-hyperpigmentation skin, should be of great importance. Consequently, there should be an enhanced interest to study bioactivity that might be useful in modern formulations (Moon *et al*., 2010; Thring *et al*., 2009).

I.3.2.2 Acetylcholinesterase in the skin – Non-neuronal cholinergic system

Acetylcholinesterase (EC 3.1.17) is involved in the termination of impulse transmission by rapid hydrolysis of the neurotransmitter acetylcholine (ACh) (Čolović *et al*., 2013). This reaction is most commonly known in numerous cholinergic pathways in the central and peripheral nervous systems. The enzyme inactivation, induced by various inhibitors, leads to acetylcholine accumulation, hyperstimulation of nicotinic and muscarinic receptors, and disrupted neurotransmission (Čolović *et al*., 2013; Filho *et al*., 2006).

Acetylcholinesterase (AChE) is a serine hydrolase found in many types of conducting tissue: nerve and muscle, central and peripheral tissues, motor and sensory fibers, and cholinergic and non-cholinergic fibers (Čolović *et al*., 2013).

Reversible AChE inhibitors play an important role in pharmacological manipulation of the enzyme activity. These inhibitors include compounds with different functional groups

(carbamate, quaternary or tertiary ammonium group), and have been applied in the diagnostic and/or treatment of various diseases such as *myasthenia gravis*, Alzheimer disease, post-operative ileus, bladder distention, glaucoma, as well as antidote to anticholinergic overdose (Čolović *et al*., 2013; Valasani *et al*., 2013).

During the past years it has become apparent that ACh is far from being exclusive of the nervous system (Kirkpatrick *et al*., 2001). In 2006 Schlereth demonstrated for the first time the *in vivo* release of non-neuronal acetylcholine from the human skin by dermal microdialysis (Schlereth *et al*., 2006).

Several studies have been performed reporting that the human skin contains resident and transiently residing cells part of the extra- or non-neuronal cholinergic system, establishing the skin as active source and target of neurotransmitters and hormones (Kurzen *et al*., 2007; Schlereth *et al*., 2006). The non-neuronal cholinergic system is implicated in many skin functions, such as growth, differentiation, adhesion and motility, and barrier formation.

Indeed, ACh is produced in keratinocytes, endothelial cells and most notably in immune competent cells invading the skin at sites of inflammation (Kurzen *et al*., 2007). Therefore, there has been an increased interest on the non-neuronal cholinergic system as a regulator of skin physiology, and pathophysiology (Kurzen *et al*., 2007; Schlereth *et al*., 2006).

The first report on the skin non-neuronal production of ACh goes back to 1983, when Mark and colleagues performed parasympathetic denervation of the rat parotid gland, and still registered a continued production of high amounts of ACh in salivary glands (Mark *et al*., 1983). More recently, there was a study reporting skin lesions and reactions after therapy with acetylcholinesterase inhibitors (Golüke *et al*., 2014), linking with the literature information.

Due to the underestimated endocrine action of choline on ACh receptors present in the different non-neuronal cholinergic systems, there are few reports on the effects of choline deficiency or excess on skin physiology (Kurzen *et al*., 2007). However, through the recent years many studies have been developed to better understand the role of the non-neuronal cholinergic system on inflammatory skin diseases (Grando *et al*., 2012).

Previous studies have proven the capacity of some *Plectranthus* extracts, as well as of some of their isolated compounds, to inhibit AChE. In fact, aqueous extracts of *P. ecklonii*, *P. grandidentatus*, *P. ornatus*, *P. porcatus*, and *P. saccatus* resulted in high AChE inhibition probably because they are also the ones with the highest content in rosmarinic acid, suggesting that this compound may be responsible for the reported activity (Rijo *et al*., 2014a).

Moreover, rosmarinic acid was reported with AChE inhibition activity (IC_{50} of 527.8 ± 27.7 µM) (Kubínová *et al*., 2014). Being the main compound in the extracts of *Plectranthus* species, it would be very interesting to focus on less known *Plectranthus* spp. constituents, and search for AChE inhibition biological activity.

I.3.3 Anti-inflammatory activity

Drugs in the market such as nonsteroidal anti-inflammatory drugs (NSAID) have a number of adverse side effects, including gastrointestinal discomfort, inhibition of platelet aggregation, and liver and kidney toxicity (Rijo *et al*., 2013a; Crofford, 2013).

Many inflammatory diseases are associated with the synthesis of prostaglandins, which are also responsible for the pain and fever outcomes. The primary enzyme responsible for prostaglandins synthesis is the membrane-associated cyclooxygenase (COX) (Matu and van Staden, 2003).

The cyclooxygenase enzyme has two isoforms, the constitutive enzyme COX-1 responsible for the synthesis of pro-aggregate thromboxane in blood platelets, and the inducible enzyme COX-2 that synthetizes prostaglandins in acute inflammation, and is cytokine-inducible and expressed mainly in a wide range of inflammatory cells (Huss *et al*., 2002; Rijo *et al*., 2013a).

Modulation of the activity of the enzyme by NSAID implies that the inflammation process can be modified (Amessis-Ouchemoukh *et al*., 2014; Huss *et al*., 2002). Being a bi-functional enzyme, COX first catalyzes oxygen to arachidonic acid (AA) to produce hydroperoxide prostaglandin G_2, and then by a peroxidase reaction, reduces the hydroperoxide to an alcohol (Amessis-Ouchemoukh *et al*., 2014).

Celecoxib, for example, is a selective COX-2 inhibitor (IC_{50} values of 40 nM for COX-2 and 15 μM for COX-1(Cao *et al*., 2010)), and has an improved safety profile in comparison with traditional NSAID that inhibit both cyclooxygenases (Huss *et al*., 2002; Ong *et al*., 2007; Rijo *et al*., 2013a).

A variety of *in vitro* methodologies have been used to assess selective COX-2 inhibitors, is schematized in Figure 8. One method examines the effect of inhibitors on mRNA and protein levels, along with the effect of enzymatic activity, using cell-based assays. Another strategy involves identifying inhibitors that affect the isolated enzyme. The latter can be done either continuously, performing oxygen measurements, or non-continuously, using a stop-time assay to detect the produced prostaglandins through methods such as RIA, ELISA, HPLC, or radiotracer (Huss *et al*., 2002).

Also, the *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine (TMPD) oxidation assay has been used to evaluate potential new COX inhibitors in a microplate format. TMPD is a high oxidizable compound that serves as a reducing co-substrate for heme peroxidases. This works as an artificial electron donor that undergoes co-oxidation by PGG_2 producing a blue product, detected at 590 nm (Petrovic and Murray, 2010).

Although this is an indirect method, the oxidation of TMPD has been shown to accurately reflect the rate of conversion of arachidonic acid to PGH_2 (Petrovic and Murray, 2010).

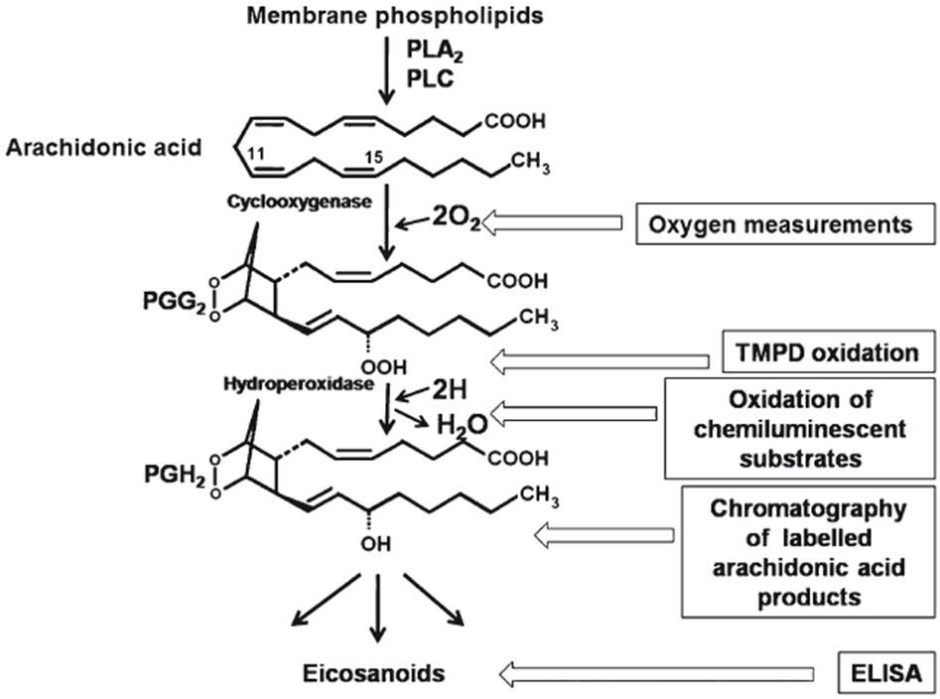


Figure 8 Cyclooxygenases metabolic pathway and most common strategies to evaluate COX inhibition. PLA_2 –Phospholipases A_2; PLC - Phospholipases C; PGG_2 – Prostaglandin G_2; PGH_2 –Prostaglandin H_2; TMPD - *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine; ELISA -Enzyme-linked immunoassay. Adapted from (Petrovic and Murray, 2010).

Under inflammatory pathogenesis nitric oxide (NO) is one of the key signaling molecules by over-expression of inducible NO synthase in macrophages and neutrophils (Sharma *et al*., 2008; Wang and Leigh, 2006). This over-expression is caused by pro-inflammatory mediators such as IFN-γ, TNF-α, leukotrienes, and most prostaglandins produced by COX enzymes (Coleman, 2001).

However, the best recognized inducer of inflammation is lipopolysaccharide (LPS) of *Escherichia coli*, that develops systemic inflammatory response syndrome (SIRS) in the course of sepsis due to Gram-negative bacteria (Guzik *et al*., 2003).

NO is synthesized by three isoforms of the nitric oxide synthase (NOS): endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (Boora *et al*., 2014). iNOS is constitutively expressed only in some tissues, but is synthesized in response to inflammatory or pro-inflammatory mediators (Cirino *et al*., 2006; Coleman, 2001; Dawson and Dawson, 1995; Guzik *et al*., 2003).

Nitric oxide is produced from L-arginine aminoacid and oxygen by conversion into NO and L-citrulline through a five-electron oxidative process (Scheme 1) (Boora *et al*., 2014; Dhawan, 2014). It is a highly reactive molecule with one unpaired electron, therefore considered a free radical (Dhawan, 2014; Sharma *et al*., 2008). The production of NO requires the presence of many cofactors such as FAD, FMN, NADPH, tetrahydrobiopterin, and a heme group (Cirino *et al*., 2006; Dawson and Dawson, 1995; Dhawan, 2014).

$$\text{NOS + L-arginine + } O_2^{\bullet -} \text{ + NADPH} \rightarrow NO^{\bullet} \text{ + L-citrulline + NADPH}$$

$$\text{NOS (Fe(II)heme) + } O_2^{\bullet -} \rightarrow \text{NOS(Fe(III)heme) + } O^{\bullet -}$$

Scheme 1 Production of nitric oxide

There are several methods to measure NO in biological systems, one of these involves the use of the Griess reaction (Boora *et al*., 2014; Ridnour *et al*., 2000).

The Griess method detects nitrite formed by the spontaneous oxidation of NO under physiological conditions (Boora *et al*., 2014; Sun *et al*., 2003). In this reaction, nitrite is first treated with a diazotizing reagent, like sulfanilamide in an acidic media to form the transient diazonium salt. This intermediate reacts with a coupling reagent, *N*-naphthyl-ethylenediamine (NED), to form a stable azo-compound. The intense purple color of the product allows high sensitivity colorimetric detection at 545 nm, with a detection limit of approximately 0.5 µM (Bryan and Grisham, 2007; Sun *et al*., 2003).

There are some drugs known to inhibit NO production, but currently the most useful drugs are arginine analogues competing for NOS (Sharma *et al*., 2008), such as ω-nitro-L-arginine methyl ester (L-NAME) (Wang and Leigh, 2006).

The NSAID chronic use causes intense inhibition of COX-1, and that is mainly responsible for the side effects previously stated. In addition, there are still some loopholes regarding NO molecular mechanisms of action, its target molecules and cells, and its role in infection and immunologically mediated diseases (Guzik *et al*., 2003).

Several natural products concerning *Plectranthus* plants have been reported for their anti-inflammatory activity (Chiu *et al*., 2012; Lukhoba *et al*., 2006; Rijo *et al*., 2013a). *P.barbatus* and *P. amboinicus* are used to prevent or alleviate inflammation, according to their ethnobotanical reports (Lukhoba *et al*., 2006; Thirugnanasampandan *et al*., 2014).

Forskolin compounds mainly isolated from *P. barbatus*, are specially notorious for their anti-inflammatory properties (Klatz and Goldman, 2003; Rijo *et al*., 2005). In addition, phytosterols such as β-sitosterol and stigmasterol obtained from *P. ecklonii*, triterpenes (α- and β-amyrins, oleanolic and ursolic acids), and polyphenols were validated for anti-inflammatory purposes (Ghimeray *et al*., 2015; Pereira *et al*., 2015; Sergent *et al*., 2010). The pentacyclic triterpene oleanolic acid, also inhibited COX-2-catalyzed PGE_2 biosynthesis (IC_{50} 87 µM) (Huss *et al*., 2002). This highlights the need to investigate the potential of *Plectranthus* spp. medicinal characteristics providing identification of new anti-inflammatory agents.

I.3.4 Anti-*mycobacterium tuberculosis* activity

According to the World Health Organization (WHO), tuberculosis (TB) is one of the major causes of death worldwide from an infectious disease. There are 8.7 million new cases

of active TB annually, causing an estimated 1.4 million deaths (Sandhu, 2011). The causative agent, *Mycobacterium tuberculosis* (Mtb) has evolved elaborate survival mechanisms in humans, allowing it to remain in a clinically latent infection state, constantly engaging the immune system, with the possibility of progress to the active disease (Meena and Rajni, 2010).

Despite evidence that TB is slowly declining, the emergence and spread of Mtb multidrug-resistant strains represents a major challenge to the global control of the disease. Treatment regimens for drug-susceptible TB are onerous, requiring a minimum of six months of treatment with four anti-tubercular drugs. Therefore, multi drug-resistant (MDR), extensively drug resistant (XDR) and totally drug resistant (TDR) forms are much more difficult to treat (Rijo *et al*., 2010). In these circumstances, the identification of new drugs remains nowadays an essential achievement in order to potentiate tools controlling Mtb infection.

The strain H37Rv of Mtb is a harmful pathogen and some studies revealed that to achieve successful infection, Mtb must rely on macrophages for its replication and, more importantly, the macrophage should remain viable. According to the literature, Mtb has several survival strategies: (a) the inhibition of phagosome-lysosome fusion; (b) the inhibition of phagosome acidification; (c) the recruitment and retention of tryptophan-aspartate containing coat protein on phagosomes to prevent their delivery to lysosomes; and (d) the expression of members of the host-induced repetitive glycine-rich protein family of proteins (Meena and Rajni, 2010).

To better understand if Mtb is inhibiting phagolysosome biogenesis by arresting phagosome maturation, two autophagy markers can be studied using western blot and immunofluorescence experiments (Romagnoli *et al*., 2012). Microtubule-associated protein 1 light chain 3 (LC3) is translocated from the cytosol to the auto-phagosome membrane, and the autophagy adaptor protein p62/SQSTM1 (p62) works in the initiation and progression of auto-phagosome formation, by mediating ubiquitination (Komatsu and Ichimura, 2010; Seto *et al*., 2013).

Other strategy can rely on measuring the concentration of viable bacteria in culture by the quantification of colony-forming units (CFU) per unit volume of culture (Peñuelas-Urquides *et al*., 2013), by plating of serial dilutions (Sieuwerts *et al*., 2008). Although the most reliable method, this might be time consuming since a primary culture is obtained in 2-4 weeks, and antibiotic susceptibility is determined after an additional 2-4 weeks (Ghodbane *et al*., 2014; Pathak *et al*., 2012). Moreover, all Mtb research must be performed at a biologic safety level 3 laboratory, taking all safety precautions (Ghodbane *et al*., 2014).

Researching for natural products active against Mtb has increased with the development of easier, faster, and safer screening techniques (Araujo *et al*., 2014). Indeed, flavones, flavonoids, and related metabolites have been reported to exhibit mild activity (Rijo *et al*., 2010). The main mechanism is associated with the quinone ability to accept electrons and

producing ROS, causing cell damage, as has been described in previous section I.2.1.2 (Rijo *et al*., 2014b; Maria Fátima Simões, 2010; Coutinho *et al*., 2009; Abdel-Mogib *et al*., 2002; Rijo *et al*., 2010, 2011).

In 2010, Rijo and co-workers (Rijo *et al*., 2010) reported that royleanone abietanes were highly active against MDR-Mtb strains, regarding 6β,7α-dihydroxyroyleanone, horminone, 6,7-dehydroroyleanone, (MIC 12.5 mg/mL), and 7α-acetoxy-6β-hydroxyroyleanone (MIC 3.12 mg/mL), when compared to first-line anti-tuberculous drugs isoniazid (MIC 4 mg/mL) and rifampicin (MIC 16 mg/mL) against the MDR-Mtb strain (Rijo *et al*., 2010).

These results first exalted that royleanone abietanes isolated from *Plectranthus* spp. are highly potent against MDR-Mtb strains, thus proving their worth in developing new derivatives in further investigations. However, their cytotoxicity could put this anti-Mtb activity in jeopardy.

On the other hand, new findings reported the existence of two genes found in a small operon, Rv3377c and Rv3378c (Layre *et al*., 2014; Mann and Peters, 2012; Nakano *et al*., 2011), specific to virulent *Mycobacterium* strains (Hoshino *et al*., 2011). The Rv3377c translates a diterpene cyclase class II that generates tuberculosinyl diphosphate (TPP), and Rv3378c encodes a diterpene synthase that acts on TPP to produce tricyclic diterpenes, responsible for Mtb phagosomal arrest, named tuberculosinol and isotuberculosinol (Layre *et al*., 2014; Mann and Peters, 2012; Nakano *et al*., 2011). These compounds are produced in an equimolar mixture, and they both individually and synergistically inhibit the opsonized zymosan particles phagocytosis (Hoshino *et al*., 2011; Mann and Peters, 2012; Nakano *et al*., 2011). Although the increasing interest on tuberculosinol for TB therapy, its stereochemistry has not been clearly established (Buter *et al*., 2016).

Interestingly, these compounds were found to be very similar to the halimane diterpenes isolated from *Plectranthus* spp. Therefore, it seems appropriate to find new enantiomeric derivatives from *Plectranthus* spp. with higher affinity than TPP to the diterpene synthase encoded by Rv3378c (Chan *et al*., 2014; Hoshino *et al*., 2011). The derivative would then interact with the binding site, thus inhibiting the production of tuberculosinols, ultimately avoiding the phagocytosis suppression (Layre *et al*., 2014; Mann and Peters, 2012).

I.4 Objectives

Concerning all the biological activities described for the secondary metabolites of Plectranthus spp., it was decided for this thesis to perform a wide biological screening for some *Plectranthus* species regarding isolated compounds and plant extracts. The focus of the work compiled in this report concerned the following main goals:

1) Extracts preparation from the plant material *Plectranthus grandidentatus, Plectranthus ecklonii, Plectranthus ornatus, Plectranthus madagascariensis, Plectranthus porcatus, Plectranthus neochilus,* and *Plectranthus prostratus*;

2) *In vitro* antioxidant study of the acetone, ethyl acetate, and methanol extracts through the DPPH radical scavenging assay;

3) Explore the anti-inflammatory activity of some *Plectranthus* spp. isolated compounds by means of nitric oxide quantification, on lipopolysaccharide-stimulated mouse monocyte macrophages cell line;

4) Assess isolated compounds and plant extracts at *in vitro* inhibition of skin-related enzymes
    i) Acetylcholinesterase enzyme inhibition using the Ellman colorimetric assay;
    ii) Tyrosinase, elastase, and collagenase inhibition, using adjusted colorimetric assays;

5) Evaluate *Mycobacterium tuberculosis* growth after macrophage infection and treatment with selected isolated compounds, using the colony-forming unit assay.

II. EXPERIMENTAL SECTION

II.1 Reagents and materials

2,2-diphenyl-1-picrylhydrazyl (DPPH), acetylcholinesterase (AChE), acetylcholine iodide ≥ 98% (AChI), L-tyrosine, kojic acid, tyrosinase from mushroom, *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide (SANA), *N*-[3-furyl-acryloyl]-Leu-Gly-Pro-Ala (FALGPA), collagenase from *Clostridium histolyticum* type IA, Sodium nitrite, lipopolysaccharide (LPS) from *Escherichia coli*, sulphanilamide, *N*-(1-naphthyl)-ethylenediamine dihydrochloride (NED), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), PBS 0.1% Triton X, and PBS 0.05% Tween 80 were purchased from Sigma-Aldrich. Tris-hydroxymethylaminomethane (Tris) base buffer and 5,5'-dithio-bis-(2-nitrobenzoic acid) (Ellman reagent, DTNB) were bought from VWR International Prolabo. 1,2,3,4-Tetrahydro-5-aminoacridine (Tacrine) was obtained from Cayman Chemical Company. Elastase from porcine pancreas was acquired from Alfa Aesar. Tricine buffer was purchased from Amresco. Phosphoric acid, dimethyl sulphoxide (DMSO) and methanol was bought from Merck; RPMI, fetal bovine serum (FBS), trypsin, L-glutamine, and penicillin/streptomycin were provided by lonza. RPMI 1640 medium was from BioWhittaker. Anti-CD14 conjugated magnetic microbeads was from Miltenyi. Lymphoprep was obtained from Cedarlane. Middlebrook 7H10 agar plates were obtained from BD bioscience, 262710. *Mycobacterium tuberculosis* H37Rv (ATCC 27294; American Type Culture Collection, biologic safety level 3) and murine leukemic monocyte-macrophage cell line (RAW 264.7, biologic safety level 2) were obtained from Faculty of Pharmacy and Centre for Neurosciences and Cell Biology (University of Coimbra, Portugal). CytoTox® 96 Non-Radioactive Cytotoxicity Assay kit was from Promega.

II.2 Equipment

For the plants extracts preparation, a microwave oven at 2.45GHz, ultrasonic apparatus from VWR, rotary evaporator from IKA and a lyophilizer FreeZone 25 from Labconco were required. For the compounds isolation methodologies, melting-points were determined on a Kofler block, optical rotations were measured on a Perkin-Elmer 241 MC polarimeter, UV spectra were recorded on a Perkin-Elmer Lambda 2 UV/vis spectrophotometer, IR spectra were obtained on a Perkin-Elmer Spectrum One spectrophotometer, NMR spectra were recorded on a Varian INOVA-400 Spectrometer, mass spectra were obtained in a Hewlett-Packard 5973 spectrometer and electro spray ionization on a Hewlett-Packard 1100 spectrometer, LC/MS was performed in Agilent 6520 Accurate-Mass QTOF apparatus, and elemental analysis in a LECO CHNS-932 apparatus. For the antioxidant assay, the absorbance values were read in a UV-Vis spectrometer. The enzymatic assays required a microplate reader from Thermo-Fisher Scientific, and incubators from Memmert and Heidolph. Regarding the anti-inflammatory assays, biosafety level 2 cabinet from Telstar was used, as

well as a CO_2 incubator from Binder, inverted microscope from Motic, and a microplate reader from BioTek. Concerning the assays for *Mycobacterium tuberculosis* assays, biosafety level 3 cabinet from Biobase, CO_2 incubator, inverted microscope and microplate reader were utilized.

II.3 Plant material and preparation of extracts

Plectranthus spp. medicinal plants from South Africa were cultivated in Instituto Superior de Agronomia campus (Lisbon). Eight *Plectranthus* species were studied: *Plectranthus grandidentatus* Gürke, *Plectranthus ecklonii* Benth., *Plectranthus ornatus* Codd., *Plectranthus madagascariensis* (Pers.) Benth. *Plectranthus porcatus* van Jaarsv. & P.J.D.Winter, *Plectranthus neochilus* Schltr., *Plectranthus prostratus* Gürke and *Plectranthus saccatus* Benth. Extraction methods were performed according to previously established literature procedures, with slight modifications (Pereira *et al*., 2015).

The organic extracts were prepared using methanol, ethyl acetate, and acetone as solvents. The extracts were obtained using 10 ± 0.010 g of the aerial parts of air dried and powdered plants, in 200 mL of the corresponding organic solvents. Each was submitted to sonication at room temperature, for 1 hour. The organic extractions were filtered to round-bottomed flasks and were set in the rotary evaporator for vacuum evaporation at 40-50ºC. The sample size contained twenty-one organic extracts. The crude extracts were stored at 20 mg/mL in DMSO.

The aqueous extracts of the medicinal plants were performed with 10 ± 0.010 g of the aerial parts of dried and powdered plants, in 150 mL of bi-distilled water (Milli-Q). The mixtures were subject to microwave-assisted extraction for 3 minutes, at a continuous irradiation of 2.45 GHz. The aqueous extractions were also filtered, and prepared for lyophilisation, being separated into 1 mL aliquots (in triplicate) and frozen at −20ºC, resulting in 7 aqueous extracts in triplicate. After lyophilisation the extracts were weighed and stored at 10 mg/mL in bi-distilled water (Milli-Q) at −20ºC.

II.4 Isolated compounds

The studied *Plectranthus* plants compounds were previously obtained and characterized by the partners in the CBIOS laboratory of Food Science and Phytochemistry Laboratory (Universidade Lusófona de Humanidades e Tecnologias, Lisboa, Portugal).

The samples of the isolated compounds for the screening were obtained by a phytochemical study that was previously performed in the described plants, using bioguided studies. The structural elucidation of the bioactive metabolites was based in physicochemical data (melting point, specific rotation), mostly spectroscopic data UV, IR, 1D- and 2D- ^{1}H and ^{13}C RMN spectra, mass spectra, elementary analysis and comparison with bibliographic data.

The compounds will be further denoted for their internal number described in Table 3.

Table 3 Terpene, phytosterols, and polyphenols isolated from the described *Plectranthus* spp. plants, and correspondent sample numbers, prepared at 10 mg/mL in DMSO.

Plant material	Isolated compounds	Compound nr.
***P. grandidentatus* (PG)**	7α-acetoxy-6β-hydroxyroyleanone	1
***P. madagascariensis* (PM)**	6β,7α-dihydroxyroyleanone	2
	6,7-dehydroroyleanone	3
***P. ecklonii* (PE)**	Parvifloron D	4
	3β-stigmast-5-en-3-ol Stigmasta-5,22*E*-dien-3β-ol	15 Mixture 1:1
	Oleanolic acid Ursolic acid	16 Mixture 1:4
***P. porcatus* (PP)**	(13S,15S)-6β,7α,12α,19-tetrahydroxy-13β,16-cyclo-8-abietene-11,14-dione	6
***P. ornatus* (PO)**	(11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid	5
	1α,6β-diacetoxy-8α,13*R**-epoxy-14-labden-11-one	7
	(11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic methyl ester	8
	(11*R**,13*E*)-15-butyryloxyhalima-5,13-dien-11-ol	9
	(11*R**,13*E*)-halima-5,13-diene-11,15-diol	10
	1,6-di-*O*-acetylforskolin 1,6-di-*O*-acetyl-9-deoxyforskolin	13 Mixture 1:1
***P. neochilus* (PN)** ***P. ornatus* (PO)**	α-amyrin β-amyrin	14 Mixture 3:1
***P. saccatus* (PS)**	Chlorogenic acid	11
***Plectranthus* spp.**	Rosmarinic acid	12

II.5 Antioxidant activity

Radical scavenging assay with 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The free radical scavenging activity of the organic extracts (1.0 mg/mL in DMSO) was evaluated using the DPPH assay according to the method used beforehand (Martins *et al*., 2013; Rijo et at., 2014a). All of the extracts were dissolved in ethanol 70% (w/v), mixed with

DPPH solution (100 mM in ethanol) and incubated at room temperature for 30 minutes, in the dark. Each sample was assayed in triplicate.

The absorbance was monitored at 517 nm against a blank containing the same concentration of the organic extracts in ethanol, and the scavenging activity (%) was determined using Equation 1.

Equation 1: $$\text{Scavenging activity (\%)} = \text{Abs control} - \left(\frac{\text{Abs sample-Abs blank}}{\text{Abs control}}\right) \times 100$$

II.6 *In vitro* inhibition of skin-related enzymes

II.6.1 Anti-acetylcholinesterase enzymatic assay

The acetylcholinesterase (AChE) inhibition assay was performed according to previous methodologies (Rijo *et al*., 2014a). For this assay the aqueous extracts have been previously studied in other work groups, hence only organic extracts were tested to evaluate preliminary bioactivity.

The Ellman reagent (3 mM) was prepared in 50 mM HEPES buffer (pH 8.0) supplemented with 20 mM $MgCl_2$ and 50 mM NaCl. The substrate AChI was prepared in bi-distilled water at 15 mM, and AChE was dissolved in 50 mM HEPES buffer (pH 8.0), in order to obtain 1 mL aliquots of 1000 U.

The assay was performed with 98 µL of HEPES buffer, 30 µL of the samples (0.1 mg/mL) and 7.5 µL of acetylcholinesterase (50 U). These were incubated for 15 minutes at 25ºC before adding 22.5 µL of acetylcholine iodide and 142 µL DTNB, to start the reaction.

Positive control was performed with 3 µM tacrine (IC_{50} = 184 nM, (Jin, 2014)), and negative controls with the respective sample solvent at the same concentration tested.

Absorbance was measured at 405 nm, immediately after starting the reaction, and then every 30 seconds for 3 minutes, in a 96-well microplate reader.

All assays were performed in triplicate, and the inhibition percentage was determined using the following equations **2a)** and **2b)**.

2a) $$\textbf{Velocity reaction of control or inhibitor} = \frac{\text{Corrected absorbance (nm)}}{\text{time (min)}}$$

2b) $$\textbf{Inhibitor activity (\%)} = 100 - \left(\frac{100 \times \text{Velocity reaction of inhibitor}}{\text{Velocity reaction of control}}\right)$$

For the AChE activity, the absorbance increase was registered by the equation **2a)** for the velocity reaction of control (ΔAbs_{405nm}/min), which should be in the linear range. The results are expressed has percentage inhibition (%) of each sample tested.

II.6.2 Anti-tyrosinase enzymatic assay

The tested compounds and extracts were assayed for the anti-tyrosinase activity with modifications (Moon *et al*., 2010). For this assay all the organic and aqueous extracts were assessed, as well as isolated compounds **(1-7)** and **(11-12)**.

The substrate L-tyrosine 0.5 mM was prepared in phosphate buffered saline (PBS) 50 mM (pH 6.8), and kojic acid 0.8 mM (IC_{50} = 43.7 µM, (Yamauchi *et al*., 2011)) was used as positive control. Negative controls were performed with the sample solvent at same concentration.

The assay was performed in 180 µL L-tyrosine and 10 µL of the tested samples (50 µg/mL) incubated for 5 minutes at 37ºC, before starting the reaction with 10 µL tyrosinase (5000 U). The reaction mixture was incubated for 5 minutes more at 37ºC and the absorbance read at 450 nm for 10 minutes (reading every 2 minutes) in a 96-well microplate reader.

All assays were performed in triplicate, and the inhibition percentage was determined using the equations **3a)** and **3b)**. The absorbance increase was registered by the equation **3a)** for tyrosinase velocity reaction of negative control (ΔAbs_{450nm}/min), which had linear increase. The results are expressed has percentage inhibition (%) of each sample tested.

3a) $$\textbf{Velocity reaction of control or inhibitor} = \frac{\text{Corrected absorbance (nm)}}{\text{time (min)}}$$

3b) $$\textbf{Inhibitor activity (\%)} = 100 - \left(\frac{100 \times \text{Velocity reaction of inhibitor}}{\text{Velocity reaction of control}}\right)$$

II.6.3 Anti-collagenase enzymatic assay

The anti-collagenase enzymatic assay was optimized based on several methods reported in literature (Wahab *et al*., 2014; Thring *et al*., 2011, 2009; Van Wart and Steinbrink, 1981). In this method the organic and aqueous extracts were tested for collagenase inhibition, as well as the compounds **(1-7)** and **(11-12)**.

The synthetic substrate *N*-[3-furyl-acryloyl]-Leu-Gly-Pro-Ala (FALGPA) 0.1 mM was dissolved in tricine buffer 50 mM (pH 7.5) supplemented with 400 mM NaCl and 10 mM $CaCl_2$, knowing that 1 U hydrolyzes 1 µmol of FALGPA per minute, at 25ºC, in the presence of calcium ions.

Collagenase was prepared in the assay buffer at 1 U, and 40 µM of epigallocatechin gallate (EGCG) was used as a positive control (IC_{50} 0.9 mM (Wittenauer *et al*., 2015)), whereas negative controls were the correspondent samples solvent at same concentration.

The assay mixture containing 80 µL of tested samples (0.1 mg/mL) and 100 µL collagenase was incubated for 10 minutes at 37ºC, before starting the reaction with 20 µL

FALGPA. The assay was performed in triplicate and absorbance read at 405 nm for 10 minutes, continuously.

The inhibition percentage was determined using the equations **4a)** and **4b)**. For the collagenase activity, the absorbance decrease was registered by the equation **4a)** for the velocity reaction of negative control (ΔAbs_{405nm}/min), which was in the linear range. The results are expressed has percentage inhibition (%) of each sample tested.

4a) $$\textbf{Velocity reaction of control or inhibitor} = \frac{\text{Corrected absorbance (nm)}}{\text{time (min)}}$$

4b) $$\textbf{Inhibitor activity (\%)} = 100 - \left(\frac{100 \times \text{Velocity reaction of inhibitor}}{\text{Velocity reaction of control}}\right)$$

II.6.4 Anti-elastase enzymatic assay

The anti-elastase enzymatic assay was based on spectrophotometric methods described in literature, with some modifications (Thring *et al*., 2011; Wahab *et al*., 2014; Jung *et al*., 2014; Bieth *et al*., 1974). For this assay all the organic and aqueous extracts were tested, as well as the following isolated compounds: **(1-7)** and **(13-17)**.

N-succinyl-Ala-Ala-Ala-*p*-nitroanilide (SANA) 1 mM was used as substrate, dissolved in Tris-HCl buffer 50 mM (pH 8.0), knowing that 1 U enzyme converts 1 µmol of SANA per minute in this buffer at 25ºC. Ursolic acid at 0.1 mg/mL was the positive control (IC_{50} = 31 µg/mL, (Masuda *et al.,* 2009)), and negative controls were the respective samples solvent at the same tested concentration.

Elastase (6 U) and the samples (0.1 mg/mL) were added, and the mixture was incubated at 25ºC for 10 minutes. The reaction was initiated by adding 20 µL of SANA and 150 µL of Tris-HCl buffer.

Absorbance was measured at 405 nm immediately after starting the reaction, and then every 30 seconds for 3 minutes, in a 96-well microplate reader.

All assays were performed in triplicate, and the inhibition percentage was determined using the equations **5a)** and **5b)**. For elastase activity, the absorbance increase is registered by the equation **5a)** for the velocity reaction of negative control (ΔAbs_{405nm}/min) that was in the linear range. The results are expressed has percentage inhibition (%) of each sample tested.

5a) $$\textbf{Velocity reaction of control or inhibitor} = \frac{\text{Corrected absorbance (nm)}}{\text{time (min)}}$$

5b) $$\textbf{Inhibitor activity (\%)} = 100 - \left(\frac{100 \times \text{Velocity reaction of inhibitor}}{\text{Velocity reaction of control}}\right)$$

II.7 Anti-inflammatory activity

This research was developed at the Centre for Marine Sciences (CCMAR), at the MARbiotech laboratories, University of Algarve, Faro, Portugal, under the supervision of Doctor Luísa Custódio.

II.7.1 Cytotoxicity assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on RAW 264.7 macrophage cell line, as previously described elsewhere (Rodrigues *et al*., 2014, 2016). Isolated compounds tested were **(1)**, **(3-4)**, **(6-8)**, and **(12-15)**.

The cell line was maintained in RPMI culture medium supplemented with 10% FBS, 1% L-glutamine (2 mM), and 1% penicillin (50 U/mL) / streptomycin (50 µg/mL), at 37°C in a humidified atmosphere with 5% CO_2. The cells were re-suspended in medium every 48 hours with a cell scraper, until approximately 80% confluency was reached.

After reaching the desired confluency, 1.0×10^3 cells/ well were plated in a 96-well plate and incubated during 24 hours (overnight) in RPMI medium at 37ºC.

All samples were tested at 8 concentrations, comprising 100, 50, 25, 12.5, 10, 5, 2.5, and 1 µM. For each sample 100 µL of compounds was added to the cells, and incubated at 37ºC throughout 24 hours. Control cells were treated only with DMSO at 0.5%, and with RPMI medium (100% cell viability).

Afterwards, 20 µL of MTT (5 mg/mL in PBS buffer) was added and incubated for 2 hours. Finally, 150 µL of DMSO was added to dissolve the formazan crystals, and the absorbance was measured at 590 nm in a microplate reader.

Results were expressed as cell viability (%) in comparison to the controls. Non-cytotoxic samples were considered if cell viability was up to 80%, compared to negative controls RPMI medium and DMSO 0.5%.

II.7.2 Nitric oxide quantification in LPS-stimulated RAW 264.7 cells treated with non-cytotoxic compounds

The anti-inflammatory properties of the isolated compounds **(1)**, **(3-4)**, **(6-8)**, and **(12-15)** were accessed as previously described (Rodrigues *et al*., 2014, 2016) at the non-cytotoxic concentrations. Mainly, all compounds were used, with exception for isolated compounds **(1)** and **(4)**. RAW 264.7 cells were plated in a 96-well microplate at a density of 2.5×10^5 cells/ well with RPMI medium, and allowed to adhere, during 24 hours.

The ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) was used as a positive control, at IC_{50} value of 29 µg/mL (Rodrigues *et al*., 2016).

After this time the medium was removed, 50 µL of lipopolysaccharide (LPS) (100 ng/mL) and 50 µL of the testing samples, were dissolved in a serum- and phenol-free RPMI medium, and added to each well.

After 24 hours of incubation at 37ºC in a humidified atmosphere with 5% CO_2, the NO production in cell culture medium was measured spectrophotometrically by the Griess method.

Briefly, the culture supernatants (approximately 100 µL) were removed and mixed with 100 µL of Griess reagent, which comprised 1% (w/v) sulphanilamide, plus 0.1% of NED, and 2.5% (v/v) of phosphoric acid. The mixtures were incubated for 20 minutes at room temperature in the dark, and absorbance was measured at 540 nm on a microplate reader.

The NO concentration was determined using a calibration curve obtained with several known concentrations (1.6, 3.1, 6.25, 12.5, 25, 50, and 100 µM) of sodium nitrite used as standard. Results were expressed as NO production (µM) of the LPS-stimulated RAW 264.7 cells.

II.8 *Mycobacterium tuberculosis* H37Rv growth assay

Research developed under the Short-Term Scientific Mission of COST action CM1407: "Natural diterpenoids as potential anti-tubercular drugs", developed at the National Institute for the infectious Disease Lazzaro Spallanzani, Laboratorio di Biologia Cellulare e Microscopia Elettronica, Rome, Italy, under the supervision of Doctor Gian Maria Fimia and Doctor Alessandra Romagnoli.

II.8.1 Peripheral blood mononuclear cell (PBMC) isolation

The PBMC isolation was performed as previously described (Romagnoli *et al*., 2012) this experiment, approximately 50 mL of human blood from random healthy volunteers from the blood bank of University "La Sapienza," Rome, Italy (who provided written informed consent, approved by the ethical committee at the National Institute for Infectious Diseases), was used and diluted (10 mL) into five Falcon tubes containing 20 mL of sterile PBS buffer. These solutions were added to another set of five tubes, containing Lymphoprep to perform Ficoll gradient separation, in order to separate the blood components into plasma, mononuclear cells and red blood cells.

The blood containing Lymphoprep was centrifuged at 2200 rpm for 20 minutes to separate the layers, and the mononuclear cells layers were removed.

For cell count, performed twice, 10 µL of PBMC was added in two Eppendorf containing 190 µL of acetic acid that lysed the remaining red blood cells, thus only PBMC were counted.

After counting the cells, a new centrifugation at 900 rpm was performed to remove platelets. Then 2.5×10^5 cells/ mL were mixed on separation buffer.

The anti-CD14 magnetic microbeads were added in 50 µL/100×10^6 cells, for 30 minutes at 4°C. These beads attached specifically to the monocytes $CD14^+$ membrane receptors. Afterwards, the samples were centrifuged for 10 minutes and re-suspended in 3 mL of PBS buffer.

An LS column was used to isolate the monocytes. The resulting sample was again centrifuged for 10 minutes and human serum 2% was added to the RPMI FBS 10%, L-Glutamine medium (supplemented medium). The resulting cells were counted again with acetic acid, and 5.0×10^5 cells were plated in 48-well plate, using 3 wells for negative control/blank, with supplemented medium. The plated cells were incubated at 37ºC in a humidified 5% CO_2 atmosphere, during 4 days for cell adhesion.

II.8.2 Lactate dehydrogenase (LDH) cytotoxicity assay

In order to evaluate cytotoxicity from compounds **(1-10)** exposure to macrophages, the lactate dehydrogenase assay was performed, using the CytoTox® 96 Non-Radioactive Cytotoxicity Assay kit.

After 48 hours of cell adhesion, the cells were treated with the compounds according to *Mycobacterium tuberculosis* (Mtb) H37Rv strain minimum inhibitory concentrations, obtained previously, described in Table 4 (Rijo *et al*., 2010, 2011).

Besides the LDH positive control from the kit, representing 100% of LDH release, staurosporine (STS) (IC_{50} 250 nM (Schiller, 2004)) isolated from *Streptomyces staurospores*, a protein kinase inhibitor that induces DNA fragmentation and apoptosis, was also used as a positive control for the cytotoxicity assay, and was tested at 2 µM.

The positive controls for Mtb infection ethambutol (ETAN) and isoniazid (INH), were also studied, in a concentration 10-fold the MIC values (1 µg/mL, and 40 µg/mL).

The supernatants were prepared in two dilutions (1:10 and 1:100). Next, the substrate mixture was prepared using the assay buffer according to fabricant instructions, and 50 µL was added to each well and incubated for 30 minutes in the dark. Also, a maximum LDH release control was performed by freezing the plates at −80ºC and heating at 50ºC.

After incubation, 50 µL of stop solution was added and absorbance read at 490 nm in a microplate reader.

Table 4 Minimum inhibitory concentration (MIC) values (µg/mL). The MICs were used as baseline for the LDH cytotoxicity test macrophages derived from PBMC (Rijo *et al*., 2010, 2011).

Compounds	Concentration (µM)	Molar mass (g/mol)	MIC Mtb (µg/mL)
7α-acetoxy-6β-hydroxyroyleanone **(1)**	64.1	390.20	
6β,7α-dihydroxyroyleanone **(2)**	71.8	348.19	25
6,7-dehydroroyleanone **(3)**	79.5	314.42	
Parvifloron D **(4)**	35.9	434.52	15.6
11*R**-acetoxy-halima-5,13*E*-dien-15-oic acid **(5)**	69.1	362	
(13*S*,15*S*)-6β,7α,12α,19-tetrahydroxy-13β,16-cyclo-8-abietene-11,14-dione **(6)**	25	364.43	
1α,6β-diacetoxy-8α,13*R**-epoxy-14- labden-11-one **(7)**	59.4	420.54	25
(11*R**,13*E*)-11-acetoxyhalima-5,13-dien-15-oic acid methyl ester **(8)**	71.8	348	
(11*R**,13*E*)-15-butyryloxyhalima-5,13-dien-11-ol **(9)**	66.5	376	
(11*R**,13*E*)-halima-5,13-diene-11,15-diol **(10)**	81.7	306	

II.8.3 Macrophage infection with *Mycobacterium tuberculosis*, and treatment with compounds

After obtaining the cytotoxicity results, macrophages were again isolated from PBMC, and were infected in a Biosafety Level 3 laboratory, according to previous described (Romagnoli *et al*., 2012). Macrophages were infected with Mtb at a multiplicity of infection (MOI) of 1.

Treatment with the compounds **(5)** and **(7-10)** at the MIC values (25 µg/mL) started 2 hours after infection and kept until the end of the experiment, according to MIC values in Table 4. Ethambutol and isoniazid were used as positive controls at 10-fold the MIC isoniazid (INH) at 1 µg/mL, and ethambutol (ETAN) at 40 µg/mL.

After 48-hours of treatment with the selected compounds, the infected cell cultures were lysed in PBS 0.1% Triton X, in order to release intracellular Mtb. Serial dilutions were prepared in PBS 0.05% Tween 80. Aliquots (50 µL) of each Mtb dilution were plated (in triplicate), to determine the intracellular bacterial load with CFU counts. Viable bacteria were evaluated after 13 days of incubation at 37°C. Values are expressed as the logarithm of CFU/mL, at the dilution 10^{-3}. The intracellular amount of CFU at time zero was assessed to make sure that all wells were infected with the same bacterial concentration and that the infecting dose was as expected. After the assay, Mtb was heat killed at 80°C for 1 hour, as previously described (Romagnoli *et al*., 2012).

II.9 Statistical analysis

Data comparisons were conducted with one-way analysis of variance (ANOVA) followed by post hoc Tukey honest significant difference test, for pairwise comparisons. Analyses and graphical presentation were performed with the GraphPad Prism Software Version 5 (GraphPad Software, Inc., San Diego, CA, USA). Values of $p<0.05$ were statistically significant. Results are presented as mean ± standard deviation (SD).

III. RESULTS AND DISCUSSION

III.1 *Plectranthus* spp. extracts

In this work eight *Plectranthus* species were studied, *P. grandidentatus* Gürke, *P. ecklonii* Benth., *P. ornatus* Codd., *P. madagascariensis* (Pers.) Benth. *P. porcatus* van Jaarsv. & P.J.D.Winter, *P. neochilus* Schltr., *P. prostratus* Gürke and *P. saccatus* Benth.. Extracts of the aerial parts of the plant material dried and powdered, resulted in twenty-one bioactive organic extracts, and seven aqueous extracts. The organic extracts were prepared with three solvents, namely acetone, methanol, and ethyl acetate. Ultrasound was the selected extraction methodology for its efficiency (Rijo et al., 2014a). The aqueous extracts were obtained using the microwave extraction, since it has been proven to have additional advantages in terms of short duration, and high efficiency to recover the bioactive compounds (Rijo et al., 2014).

The amount of dry weights of each plant are shown in Table along with the resulting yields. The extraction resulted in approximately the same low dry residue for the microwave methods between 0.26% (mg/g) for *P. porcatus* and 0.43% (mg/g) for *P. ornatus*. Higher yields were indeed obtained for organic extracts, particularly on *P. ornatus* acetone extract that resulted in 59.9% (mg/g) of dry extract yield. Considering yield data (Table), with exception for *P. ornatus*, methanol was the most effective solvent for this extraction methodology.

Table 5– Extraction solvent, technique and obtained yields for each *Plectranthus* spp.

Plant	Solvent	Technique	Dry residue (±0.01g)	Yield % (mg/g)
P. madagascariensis	Water	Microwave	0.031	**0.31**
	Acetone	Ultrasound	0.203	2.03
	Methanol		0.823	8.21
	Ethyl Acetate		0.236	2.35
P. neochilus	Water	Microwave	0.032	**0.32**
	Acetone	Ultrasound	0.430	4.26
	Methanol		1.040	10.4
	Ethyl Acetate		0.552	5.51
P. porcatus	Water	Microwave	0.026	**0.26**
	Acetone	Ultrasound	0.662	6.59
	Methanol		1.233	12.3
	Ethyl Acetate		0.704	7.0
P. prostratus	Water	Microwave	0.033	**0.33**
	Acetone	Ultrasound	0.819	8.16
	Methanol		1.189	11.82
	Ethyl Acetate		0.951	9.48
P. grandidentatus	Water	Microwave	0.028	**0.27**
	Acetone	Ultrasound	0.237	2.37
	Methanol		0.686	6.76
	Ethyl Acetate		0.247	2.45
P. ecklonii	Water	Microwave	0.034	**0.34**
	Acetone	Ultrasound	0.384	3.83
	Methanol		0.949	9.45
	Ethyl Acetate		0.332	3.32
P. ornatus	Water	Microwave	0.043	**0.43**
	Acetone	Ultrasound	6.0	59.9
	Methanol		1.059	10.6
	Ethyl Acetate		0.924	9.15

III.2 Organic extracts antioxidant activity by scavenging of DPPH radical

The antioxidant capacity of the organic extracts was evaluated using the DPPH radical scavenging assay (Rijo et al., 2014a) (Section II.5). Several studies report the antioxidant activity of *Plectranthus* plants through radical scavenging, and DNA protection from oxidative stress properties (Rijo et al., 2012; Rosa et al., 2015). As shown in Figure 9, methanol extracts held the highest scavenging activity (20-76%), among the plants, except for ethyl acetate extract of *P. grandidentatus* (62.3 ± 0.43% p<0.0001).

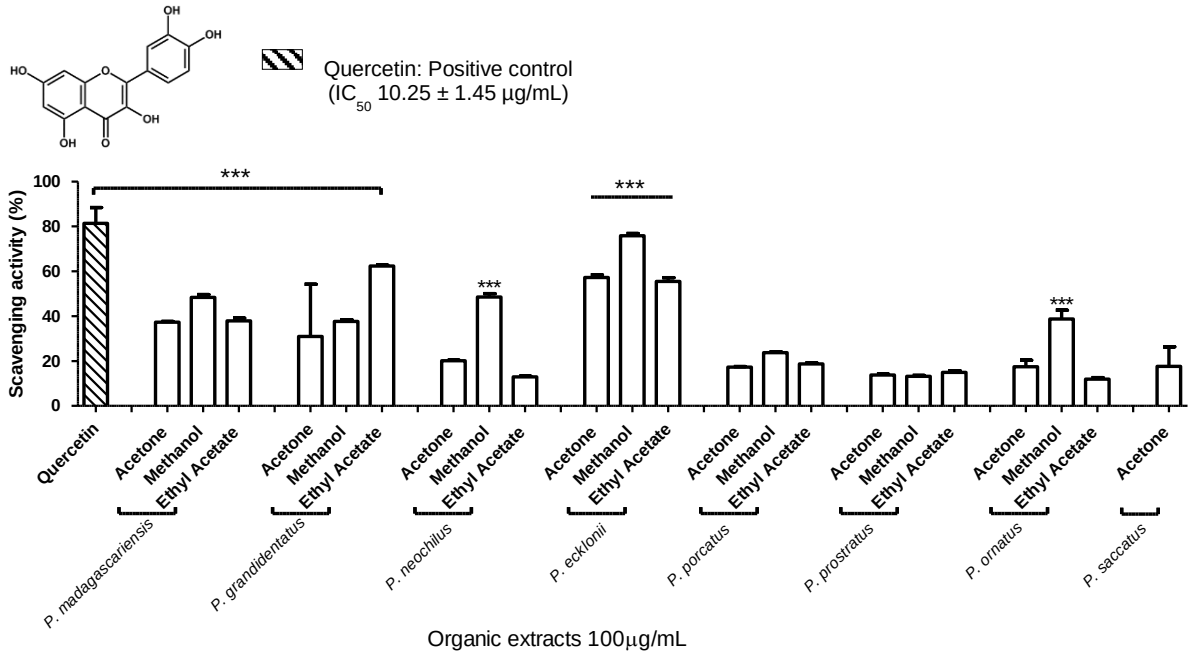


Figure 9 *In vitro* antioxidant activity by the DPPH radical scavenging assay of the *Plectranthus* spp. organic extracts. Twenty-two organic extracts were tested at 100 µg/mL, in triplicate, for their ability to scavenge the DPPH free radical. The results are presented as means percentage values, considering the absorbance of Quercetin as the positive control. Data are expressed as the mean ± SD (n=3) **p<0.005 ***p<0.0001 *vs* negative control (DPPH in ethanol). Values were determined by one-way ANOVA followed by Tukey HSD comparison test.

The observed results for the methanol extracts is possibly related with the high contents of polyphenols in these extracts, that are known for their antioxidant activity (Quy Diem *et al*., 2014). Nevertheless, there were recent discoveries on the abietane diterpenes with antioxidant properties, particularly concerning Parvifloron D **(4)** and 7α-acetoxy-6β-hydroroyleanone **(1)** (Rosa et al., 2015). The antioxidant results of *P. ecklonii* (methanol, 75.9 ± 1.02% p<0.0001), *P. madagascariensis* (methanol 48.4 ± 1.23% p<0.0001), and *P. grandidentatus* (ethyl acetate 62.3 ± 0.43% p<0.0001), can possibly be explained by the mainly present compounds with a abietane backbone that have antioxidant activity (Rosa *et al*., 2015).

Additionally, PE ethyl acetate (55.5 ± 1.66% p<0.0001) and acetone (56.9 ± 1.45% p<0.0001) extracts resulted in relatively increased radical scavenging activity, in comparison to the remaining plants extracts that held high values only for the methanol extract. In fact, PG

and PE had the most similar antioxidant activity with quercetin at 89 ± 2.5% (structural representation in Figure 9), a potent antioxidant compound.

Since the organic extracts have been studied for their notorious amounts of polyphenol compound and abietane diterpenes compounds, identified in *Plectranthus* spp. plants, this assay validates their aptitude for the conversion of the DPPH free radical into a more stable molecule, thus stopping the radical chain reaction.

III.3 Skin-related enzymatic inhibitions *in vitro*

Plectranthus spp. natural products have shown antioxidant activity (Section III.2), and ROS can initiate complex molecular pathways for melanogenesis (Tu and Tawata, 2015), and activation of ECM proteases such as collagenase and elastase (Ghimeray *et al*., 2015).

Several studies have been performed reporting that the human skin contains resident and transiently residing cells part of the extra- or non-neuronal cholinergic system, establishing the skin as active source and target of neurotransmitters and hormones (Kurzen *et al*., 2007; Schlereth *et al*., 2006).

Therefore, to investigate if *Plectranthus* spp. could be useful as promising approach to prevent extrinsic skin aging, several *in vitro* assays were developed for anti-acetylcholinesterase, -tyrosinase, -collagenase, and -elastase activities.

III.3.1 Organic extracts AChE inhibition *in vitro*

There have been previous studies (Rijo *et al*., 2014a) proving the capacity of aqueous *Plectranthus* extracts, as well as of some of their isolated compounds, to inhibit AChE. In fact, the aqueous extracts of *P. ecklonii*, *P. grandidentatus*, *P. ornatus*, *P. porcatus*, *P. barbatus*, and *P. saccatus* were reported to interfere with AChE activity. Moreover the eminent AChE inhibition can be probably be related to high contents in rosmarinic acid (IC_{50} 527.8 ± 27.7 µM), that has been previously tested (Rijo *et al*., 2014a).

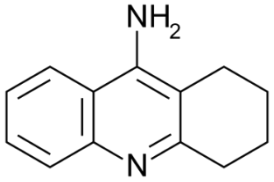


Figure 10 Structural representation of tacrine, a known AChE inhibitor.

The majority of AChE inhibitors reported so far have alkaloid characteristics, such as tacrine represented in Figure 10, and there are few terpene-like inhibitors reported. The aqueous extracts have been the focus of AChE inhibitory assays (Rijo *et al*., 2014a), so in order to find potential inhibitors identities, only the organic extracts were tested for this assay.

This *in vitro* enzymatic assay was performed by measuring the colorimetric product obtained from the reaction of thiocholine produced by AChE, with the Ellman reagent (DTNB), which was proportional to the AChE activity. The results are shown in Table 6 and represent

the inhibition percentage of AChE, calculated according to equation 2b) at section II.6.1 in comparison with the enzymatic activity of the negative control (ΔAbs_{405nm}/min).

These results demonstrate that the *Plectranthus* spp. organic extracts do not exert inhibitory properties in acetylcholinesterase *in vitro* (Table 6). In truth, with exception for the *P. porcatus* (PPP) and *P. prostratus* (PP) extracts from all solvents, and *P. ornatus* (PO) acetone extract, the inhibition of AChE was probably due to the DMSO interference with the enzyme (17.0 ± 0.05% of AChE inhibition). Even though, the inhibition percentage on AChE did not exceeded more than 23%, and are very low comparing to the positive control (tacrine) that could inhibit AChE by 95.65 ± 2.89%.

Table 6 *In vitro* acetylcholinesterase enzymatic assay of the organic extracts obtained from seven *Plectranthus* spp. plant material. The samples represent the extracts reconstructed in DMSO at 10 mg/mL, and tested at 10 µg/mL from serial dilutions in HEPES buffer 50 mM (pH 8.0), until reaching 0.1% (v/v) of DMSO. Values represent mean ± standard deviation (SD) of the assays performed in triplicate.

Plant material	Sample	AChE inhibition ± SD (%)
P. ornatus	Acetone	**22.81 ± 2.31**
	Ethyl acetate	< 17
	Methanol	< 17
P. porcatus	Acetone	**19.93 ± 2.07**
	Ethyl acetate	**22.76 ± 0.80**
	Methanol	**18.47 ± 1.67**
P. prostratus	Acetone	**19.27 ± 0.12**
	Ethyl acetate	**18.41 ± 1.21**
	Methanol	**20.49 ± 1.21**
P. ecklonii	Acetone	< 17
	Ethyl acetate	< 17
	Methanol	< 17
P. neochilus	Acetone	< 17
	Ethyl acetate	< 17
	Methanol	<17
P. grandidentatus	Acetone	< 17
	Ethyl acetate	< 17
	Methanol	< 17
P. madagascariensis	Acetone	< 17
	Ethyl acetate	< 17
	Methanol	< 17
Negative control	DMSO 0.1% [a]	17.0 ± 0.05
Positive control	Tacrine [b]	**95.65 ± 2.89**

[a] The negative control: DMSO at 0.1% (v/v) with HEPES buffer 50mM (pH 8.0);

[b] Positive control: Tacrine 3.0 µM.

As for PO acetone extract, the outcome of AChE mild inhibition was probably due to the presence of some labdane diterpenes with forskolin-like structure. These compounds have not been previously studied on AChE inhibition due to their low quantity, but were isolated in higher yields from PO acetone extract, as previously described elsewhere (Rijo *et al*., 2005). Also, it is known that they activate adenylate cyclase in cooperation with a wide range of neurotransmitters (Bone and Mills, 2013), what could justify their further studies.

Regarding *P. porcatus*, there is only one phytochemical study providing information on the main isolated compound from the acetone extract (Maria Fátima Simões, 2010), namely (13*S*,15*S*)-6β,7α,12α,19-tetrahydroxy-13β,16-cyclo-8-abietene-11,14-dione **(6)** as shown in Table 2. On the other hand, *P. prostratus* simply has literature report on ethnomedical uses (Lukhoba *et al*., 2006). Therefore, more phytochemical studies for these two *Plectranthus* spp. are necessary to identify the causative agents for the registered mild AChE inhibition of *Plectranthus* spp. organic extracts.

III.3.2 *Plectranthus* spp. extracts and isolated compounds in tyrosinase inhibition *in vitro*

In the *in vitro* anti-tyrosinase assay, the enzymatic activity was evaluated by using L-tyrosine as substrate, and detecting the produced chromophore (L-DOPA) at 450 nm (Yamauchi *et al*., 2011).

The data from this *in vitro* assay represented as the inhibition percentage of tyrosinase, calculated with the equation 3b) at section II.6.2, in comparison with the enzymatic activity of the negative control (ΔAbs_{450nm}/min).

According to the results expressed in Figure 11, methanol (25-66%) and acetone (27-68%) extracts had the highest tyrosinase inhibition ($p<0.05$).

This is the first report on the activity of the *Plectranthus* spp. organic extracts over tyrosinase activity (14.73% to 75.72%), and the results are very promising when compared with 92.87 ± 7.28% inhibition held by kojic acid. For *P. grandidentatus* (PG), the acetone extract caused 67.96 ± 3.55% of tyrosinase inhibition, *P. ecklonii* (PE) methanol extract showed 65.95 ± 3.42%, and *P. saccatus* (PS) acetone held 56.42 ± 5.68% of anti-tyrosinase activity.

In fact, (PE) and (PG) had shown the highest anti-tyrosinase activity *in vitro*, and these were also the plants that showed increased antioxidant activity. These results could be possibly explained by the abietane diterpenes, mainly present in organic extracts of these two plants (Rijo et al., 2012; Rosa et al., 2015).

Pursuing a more extensive comprehension of the agents causing tyrosinase inhibition, the aqueous extracts obtained from *Plectranthus* spp. plants, were additionally tested, and the results are expressed in Figure 12.

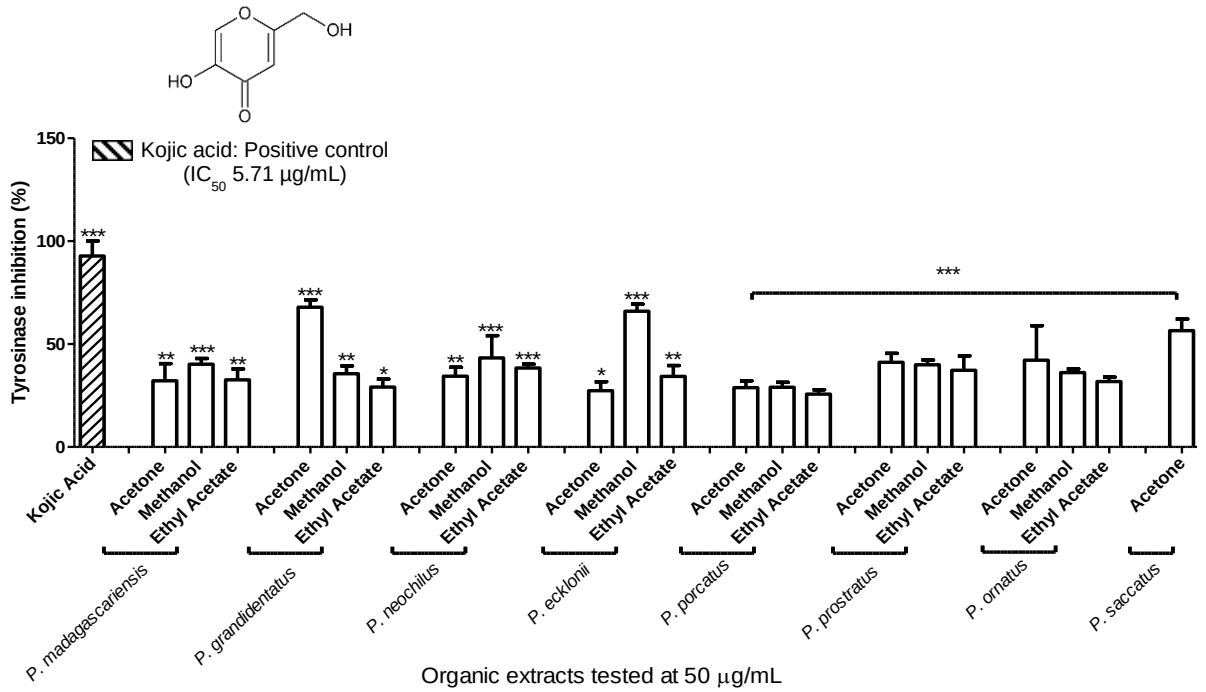


Figure 11 *In vitro* enzymatic assay on tyrosinase inhibition of *Plectranthus* spp. organic extracts. The assay uses L-tyrosine as substrate and detects the production of L-DOPA. Twenty-two organic extracts were tested at 50 µg/mL, in triplicate, for their ability to inhibit tyrosinase. The results are presented as means percentage values, considering the absorbance of kojic acid as the positive control. Data are expressed as the mean ± SD (n=3) **p*<0.05 ***p*<0.005 ****p*<0.0001 *vs* negative control (DMSO 0.5% (v/v) in PBS buffer). Values were determined by one-way ANOVA followed by Tukey HSD comparison test.

In contrast with the results on Figure 11, the aqueous extracts on the represented Figure 12 had a less effect on tyrosinase (*p*>0.05) thus lower inhibitory percentages were obtained, except for *P. porcatus* (*p*<0.0001). The aqueous extract of *P. porcatus* (PP) was able to inhibit tyrosinase by 65.04 ± 8.67%, and in comparison, with kojic acid (92.87 ± 7.28%) this is a very curious result.

Actually, PP aqueous extracts obtained with microwave extraction have been previously evaluated by HPLC profile, concerning polyphenols quantification (Rijo *et al*., 2014a). From those studies, it is known that PP aqueous extracts have some amounts of rosmarinic and caffeic acids, but it was PE and PS that were reported with the highest polyphenol content (Rijo *et al*., 2014a). Therefore it can only be suggested that the extract activity was probably due to a synergistic effect of the present compounds.

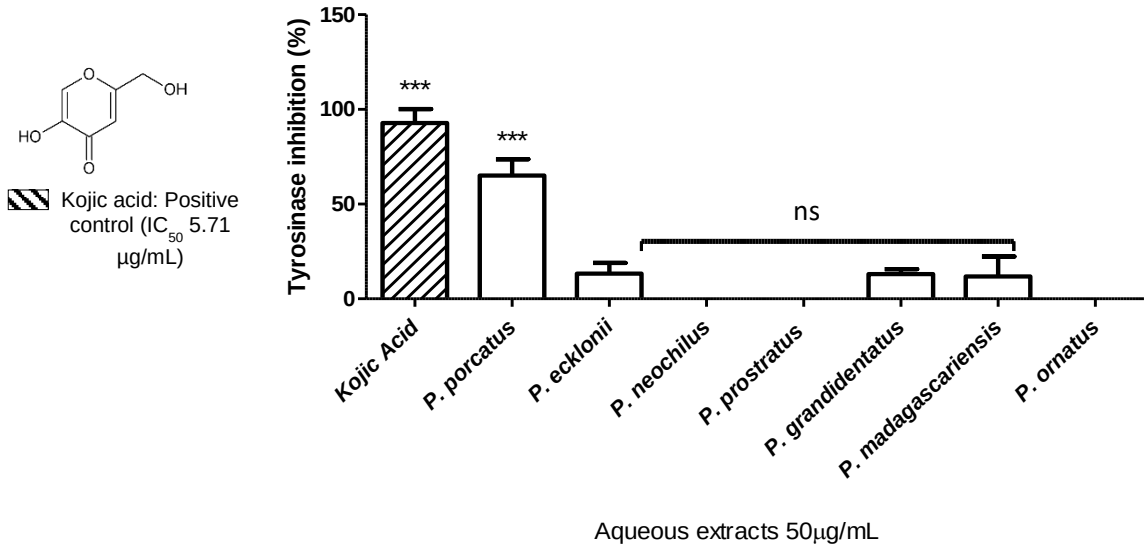


Figure 12 *In vitro* enzymatic assay on tyrosinase inhibition of *Plectranthus* spp. aqueous extracts. The assay uses L-tyrosine as substrate and detects the production of L-DOPA. Seven aqueous extracts were tested at 50 µg/mL, in triplicate, for their ability to inhibit tyrosinase. The results are presented as means percentage values, considering the absorbance of kojic acid as the positive control. Data are expressed as the mean ± SD (n=3) ns: not significant ***p<0.0001 *vs* negative control (PBS buffer). Values were determined by one-way ANOVA followed by Tukey HSD comparison test.

To better understand the extracts obtained results, one additional assay was performed to evaluate which natural products present in the organic and aqueous extracts, could be responsible for the exhibited anti-tyrosinase activity. For this reason, previously isolated compounds from both the organic and aqueous extracts were tested, for their ability to inhibit tyrosinase *in vitro*. The results are shown in Figure 13.

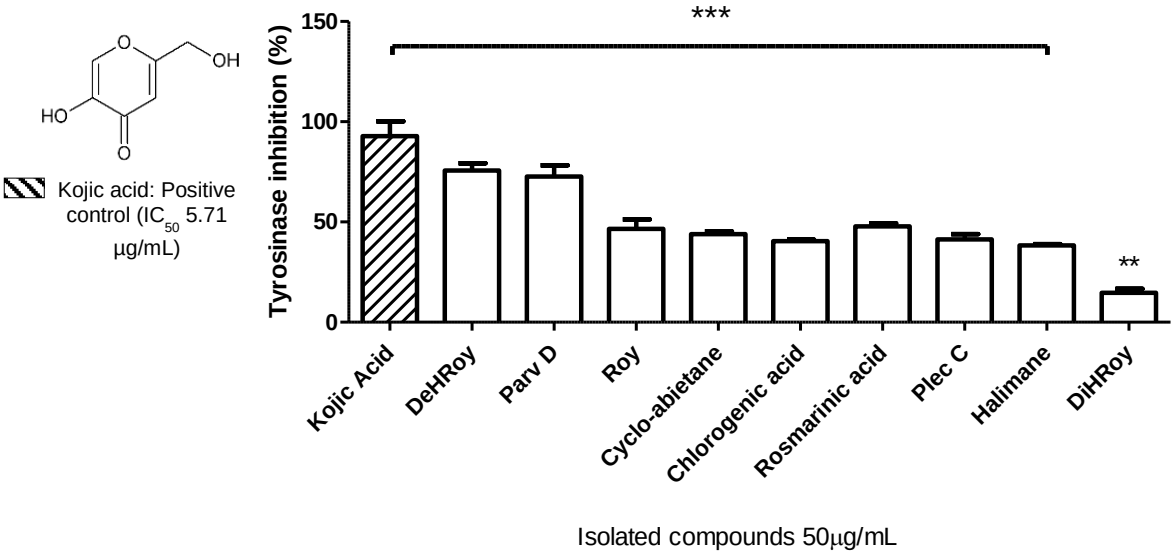


Figure 13 *In vitro* enzymatic assay on tyrosinase inhibition of *Plectranthus* spp. isolated compounds. The assay uses L-tyrosine as substrate and detects the production of L-DOPA. Nine compounds were tested at 50 µg/mL, in triplicate, for their ability to inhibit tyrosinase. Rosmarinic **(12)** and chlorogenic **(11)** acids are mostly isolated from aqueous extracts, and the remaining **(1-7)** are obtained from organic extracts. The results are presented as means percentage values, considering the absorbance of kojic acid as the positive control. Data are expressed as the mean ± SD (n=3) **p<0.005 ***p<0.0001 *vs* negative control (PBS buffer, and DMSO 0.5% (v/v) in PBS buffer). Values were determined by one-way ANOVA followed by Tukey HSD comparison test. Parv D **(4)**; Roy **(1)**; Cyclo-abietane **(6)**; Plec C **(7)**; Halimane **(5)**; DeHRoy **(3)**; DiHRoy **(2)**.

The results of the isolated compounds on tyrosinase activity (Figure 13), confirm some of the the high inhibition values previously found for the extracts. Notably, the abietane diterpenes **(1)**, **(3)**, and **(4)** present in PG, PM, and PE (main organic extracts) seem to be highly active against tyrosinase activity in more than 46% up to 75%.

Moreover, it is possible to better understand the effect of PP aqueous extract, according to the result of compound **(6)** holding 43.97 ± 1.34% of tyrosinase inhibition, although, this is a compound mainly isolated from the acetone extract of PP that showed low tyrosinase inhibition in Figure 12 (28.81 ± 3.41, $p>0.05$). It is possible though, that compound **(6)** can be present in the extract, since the microwave aqueous extraction has a higher efficiency in recovering bioactive compounds (Rijo *et al*., 2014c). Additionally, rosmarinic **(12)** and chlorogenic acids **(11)** were able to inhibit tyrosinase by 47.87 ± 1.41%, and 40.39 ± 0.74%, respectively. Knowing that these compounds are not present in high amounts in PP aqueous extracts, it can only be suggested that the extract activity was probably due to the abietane compound, cyclo-abietane **(6)**.

Overall, these results suggest that *Plectranthus* spp. natural product (polyphenols and abietane diterpenes) are notably able to inhibit tyrosinase almost as efficient as positive control kojic acid.

The mechanisms of inhibition were not studied, nor the type of inhibition exerted. The most potent tyrosinase inhibitors, such as kojic acid, act in the enzyme active site through metal chelation (Chen *et al*., 2015), thus the catalytic activity is significantly impaired. Likewise, hydroquinones and phenols (that are part of the chemical structure of abietane diterpenes, rosmarinic acid, and chlorogenic acid), have been recognized for their chelating ability (Chen *et al*., 2015), which can probably explain their elevated anti-tyrosinase activity. Actually, due to the presence of polyphenolic acids compounds such as chlorogenic acid and quercetin, other plants have been used for the treatment of skin depigmentation (Ghimeray *et al*., 2015). Therefore, it is possible to suggest that *Plectranthus* spp. natural products could be useful in a synergistic use for both antioxidant and anti-pigmentation skin treatment.

III.3.3 *Plectranthus* spp. extracts and isolated compounds in collagenase inhibition *in vitro*

The *in vitro* assay to examine collagenase (ChC) activity, obtained from *Clostridium histolyticum*, was performed using FALGPA as substrate.

Several studies have reported the catalytic activity of collagenase in collagen breakdown, leading to skin damage and wrinkles (Ghimeray *et al*., 2015; Thring *et al*., 2009; Wahab *et al*., 2014). In this thesis, the ChC activity was measured by the decrease in absorbance of

FALGPA hydrolysis, and the relative inhibition was calculated according to equation 4b) described in section II.6.3.

The results expressed in Figure 14 represent the enzymatic assay, performed with twenty-one organic extracts from *Plectranthus* spp., that showed mild to high ChC inhibition (from 28-76%, p<0.0001). The plant extract with the highest anti-collagenase activity was *P. neochilus* (PN) methanol extract holding 76.43 ± 2.09%, in comparison with the positive control (EGCG) that inhibited ChC by 93.09 ± 5.27%. In addition, the organic extracts of *P. madagascariensis* (PM)*, P. grandidentatus* (PG), and *P. ecklonii* (PE) were effective at inhibiting ChC in more than 60%.

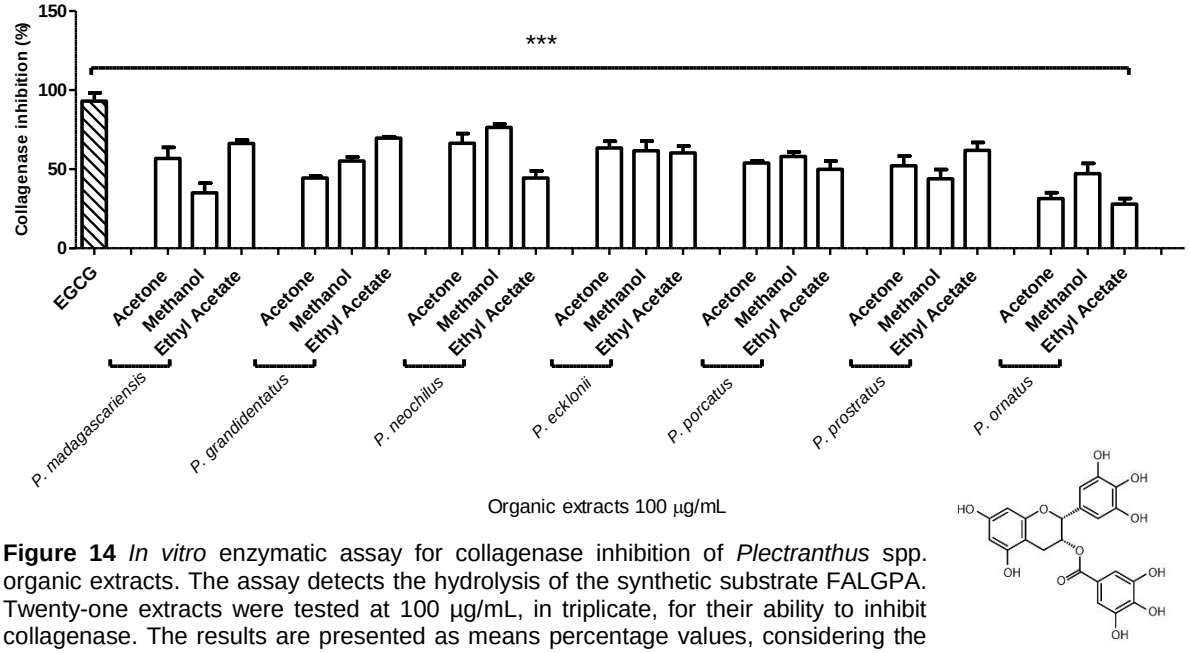


Figure 14 *In vitro* enzymatic assay for collagenase inhibition of *Plectranthus* spp. organic extracts. The assay detects the hydrolysis of the synthetic substrate FALGPA. Twenty-one extracts were tested at 100 µg/mL, in triplicate, for their ability to inhibit collagenase. The results are presented as means percentage values, considering the absorbance of **EGCG** as the positive control. Data are expressed as the mean ± SD (n=3) ***p<0.0001 *vs* negative control (DMSO 0.3% (v/v) in Tricine buffer). Values were determined by one-way ANOVA followed by Tukey HSD comparison test. EGCG – Epigallocatechin gallate.

The obtained results on PN organic extracts highly suggest that the anti-ChC activity observed, is mainly due to the naturally present pentacyclic triterpenes, particularly α- and β-amyrin **(14)**, previously reported for ChC inhibition (Ying *et al*., 1991). On the other hand, it is possible to recognize a high inhibition from PE organic extracts, most likely due to the presence of other pentacyclic triterpenes such as oleanolic and ursolic acids **(16)**, and/or abietane diterpenes **(1-4)**.

In fact, the compounds **(14)** and **(16)** have been widely studied for their ability to inhibit both collagenase and elastase, possibly by reversibly binding to the catalytic sites of these enzymes (Jung *et al*., 2014; Tu and Tawata, 2015; Ying *et al*., 1991).

Also, naturally present phenolic compounds have been described as collagenase inhibitors (Ghimeray *et al*., 2015). Therefore, the aqueous extracts from *Plectranthus* spp. plants

containing the larger amounts of these compounds, were studied for ChC inhibition, and the results are displayed in Figure 15.

In contrast with the organic extracts, the PN aqueous extract was the lowest ChC inhibitor with only 24.90 ± 9.37% (p>0.05), against the positive control EGCG holding inhibition of 93.09 ± 5.27%. Even though, the remaining aqueous extracts revealed more promising results, being the highest inhibition of 75.59 ± 6.5% from PE aqueous extract.

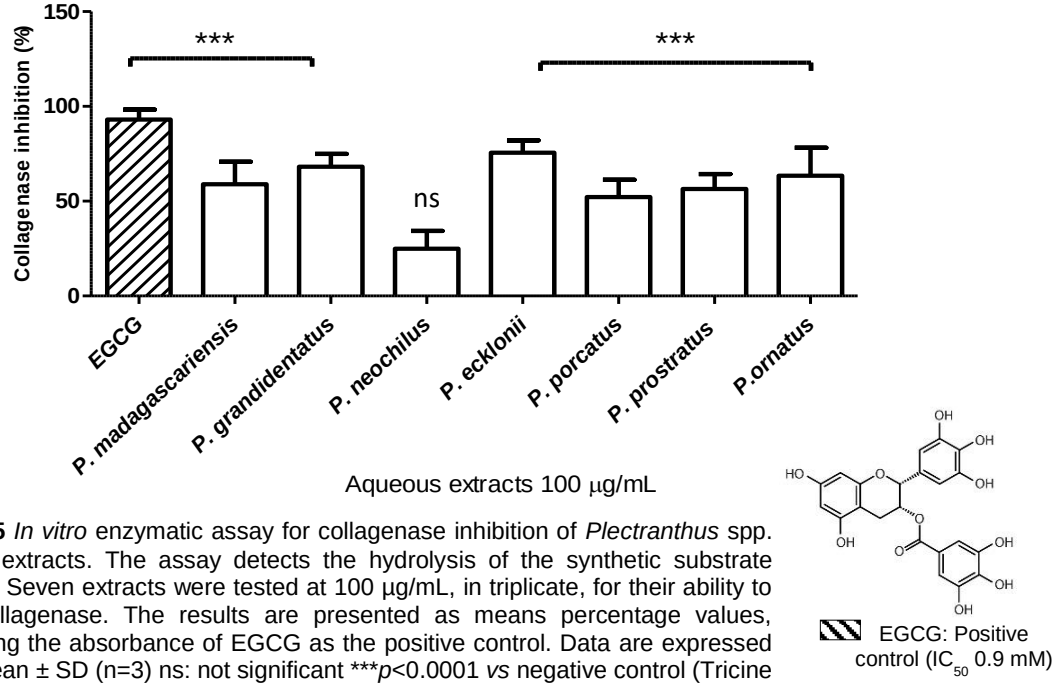


Figure 15 *In vitro* enzymatic assay for collagenase inhibition of *Plectranthus* spp. aqueous extracts. The assay detects the hydrolysis of the synthetic substrate FALGPA. Seven extracts were tested at 100 μg/mL, in triplicate, for their ability to inhibit collagenase. The results are presented as means percentage values, considering the absorbance of EGCG as the positive control. Data are expressed as the mean ± SD (n=3) ns: not significant ***p<0.0001 *vs* negative control (Tricine buffer). Values were determined by one-way ANOVA followed by Tukey HSD comparison test. EGCG – Epigallocatechin gallate.

Indeed, previous studies regarding the polyphenols quantification on *Plectranthus* spp. plants have established that PE aqueous extracts, from microwave extraction, had one of the highest content on rosmarinic acid **(12)** (Rijo *et al*., 2014a). This study helps to understand the increased collagenase inhibition reported here, since many polyphenolic compounds, such as catechin and epigallocatechin gallate (EGCG), have been reported to inhibit collagenase (Thring et al., 2009). Some suggestions in literature help understand that polyphenols can act as Zn^{2+} chelators, making the ion unavailable for catalytic activity (Jung *et al*., 2014; Thring *et al*., 2009).

The results generally showed that most of the *Plectranthus* extracts seem to exert an inhibitory effect on collagenase. To understand which compounds may be responsible for the activity shown, one more assay with the isolated compounds was performed, and the results are expressed in Figure 16.

From the obtained results, two isolated compounds with an abietane diterpene structure **(3)** and **(4)**, along with a polyphenol **(12)**, had the highest ability for collagenase inhibition

(p<0.0001). In fact, Parvifloron D **(4)** was almost as efficient as EGCG (93.09 ± 5.27%), retaining ChC activity by 84.64 ± 5.91%. This is the first report on the ChC *in vitro* inhibition by diterpenes with an abietane backbone.

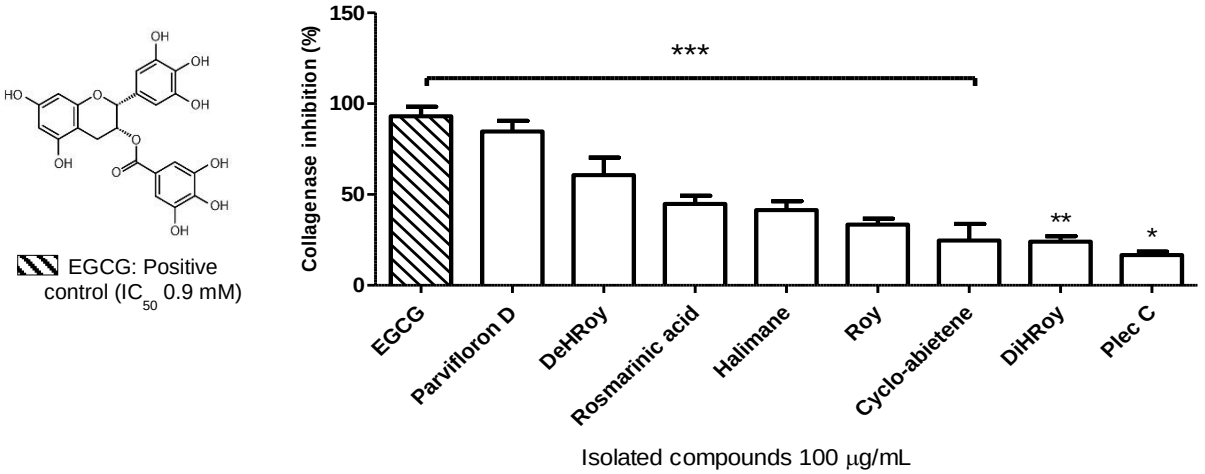


Figure 16 *In vitro* enzymatic assay for collagenase inhibition of *Plectranthus* spp. isolated compounds. The assay detects the hydrolysis of the synthetic substrate FALGPA. Eight compounds were tested at 100 μg/mL, in triplicate, for their ability to inhibit collagenase. The results are presented as means percentage values, considering the absorbance of EGCG as the positive control. Data are expressed as the mean ± SD (n=3) *p<0.05 **p<0.005 ***p<0.0001 *vs* negative control (Tricine buffer, and DMSO 0.3% (v/v) in Tricine buffer). Values were determined by one-way ANOVA followed by Tukey HSD comparison test. **EGCG** – Epigallocatechin gallate. Rosmarinic acid **(12)** is mostly isolated from aqueous extracts, and the remaining **(1-7)** are obtained from organic extracts. Parvifloron D **(4)**; DeHRoy **(3)**; Halimane **(5)**; Roy **(1)**; Cyclo-abietane **(6)**; DiHRoy **(2)**; Plec C **(7)**.

Different results were obtained for DiHRoy **(2)** and Roy **(1)**, that only inhibited ChC by 24.04 ± 3.02% (p<0.005) and 33.45 ± 3.25% (p<0.0001), which are also abietane diterpenes but with a royleanone motif, that may be lowering the inhibitory capacity. One possible explanation could be taking into consideration that ChC inhibitors mainly act by metal chelation. There is one particular feature for Parvifloron D **(4)** differently apart from the other abietane, which is a structure including different donor atoms, resulting in higher metal chelation (Rosa *et al*., 2015).

Rosmarinic acid **(12)** inhibited collagenase by 44.78 ± 4.53% (p<0.0001), suggesting the presence of other polyphenols such as chlorogenic acid or caffeic acid in the aqueous extracts to possibly justify the reported activity of Figure 15.

Besides metal chelation, polyphenols can be acting through the hydroxyl and/or benzene group, forming hydrogen bonds, or through hydrophobic interaction with ChC functional groups (Wahab *et al*., 2014). Thus, *Plectranthus* spp. plants seem to be highly promising for developing potential cosmetic agents against skin ageing induced by increased collagenase activity.

III.3.4 *Plectranthus* spp. extracts and isolated compounds in elastase inhibition *in vitro*

Besides collagen, elastin also plays an important role in the dermis matrix. Because ROS can induce the expression of proteinases, their activation (e.g., matrix metalloproteinases and serine proteases) may be involved in the lack of skin elasticity (Wahab *et al*., 2014). It has been shown, that damage to the elastic fiber network in the skin of hairless mice, was responsible for wrinkling of UVB-exposed skin (Lee *et al*., 2001).

Since elastin degradation leads to line and wrinkle formations in the skin, agents that inhibit elastase activity are ideal candidates for the treatment or prevention of skin aging (Lee *et al*., 2001; Wahab *et al*., 2014).

In this assay, elastase from porcine pancreas was used with the synthetic substrate *N*-sucynil-Ala-Ala-Ala-*p*NA (SANA), knowing that 1 U enzyme converts 1 µmol of SANA per minute with Tris-HCl buffer (pH 8.0) at 25ºC. The assay detected at 405 nm the increasing of absorbance, by the hydrolisis of SANA, producing *p*-nitroaniline.

Ursolic acid was used as a positive control, since it is known for more that two decades, that several pentacyclic triterpenoid metabolites of plant origin are great elastase inhibitors. Ursolic acid, the most potent of these compounds, was reported to have an inhibition constant of 4-6 µM (Ying *et al*., 1991).

Results for the enzymatic assay regarding the organic extracts from *Plectranthus* spp. plants are expressed in Figure 17. In contrast to what has been observed in the previous enzymatic assays developed in this thesis, the organic extracts were not good elastase inhibitors, since the maximum inhibition obtained was 42.84 ± 4.18% from *P. grandidentatus* ethyl acetate.

Overall the extracts inhibited elastase in about 30% ($p<0.0001$), with exception for *P. madagascariensis* (PM) organic extracts that held no elastase inhibition, as well as *P. grandidentatus* (PG) acetone extract ($p>0.05$). The positive control used in this assay was ursolic acid inhibited elastase by 69.85 ± 3.65%.

Additionally, *P. neochilus* (PN) and *P. ecklonii* (PE) extracts also revealed mild elastase inhibition, possibly due to the presence of different types of triterpenes such as α- and β-amyrin **(14)**, oleanolic acid and ursolic acid **(16)**.

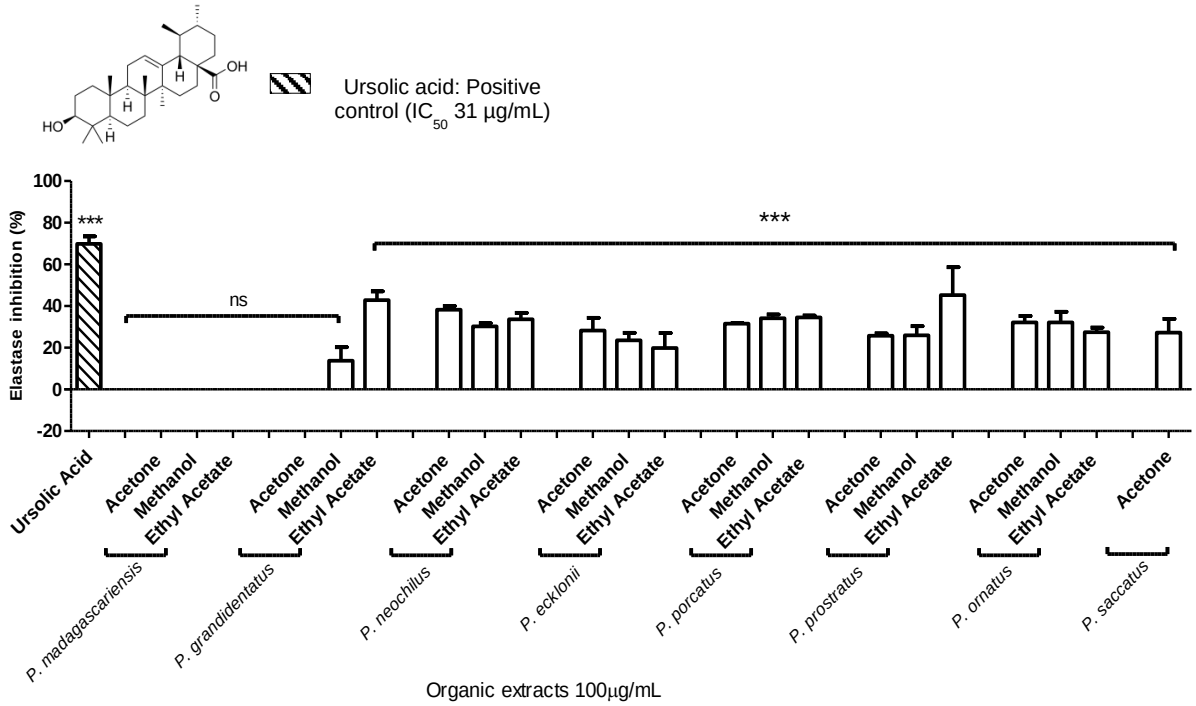


Figure 17 *In vitro* enzymatic assay for elastase inhibition of *Plectranthus* spp. organic extracts. The assay was performed using SANA as substrate, and detects the formation of *p*-nitroaniline at 405 nm. Twenty-two organic extracts were tested at 100 µg/mL, in triplicate, for their ability to inhibit elastase. The results are presented as means percentage values, considering the absorbance of ursolic acid as the positive control. Data are expressed as the mean ± SD (n=3) ns: not significant ***p<0.0001 *vs* negative control (DMSO 1% (v/v) in Tris-HCl buffer). Values were determined by one-way ANOVA followed by Tukey HSD comparison test.

Moreover, since previous studies have suggested that polyphenolic compounds have anti-elastase activity by the interaction of the hydroxyl groups with the elastase domain (Lee *et al*., 2001), the aqueous extracts were tested and the results are shown in Figure 18.

The results on Figure 18 revealed that the aqueous extracts were not effective upon elastase activity (p>0.05), being PG aqueous extract the only one holding 23.82 ± 9.99% of anti-elastase activity (p<0.0001). This result was possibly due to different and improved synergy of the polyphenols present in PG aqueous extract, in comparison with the remaining aqueous extracts (Rijo *et al*., 2014a).

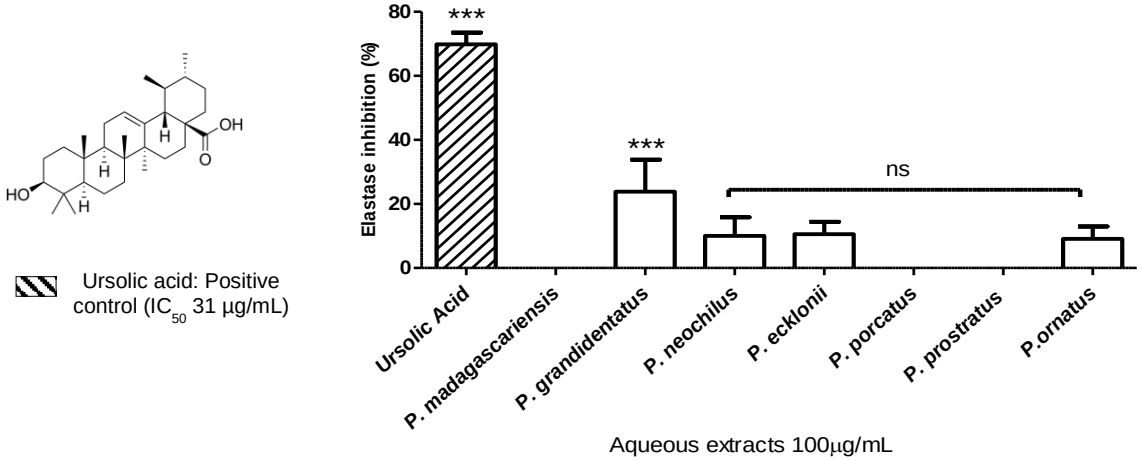


Figure 18 *In vitro* enzymatic assay for elastase inhibition of *Plectranthus* spp. aqueous extracts. The assay was performed using SANA as substrate, and detects the formation of *p*-nitroaniline at 405 nm. Seven aqueous extracts were tested at 100 µg/mL, in triplicate, for their ability to inhibit elastase. The results are presented as means percentage values, considering the absorbance of ursolic acid as the positive control. Data are expressed as the mean ± SD (n=3) ns: not significant ***p<0.0001 *vs* negative control (Tris-HCl buffer). Values were determined by one-way ANOVA followed by Tukey HSD comparison test.

The isolated compounds were further assessed for their ability to inhibit elastase, and in contrast to what was observed in the extracts (Figures 17 and 18), the tested compounds highly inhibited elastase. The results are expressed in Figure 19.

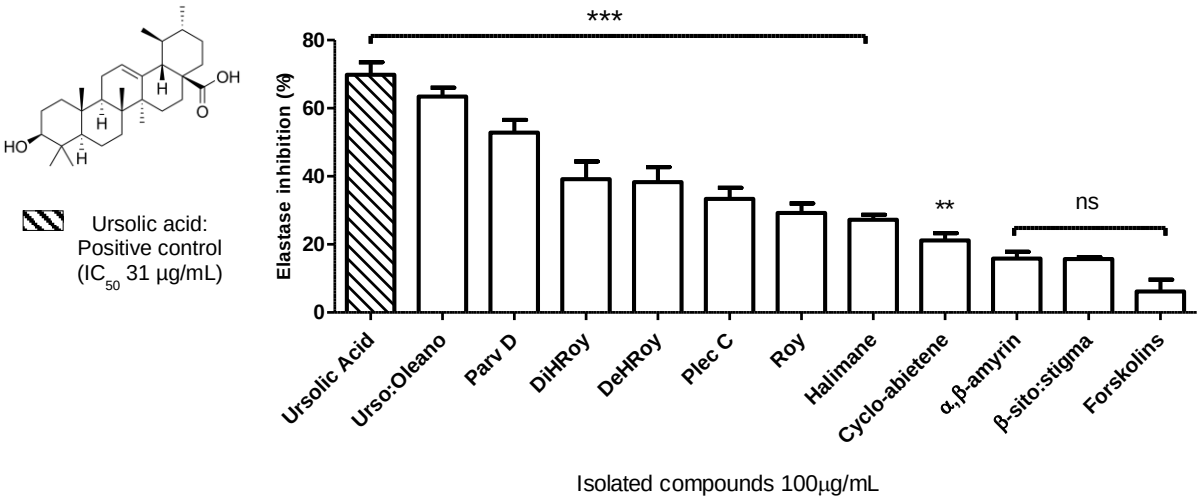


Figure 19 *In vitro* enzymatic assay for elastase inhibition of *Plectranthus* spp. isolated compounds. The assay was performed using SANA as substrate, and detects the formation of *p*-nitroaniline at 405 nm. Eleven compounds were tested at 100 µg/mL, in triplicate, for their ability to inhibit elastase. The results are presented as means percentage values, considering the absorbance of ursolic acid as the positive control. Data are expressed as the mean ± SD (n=3) ns: not significant ***p*<0.005 ****p*<0.0001 *vs* negative control (DMSO 1% (v/v) in Tris-HCl buffer). Values were determined by one-way ANOVA followed by Tukey HSD comparison test. Urso:Oleano **(16)**; Parvifloron D **(4)**; DiHRoy **(2)**; DeHRoy **(3)**; Plec C **(7)**; Roy **(1)**; Halimane **(5)**; Cyclo-abietane **(6)**; α,β-amyrin **(14)**; β-sito:stigma **(15)**; Forskolins **(13)**.

In accordance with the positive control, ursolic acid holding 69.85 ± 3.65% of elastase inhibition, the 1:4 mixture of oleanolic:ursolic acid also had an high anti-elastase activity with 63.42 ± 2.56% (*p*<0.0001). These preliminary results strengthen the literature reports obtained so far on the pentacyclic triterpenes interaction on elastase, possibly involving different subsites (Lee *et al*., 2001). In contrast, other compounds with pentacyclic triterpene similar to that ursolic acid like α- and β-amyrin **(14)**, was anticipated to have higher anti-elastase activity (*p*>0.05), according to previous studies (Thring *et al*., 2009). This is the first report on elastase inhibition of ursolic and oleanolic acids, isolated from *Plectranthus* spp. plants.

Following the triterpenes, royleanone-like diterpenes were also very effective in elastase inhibition (*p*<0.0001). These compounds such as Parvifloron D **(4)** 52.83 ± 3.76%, DiHRoy **(2)** 39.16 ± 5.18%, and DeHRoy **(3)** 38.33 ± 4.40%, have high elastase inhibitory activity. These *in vitro* inhibition assays suggest that the *Plectranthus* spp. plants with agents acting in elastase inhibition, are possible candidates for the treatment or prevention of skin photoaging.

The obtained preliminary results of the three skin-related enzymatic inhibitions *in vitro* are displayed in Table 7, suggesting that *Plectranthus* spp. natural products are promising agents for future formulations regarding hyper-pigmentation, wrinkle, and sagging of the skin.

Table 7 Results comparison for *in vitro* inhibition of tyrosinase, elastase, and collagenase skin-related enzymes screening, of isolated compounds from *Plectranthus* spp. The results are expressed as enzymatic inhibition ± standard deviation (SD).

	Enzymatic Inhibition ± SD (%)		
Isolated compounds	**Anti-Tyrosinase ****	**Anti-Elastase***	**Anti-Collagenase***
Roy (1)	**46.62 ± 4.74**	29.26 ± 2.75	33.45 ± 3.25
DiHRoy (2)	14.73 ± 2.02	**39.16 ± 5.18**	24.04 ± 3.02
DeHRoy (3)	**75.72 ± 3.61**	**38.33 ± 4.40**	**60.63 ± 9.68**
Parvifloron D (4)	**72.73 ± 5.63**	**52.83 ± 3.76**	**84.64 ± 5.91**
Halimane (5)	38.26 ± 0.63	27.27 ± 1.41	**41.50 ± 4.87**
Cyclo-abietane (6)	**43.97 ± 1.34**	21.19 ± 2.09	24.63 ± 9.18
Plectrornatine C (7)	41.29 ± 2.71	33.34 ± 3.30	16.70 ± 1.87
Chlorogenic acid (11)	40.39 ± 0.73	NT	NT
Rosmarinic acid (12)	**47.97 ± 1.41**	NT	**44.78 ± 4.53**
Forskolins (13)	NT	6.15 ± 3.49	NT
α,β-amyrin (14)	NT	15.83 ± 1.99	NT
β-sitosterol:stigmasterol (15)	NT	NT	NT
Oleanolic:Ursolic acid (16)	NT	**63.45 ± 2.56**	NT
Positive Control	**92.87 ± 7.28 / KOJ**	**69.85 ± 3.65 / URS**	**93.09 ± 5.27 / EGCG**

NT – Not Tested; **KOJ** – Kojic acid; **URS** – Ursolic acid; **EGCG** – Epigallocatechin gallate

*Samples tested at 100 µg/mL; **Samples tested at 50 µg/mL

Roy(1)– 7α-acetoxy-6β-hydroxyroyleanone; **DiHRoy(2)**– 6β,7α-dihydroxyroyleanone; **DeHRoy(3)**– 6,7-dehydroroyleanone; **Halimane(5)**– 11*R**-acetoxy-halima-5,13*E*-dien-15-oic acid; **Cyclo-abietane(6)**– (13*S*,15*R*)-6β,7α,12α,19-tetrahydroxy-13β,16-cyclo-8-abietane-11,14-dione; **Plectrornatine C(7)**– 1α,6β-diacetoxy-8α,13*R**-epoxy-14-labden-11-one; **Forskolins(13)**– 1,6-di-O-acetylforskolin+1,6-di-O-acetyl-9-deoxyforskolin (mixture 1:1).

III.4 Anti-inflammatory study on isolated compounds by NO quantification with Griess assay

Plectranthus spp. isolated compounds were tested for their ability to decrease nitric oxide (NO) production, after induced inflammation in macrophage RAW 264.7 cell line, using lipopolysaccharide (LPS). After cytotoxic screening with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (see section II.7.1), eight of the total ten compounds proceeded to anti-inflammatory assay *in vitro* (section II.7.2).

III.4.1 MTT cytotoxicity assay of isolated compounds on RAW 264.7 cells

In this study, the cytotoxicity of the tested isolated compounds was evaluated. Preceding the *in vitro* anti-inflammatory activity, the cell viability assay was performed with the compounds **(1)**, **(3-4)**, **(6-8)**, and **(12-15)**, at several concentrations (100, 50, 25, 12.5, 10, 5, 2.5 and 1 µM),

to assess the capability of RAW 264.7 macrophage cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). This reaction detected the formazan crystals produced by living cells, at 590 nm after dissolution in DMSO (Rodrigues *et al*., 2014, 2016).

The results of the MTT assay are displayed in Appendix Figure I.1. These results show the cell viability after 24-hours treatment with the compounds at several concentrations.

The graphics depict, in black bars, the concentration at which cell viability was higher than 80%, in comparison with the negative control DMSO 0.5% (v/v) in RPMI medium as 100% of cellular viability.

From Figure I.1 resulted that at the highest concentration of 100 µM, rosmarinic acid **(12)** was the single compound holding 79.49 ± 4.31 % of cell viability, followed by, halimane **(5)**, Plectrornatine C **(7)**, cyclo-abietane **(6)**, β-sitosterol:stigmasterol **(15)**, and α,-β-amyrin **(14)**, which were nontoxic at 50 µM.

Regarding DeHRoy **(3)** and forskolin type compound **(13)**, they only held up to 86.12 ± 1.30% and 94.27 ± 3.89% of respective cell viability, in concentrations lower than 10 µM, and 12.5 µM, respectively.

Finally, Parvifloron D **(4)** and Roy **(1)** exhibited low cell viability, being only safe at 1 µM (87.18 ± 11.70%), and 5 µM (80.15 ± 6.50%), respectively.

Due to some uncertainty of percentage values, observed by the elevated standard deviations of these two results, compounds **(1)** and **(4)** did not proceed to further anti-inflammatory testing.

This is the first report of cytotoxicity values for RAW 264.7 cells with the isolated compounds from *Plectranthus* spp., and the preliminary IC_{50} values are expressed in Table 8, in comparison with the positive control epimuqubilin A (Cheenpracha *et al*., 2010).

The lowest IC_{50} values belong to Parvifloron D **(4)** with 7.41 µM, and to Roy **(1)** with 9.40 µM. In comparison with the compound with lowest viability, DeHRoy **(3)** (IC_{50} 19.2 µM), compounds **(1)** and **(4)** are almost two-fold more toxic. Therefore, these two compounds did not continue to the anti-inflammatory assay, due to their elevated toxicity.

The remaining eight compounds proceeded to *in vitro* evaluation of nitric oxide (NO) production by stimulated RAW 264.7 cells.

Table 8 Cytotoxicity evaluated by the MTT assay for the isolated compounds. The results represent the IC_{50} values (µM) of isolated compounds tested on RAW 264.7 macrophage cell line. Data in bold represent the two most cytotoxic compounds.

Samples	IC_{50} (µM)
Epimuqubilin A*	37.1
Halimane (5)	47.8
Parvifloron D (4)	**7.41**
Plectrornatine C (7)	64.8
DeHRoy (3)	19.2
Cyclo-abietene (6)	>100
Forskolins (13)	43.5
Roy (1)	**9.40**
Rosmarinic Acid (12)	>100
β-sitosterol:stigmasterol (15)	>100
α-, β-amyrin (14)	>100

* Epimuqubulin A was considered as the positive control at the tested concentration of 51µM.

III.4.2 NO production upon inflammation on RAW 264.7 cells after treatment with non-cytotoxic isolated compounds

During inflammation, macrophages play an essential role in the release of pro-inflammatory factors such as NO, ROS, NF-κB, iNOS, and cycloxygenase-2 (COX-2), and pro-inflammatory cytokines like, interleukin-1β (IL-1β), interleukin-6 (IL-6), and TNF-α (Lee *et al*., 2013).

The macrophages RAW 264.7 cell line are chosen for many anti-inflammatory *in vitro* studies, since they express the TLR4 receptor that through incubation with lipopolysaccharide (LPS), can trigger production the of some inflammatory mediators (Balaji and Ramanathan, 2014), such as NO. In this context, NO production was measured in LPS-stimulated RAW 264.7 macrophage cells, after treatment with nontoxic concentrations of eight isolated compounds. Therefore, a reduction in NO production after treatment with the compounds would be indicative of the potential to attenuate an inflammatory response.

In this study, the isolated compounds were tested at the maximum concentration at which they were non-cytotoxic, defined in section III.5.1. The results on NO production are expressed in Figure 20. The results express NO quantification (µM) of LPS-stimulated RAW 264.7 macrophages, after incubation with compounds (at nontoxic concentrations), in comparison with the negative controls, DMSO 0.5% (v/v) in RPMI medium (without phenol red) without LPS, and DMSO 0.5% (v/v) supplemented with LPS, and the positive control, a known inhibitor of NO production upon inflammation, L-NAME (Wang and Leigh, 2006).

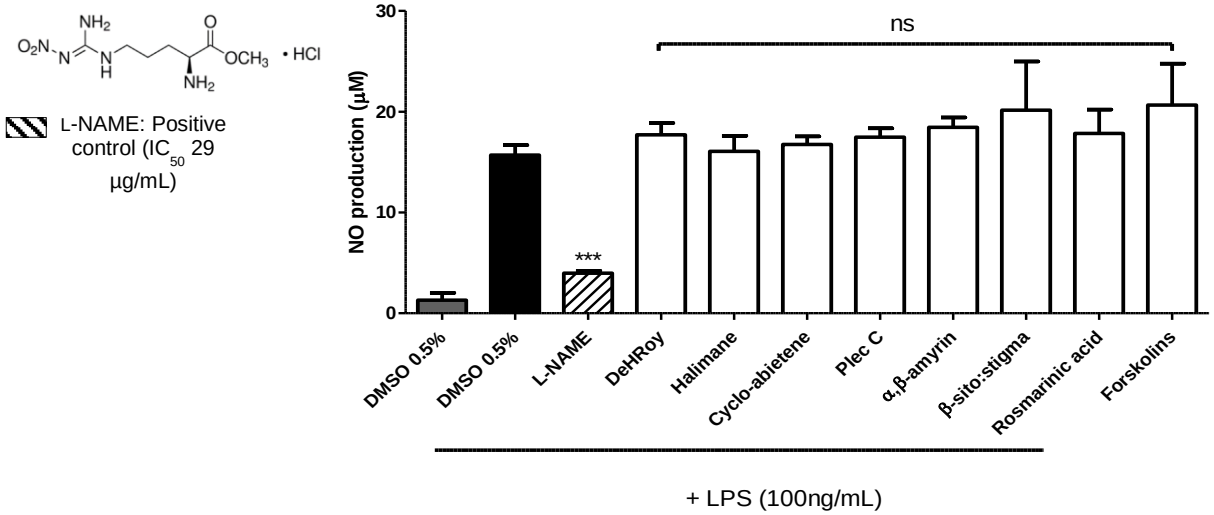


Figure 20 Anti-inflammatory assay *in vitro*, on macrophage cells RAW 264.7. The inflammation process was induced by adding LPS to the cells medium during 24 hours, together with nontoxic compounds for treatment. The medium was mixed with the Griess reagent for nitrite quantification at 540 nm. The grey bar represents the negative control (DMSO 0.5% (v/v) in RPMI without phenol red) before adding LPS, and the black bar the negative control after adding LPS. The results are presented as means concentration values, considering the absorbance of L-NAME at 29 µg/mL, as the positive control. Data are expressed as the mean ± SD (n=6) ns: not significant, ***p<0.0001 *vs* negative controls (DMSO 0.5% (v/v) in RPMI without phenol red, with and without LPS). Values were determined by one-way ANOVA followed by Tukey HSD comparison test. DeHRoy **(3)**; Plec C **(7)**; Halimane **(5)**; Cyclo-abietane **(6)**; α,β-amyrin **(14)**; β-sito:stigma **(15)**; Forskolins **(13)**; Rosmarinic acid **(12)**.

The nitrite quantification was evaluated with the Griess reaction and measured at 540 nm, using a calibration curve obtained with several solutions of known concentrations (1.6, 3.1, 6.25, 12.5, 25, 50, and 100 µM), using sodium nitrite used as standard.

With this assay it was possible to observe that the isolated compounds were not able to reduce the NO production after LPS-stimulated inflammation (p>0.05), in comparison with the positive control L-NAME.

The inflammation process generated NO at 15.70 ± 0.99 µM (negative control with DMSO 0.5% (v/v) supplemented with LPS), and the treatment with compounds maintained this NO quantities around 16.05 µM, and 20.16 µM. In truth it was observed a slight increase in NO production after treatment with some compounds, suggesting an intensification of the observed inflammation.

The obtained results in Figure 20 were far from the expected, since several natural products from *Plectranthus* plants have been reported for their anti-inflammatory activity (Chiu *et al*., 2012; Lukhoba *et al*., 2006; Rijo *et al*., 2013a). Forskolin-like compounds are specially notorious for their anti-inflammatory properties (Klatz and Goldman, 2003; Rijo *et al*., 2005). In addition, phytosterols such as β-sitosterol and stigmasterol, triterpenes (α- and β-amyrins, oleanolic and ursolic acids), here obtained from *P. ecklonii*, and polyphenols, have also been validated for anti-inflammatory purposes (Ghimeray *et al*., 2015; Pereira *et al*., 2015; Sergent *et al*., 2010).

Besides triterpenes and forskolins-like compounds, it was also expected that the polyphenolic compound tested, rosmarinic acid would demonstrate some anti-inflammatory

outcome. The polyphenolic compounds are considered to possess anti-inflammatory properties and have been proposed as an alternative natural approach to prevent or treat chronic inflammatory diseases (Sergent *et al*., 2010).

However, this is the first report on the anti-inflammatory effect by NO production tested *in vitro*, with macrophage cells, of such compounds isolated from *Plectranthus* spp. plants. Mainly, the results suggest that the compounds do not exert an anti-inflammatory response through the NO mechanism, although it does not mean that they do not possess anti-inflammatory activity at all. For sustainable conclusions, it would be necessary to further evaluate their effect on other inflammation mediators, for instance, in COX-2. This could be achieved for instance, by COX-2 enzymatic inhibition assay. The COX-2 enzymatic inhibition assay was attempted to be performed in this project, and the details are in Appendix II, although the protocol establishment is still under study.

III.5 *Mycobacterium tuberculosis* H37Rv growth with CFU assay

For the *Mycobacterium tuberculosis* H37Rv growth assay, peripheral blood mononuclear cells (PBMC) were isolated from random healthy volunteers, at the blood bank of University “La Sapienza” in Italy (Section II.8.1). The obtained monocytes from the LS column, were plated and incubated at 37ºC in a humidified with 5% CO_2 atmosphere, during 4 days for cell adhesion and differentiation.

The LDH cytotoxicity test was performed at the minimum inhibitory concentration (MIC), for each compound in *Mycobacterium tuberculosis* (Mtb), (Section II.8.2) proceeded by CFU analysis at 10^{-3} dilution after 13 days of infection and treatment (Section II.8.3).

III.5.1 LDH release (cytotoxicity assay) on macrophages derived from PBMC

The cytotoxicity assay measured the release of cytosolic lactate dehydrogenase (LDH) upon cell lysis. The enzyme LDH released in the supernatant, and by conversion of the tetrazolium salt into red formazan, was detected at 490 nm proportional to cell lysis.

This experiment was performed using previously reported minimum inhibitory concentration (MIC) for *M. tuberculosis*, described in Table 4 (Rijo et al., 2010, 2011). The experiment was performed with the CytoTox® 96 Non-Radioactive Cytotoxicity Assay kit from Promega. Staurosporine (STS) isolated from *Streptomyces staurospores*, is a protein kinase inhibitor that induces DNA fragmentation and apoptosis. Therefore, besides LDH 100% release, STS was also used as a positive control for the cytotoxicity assay, and was tested at 2 µM.

The results expressed in Figure 21, assisted the understanding on whether the compounds were cytotoxic for macrophages, so that false positives would be avoided in further studies.

Isoniazid (INH) and ethambutol (ETAN), the positive controls for Mtb infection, were also tested for their cytotoxicity to guarantee that there not toxic at a concentration 10-fold their MIC values (1 µg/mL, and 40 µg/mL, respectively).

From these results, it was observed that the positive control STS, resulted in macrophage cytotoxicity of 21.86% (p<0.0001). From the tested isolated compounds, Parvifloron D **(4)** was considered as a much more powerful cytotoxic agent that caused 52.46% of macrophage cell death, thus it did not proceed to Mtb growth assays (p<0.0001).

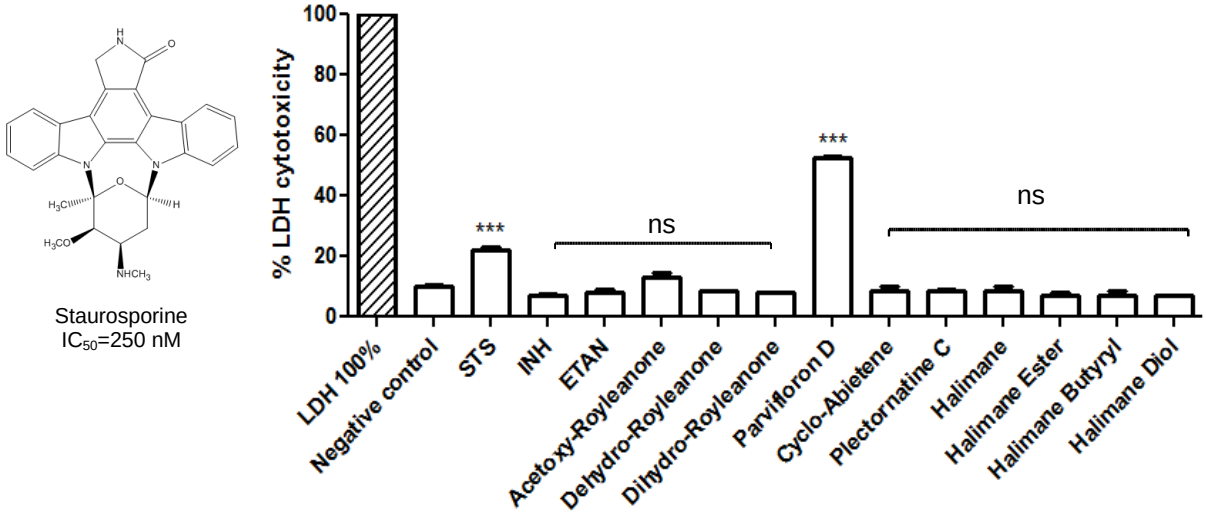


Figure 21 Cytotoxicity assay performed by LDH method of the *Plectranthus* isolated compounds. The negative control were untreated cells **(UNT)**, and two positive controls were used, 100% of LDH release (LDH positive control from kit), and staurosporine **(STS 2 µM)**. The positive control for Mtb infection isoniazid **(INH 1 µg/mL)**, and ethambutol **(ETAN 40 µg/mL)** were also tested. The absorbance measurement was performed 48-hours after treatment with controls and drugs used at MIC (25 µg/mL), with the exception of Parvifloron D **(4)** whose MIC was 15.6 µg/mL. All values represent mean ± SD (n=3). Values were determined by one-way ANOVA followed by Tukey HSD comparison test, ns: not significant, ***p<0.0001.
Acetoxy-Royleanone **(Roy, 1)**; Dehydro-Royleanone **(DeHRoy, 3)**; Dihydro-Royleanone **(DiHRoy, 2)**; Parvifloron D **(4)**; Cyclo-abietane **(6)**; Plectrornatine C **(7)**; Halimane **(5)**; Halimane ester **(8)**; Halimane butyryl **(9)**; Halimane diol **(10)**.

The remaining samples, including ETAN and INH, did not significantly revealed toxicity for macrophage cells (p>0.05), as they were similar to the negative control, the untreated cells (UNT). Thus, with exception for compound **(4)**, all samples were selected for the subsequent test of colony-forming units.

III.5.2 Colony-forming units assay for *Mycobacterium tuberculosis* H37Rv growth

Quantitative analysis of *Mycobacterium tuberculosis* (Mtb) growth via the determination of colony forming units (CFUs), from serially diluted suspensions, was the method of choice,

since this was an the initial evaluation of compounds for anti-tubercular activity (Cai *et al*., 2013).

After attaining the cytotoxicity results, monocytes were again obtained from PBMC isolation, and incubated at 37ºC in a humidified with 5% CO_2 atmosphere, during 4 days for cell adhesion and differentiation. After this time, the cells were infected with Mtb, in a biosafety level 3 laboratory. Due to the time consuming assay, and the timeline of this thesis, it was only possible to evaluate the effects on Mtb growth of compounds **(5)** and **(7-10)**.

Treatment with the compounds **(5)** and **(7-10)** started 2 hours after infection and was kept until the end of the experiment. The concentrations tested were according to the MIC values (25 µg/mL), described in Table 4. Viable bacteria were evaluated after 13 days of incubation at the 10^{-3} dilution. Values are expressed in Figure 22 as the logarithm of CFU/mL. The normalized preliminary results, expressed in Figure 22, of colony-forming units counts (dilution 10^{-3}) have shown very interesting results, especially regarding the compound a derivative of halimane **(9)**, available in the laboratory. The high efficacy in decreasing Mtb growth from compound **(9)** resulted in 2.1×10^5 CFU/mL, in comparison with the positive controls, ethambutol (2.0×10^5 CFU/mL) and isoniazid (1.2×10^5 CFU/mL), two known drugs used for Mtb infection treatment. The remaining compounds revealed approximately the same CFU counts as the non-treated control (6.0×10^5 CFU/mL).

Regarding the halimane diterpenes differences, compound **(9)** was obtained by hemi-synthesis from compound **(5)**, performing a reduction with lithium aluminum hydride. This difference can be the reason for some enantiomeric derivatives having higher affinity than TPP to the diterpene synthase encoded by Rv3378c (Chan *et al*., 2014; Hoshino *et al*., 2011). From all the compounds tested, **(9)** could be more suitable to interact with the binding site, thus inhibiting the production of tuberculosinols, ultimately avoiding the phagocytosis suppression (Layre *et al*., 2014; Mann and Peters, 2012), and leading to a decrease in CFU.

As future studies, is essential to better understand how compound **(9)** may be acting upon Mtb growth. Western blot and immunofluorescence methods can evaluate whether there is inhibition of the phagolysosome biogenesis, by arresting phagosome maturation.

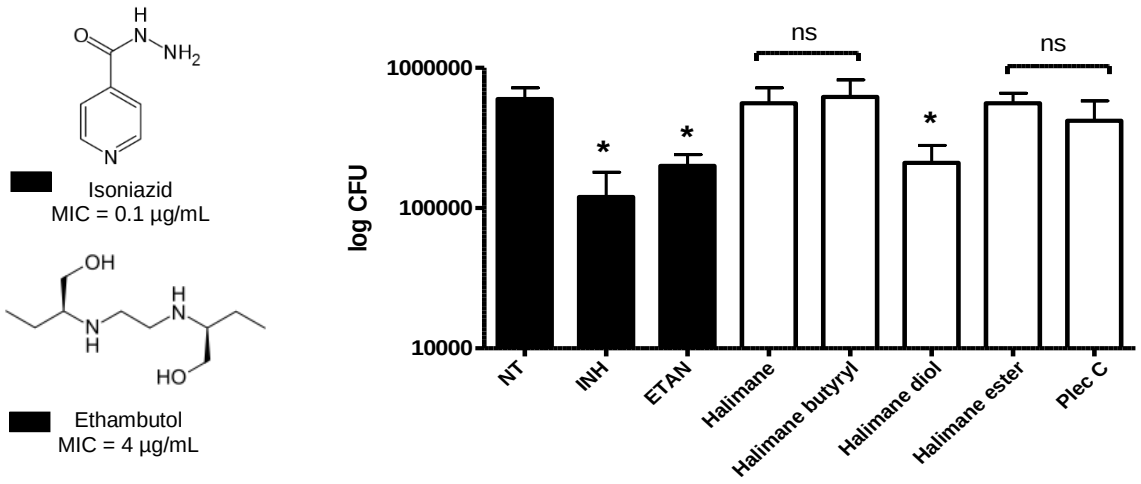


Figure 22 *In vitro* evaluation of the effect of isolated compounds on Mtb viability, on infected macrophages derived from PBMC, using the colony-forming units assay. Macrophages were treated with the positive controls, in a concentration 10-fold the MIC, isoniazid **(INH)** at 1 µg/mL, and ethambutol **(ETAN)** at 40 µg/mL, and isolated compounds at the MIC values (25 µg/mL), 2-hours after infection. In this assay *Plectranthus* isolated compounds **(5, 7-10)** were tested. The treatment was kept at 37ºC, and plated at serial dilutions of the bacterial suspensions on 7H10 agar plates. Viable bacteria were evaluated at 10^{-3} dilution, after 13 days of colony growth. The negative control were untreated **(UNT)** cells. All values represent mean ± SD (n=3). Values were determined by one-way ANOVA followed by Tukey HSD comparison test, ns: not significant, **p*<0.05. Plectrornatine C **(7)**; Halimane **(5)**; Halimane ester **(8)**; Halimane butyryl **(9)**; Halimane diol **(10)**.

In the Western blot assay, it is possible to study two autophagy markers, microtubule-associated protein 1 light chain 3 (LC3), that is translocated from the cytosol to the auto-phagosome membrane, and the autophagy adaptor protein p62/SQSTM1 (p62), that works in the initiation, and progression of auto-phagosome formation by mediating ubiquitination (Komatsu and Ichimura, 2010; Seto *et al*., 2013). For immunofluorescence assay, it would be necessary that macrophages were infected and treated with a possible lysosome inhibitor (for phagosome maturation with lysosome), and with rapamycin which is a known autophagy inducer.

IV. CONCLUSIONS AND FUTURE PERSPECTIVES

The presented thesis performed a screening of biological activities of eight *Plectranthus* spp. plants namely, *P. grandidentatus, P. ecklonii, P. ornatus, P. madagascariensis, P. porcatus, P. neochilus, P. prostratus* and *P. saccatus*. Previous isolated secondary metabolites and prepared extracts were subjected to several different assays concerning their antioxidant activity, *in vitro* inhibition of skin-related enzymes, reduction of NO concentration for anti-inflammatory assessment, and *Mycobacterium tuberculosis* H37Rv survival decrease.

All ultrasound assisted and microwave extracts (twenty-one organic extracts - methanol, ethyl acetate and acetone - and seven aqueous extracts) from each of the *Plectranthus* spp. were successfully obtained and assessed in the biological tests.

ROS can initiate complex molecular pathways for melanogenesis (pathway of tyrosinase), and activate extracellular matrix metalloproteases such as collagenase and elastase. Therefore, this is a primary evaluation of potential agents for a synergistic treatment (antioxidant and skin-related enzymatic inhibition) of skin disorders, regarding ageing, sagging, and hyper-pigmentation of the skin. The antioxidant activity by the DPPH radical scavenging assay, and *in vitro* inhibition of skin-related enzymes, namely anti-tyrosinase, -elastase, and -collagenase assays, enabled to conclude by the preliminary results that *P. grandidentatus* ethyl acetate (62.3 ± 0.43%)*, P. ecklonii* methanol (75.9 ± 1.02%)*,* and *P. madagascariensis* methanol (48.4 ± 1.23%) extracts, generally had the highest values of radical scavenging and enzymatic inhibition.

Interestingly, the isolated compounds mainly present in these plants extracts (abietane diterpenes, triterpenes and polyphenols), also revealed increased effects on tyrosinase, elastase, and collagenase inhibitions.

The AChE *in vitro* enzymatic assay was performed in order to find new inhibitors, due to the importance of AChE in the human skin, apart from its extensively known relevance in the diagnostic and/or treatment of Alzheimer disease. Although, preliminary results showed that the *Plectranthus* spp. organic extracts were not able to significantly decrease AChE activity (less than 17%), in comparison with the positive control tacrine (95.7 ± 2.89%). Even though, previous reports have shown a high AChE inhibition on some *Plectranthus* spp. plants aqueous extracts (*P. barbatus*, *P. ecklonii* and *P. saccatus*), due to rosmarinic and caffeic acids as major compounds.

In the anti-tyrosinase enzymatic assay, *P. porcatus* aqueous extract had the highest inhibition percentage (65.04 ± 8.67%), along with the organic extracts, such as *P. grandidentatus* acetone (67.9 ± 3.55%), *P. ecklonii* methanol (65.5 ±3.42%) and *P. saccatus* acetone (56.5 ± 5.68%). Since *P. porcatus* aqueous extract, has been reported to have low amounts of polyphenolic compounds, and knowing that these compounds are not present in high amounts, it could be suggested that the extract activity was probably due to the abietane compound, cyclo-abietane **(6)**.

The anti-collagenase assay of the tested extracts and compounds, had an inhibition of 28-76%. *P. neochilus* methanol extract held the highest result (75.4 ± 2.09%), whereas *P. ecklonii* aqueous extract inhibited ChC by 75.6± 6.5%. For elastase, the samples tested were not efficient overall, but the highest inhibitions were obtained from the oleanolic:ursolic acids 1:4 mixture (63.4 ± 2.56%) and Parvifloron D (52.8 ± 3.76%).

Regarding the anti-inflammatory assay of the isolated compounds, the ability to decrease NO production was measured in LPS-stimulated RAW 264.7 macrophage cells. The NO quantification evaluated by the Griess reaction, revealed that the non-cytotoxic compounds were not able to reduce the NO production (16-23 µM), after LPS-stimulated inflammation, in comparison with the normal NO production from the cells (17.7 ± 0.67 µM) and L-NAME (3.9 ± 0.2 µM). However, according to the references in this work, this is the first report on this activity by *Plectranthus* spp. isolated compounds. Supplementary studies to evaluate other inflammation mediators, for instance, COX-2 (by *in vitro* enzymatic inhibition), could be studied to understand which anti-inflammatory mechanisms are (or not) activated.

The final biological assay evaluated the Mtb H37Rv growth assay, after macrophages (derived from PBMC) infection, and treatment with isolated compounds. The first results of normalized CFU revealed very interesting results, especially regarding a halimane diterpene compound (2.1×10^5 CFU/mL), obtained by hemi-synthesis. This possible treatment with the halimane diterpene, leading to the growth inhibition of the bacteria, could be the cause of the observed CFU decrease, in comparison with isoniazid (1.2×10^5 CFU/mL) and ethambutol (2.0×10^5 CFU/mL). Nonetheless, as a future perspective regarding Mtb survival assays, it would be important to better understand the growth mechanisms of action. Western blot and immunofluorescence methods, could evaluate whether there is inhibition of the phagolysosome biogenesis, by arresting phagosome maturation.

According to the references of this thesis, this is the first report on seven *Plectranthus* spp. medicinal plants biological activities, with preliminary scientific validations upon their known ethnopharmacological uses. Although the recent renewed interest on medicinal plants and their biological applications, it is important to improve the studies on *Plectranthus* spp., to scientifically validate their uses, understand their safety, and unravel new bioactive compounds with therapeutic potential and specific targets.

REFERENCES

Abdel-Mogib, M., Albar, H.A., and Batterjee, S.M. (2002). Chemistry of the Genus *Plectranthus*. *Molecules* 7, 271–301.

Amessis-Ouchemoukh, N., Madani, K., Falé, P.L.V., Serralheiro, M.L., and Araújo, M.E.M. (2014). Antioxidant capacity and phenolic contents of some Mediterranean medicinal plants and their potential role in the inhibition of cyclooxygenase-1 and acetylcholinesterase activities. *Elsevier Industrial Crops and Products* 53, 6–15.

Araujo, R.C., Neves, F.A., Formagio, A.S., Kassuya, C.A., Stefanello, M.E., Souza, V.V., Pavan, F.R., and Croda, J. (2014). Evaluation of the anti-*Mycobacterium tuberculosis* activity and *In vivo* acute toxicity of *Annona sylvatica. BMC Complementary and Alternative Medicine* 14, 209.

Ascensão, L., Figueiredo, A.C., Barroso, J.G., Pedro, L.G., Schripsema, J., Deans, S.G., and Scheffer, J.J.C. (1998). *Plectranthus madagascariensis*: Morphology of the Glandular Trichomes, Essential Oil Composition, and Its Biological Activity*. International Journal of Plant Sciences* 159, 31–38.

Awad, A.B., Burr, A.T., and Fink, C.S. (2005). Effect of resveratrol and beta-sitosterol in combination on reactive oxygen species and prostaglandin release by PC-3 cells. *Prostaglandins, Leukotrienes and Essential Fatty Acids* 72, 219–226.

Backhouse, N., Rosales, L., Apablaza, C., Goïty, L., Erazo, S., Negrete, R., Theodoluz, C., Rodríguez, J., and Delporte, C. (2008). Analgesic, anti-inflammatory and antioxidant properties of *Buddleja globosa*, Buddlejaceae. *Journal of Ethnopharmacology* 116, 263–269.

Balaji, S.P., and Ramanathan, M. (2014). Telmisartan protects the lipopolysaccharide intoxicated RAW 264.7 cell line by deactivating NF-κB mediated inflammatory mechanism. *Journal of Pharmaceutical Sciences and Research* 5, 279-283.

Balunas, M.J., and Kinghorn, A.D. (2005). Drug discovery from medicinal plants. *Elsevier Life Sciences* 78, 431–441.

Bandaranayake, W.M. (2006). *Quality Control, Screening, Toxicity, and Regulation of Herbal Drugs.* Modern Phytomedicine: Turning Medicinal Plants into Drugs, John Wiley & Sons editions 2, 25–57.

Barrantes, E., and Guinea, M. (2003). Inhibition of collagenase and metalloproteinases by aloins and aloe gel. *Elsevier Life Sciences* 72, 843–850.

Batanouny, K.H., Aboutabl, E., Shabana, M., C., and Soliman, F. (1999). *Wild medicinal plants in Egypt : an inventory to support conservation and sustainable use*. Academy of Scientific Research & Technology of Egypt.

Baylac, S., and Racine, P. (2004). Inhibition of human leukocyte elastase by natural fragrant extracts of aromatic plants. *International Journal of Aromatherapy* 14, 179–182.

Bernardo-Gil, M.G., Roque, R., Roseiro, L.B., Duarte, L.C., Gírio, F., and Esteves, P. (2011). Supercritical extraction of carob kibbles (*Ceratonia siliqua* L.). *The Journal of Supercritical Fluids* 59, 36–42.

Bieth, J., Spiess, B., and Wermuth, C.G. (1974). The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. *Biochemical Medicine and Metabolic Biology* 11, 350–357.

Blanco, F.J., Guitian, R., Moreno, J., de Toro, F.J., and Galdo, F. (1999). Effect of antiinflammatory drugs on COX-1 and COX-2 activity in human articular chondrocytes. *The Journal of Rheumatology* 26, 1366–1373.

Bone, K., and Mills, S. (2013). *Principles and Practice of Phytotherapy: Modern Herbal Medicine 2nd edition*. Elsevier Health Sciences.

Boora, F., Chirisa, E., Mukanganyama, S., Boora, F., Chirisa, E., and Mukanganyama, S. (2014). Evaluation of Nitrite Radical Scavenging Properties of Selected Zimbabwean Plant Extracts and Their Phytoconstituents. *Journal of Food Processing 2014*.

Bryan, N.S., and Grisham, M.B. (2007). Methods to Detect Nitric Oxide and its Metabolites in Biological Samples. Free Radic Biol Med 43, 645–657.

Burmistrova, O., Simões, M.F., Rijo, P., Quintana, J., Bermejo, J., and Estévez, F. (2013). Antiproliferative Activity of Abietane Diterpenoids against Human Tumor Cells. *Journal of Natural Products* 76, 1413–1423.

Burmistrova, O., Perdomo, J., Simões, M.F., Rijo, P., Quintana, J., and Estévez, F. (2015). The abietane diterpenoid parvifloron D from *Plectranthus ecklonii* is a potent apoptotic inducer in human leukemia cells. *Elsevier Phytomedicine* 22, 1009–1016.

Buter, J., Heijnen, D., Wan, I.C., Bickelhaupt, F.M., Young, D.C., Otten, E., Moody, D.B., and Minnaard, A.J. (2016). Stereoselective Synthesis of 1-Tuberculosinyl Adenosine; a

Virulence Factor of *Mycobacterium tuberculosis*. *The Journal of Organic Chemistry* 81, 6686–6696.

Butler, M.S. (2004). The role of natural product chemistry in drug discovery. *Journal of Natural Products* 67, 2141–2153.

Cai, G., Pauli, G.F., Wang, Y., Jaki, B.U., and Franzblau, S.G. (2013). Rapid determination of growth inhibition of *Mycobacterium tuberculosis* by GC–MS/MS quantitation of tuberculostearic acid. *Elsevier Tuberculosis* 93, 322–329.

Cao, H., Yu, R., Choi, Y., Ma, Z.-Z., Zhang, H., Xiang, W., Lee, D.Y.-W., Berman, B.M., Moudgil, K.D., Fong, H.H.S., et al. (2010). Discovery of cyclooxygenase inhibitors from medicinal plants used to treat inflammation. *Pharmacological Research* 61, 519–524.

Cha, H.J., Park, M.T., Chung, H.Y., Kim, N.D., Sato, H., Seiki, M., and Kim, K.W. (1998). Ursolic acid-induced down-regulation of MMP-9 gene is mediated through the nuclear translocation of glucocorticoid receptor in HT1080 human fibrosarcoma cells. *Nature Oncogene* 16, 771–778.

Chan, H.-C., Feng, X., Ko, T.-P., Huang, C.-H., Hu, Y., Zheng, Y., Bogue, S., Nakano, C., Hoshino, T., Zhang, L., et al. (2014). Structure and Inhibition of Tuberculosinol Synthase and Decaprenyl Diphosphate Synthase from *Mycobacterium tuberculosis*. *Journal of the American Chemical Society* 136, 2892–2896.

Cheenpracha, S., Park, E.-J., Rostama, B., Pezzuto, J.M., and Chang, L.C. (2010). Inhibition of Nitric Oxide (NO) Production in Lipopolysaccharide (LPS)-Activated Murine Macrophage RAW 264.7 Cells by the Norsesterterpene Peroxide, Epimuqubilin A. *Journal of Marine Drugs* 8, 429–437.

Chen, W.-C., Tseng, T.-S., Hsiao, N.-W., Lin, Y.-L., Wen, Z.-H., Tsai, C.-C., Lee, Y.-C., Lin, H.-H., and Tsai, K.-C. (2015). Discovery of Highly Potent Tyrosinase Inhibitor, T1, with Significant Anti-Melanogenesis Ability by zebrafish *In vivo* Assay and Computational Molecular Modeling. *Nature Scientific reports* 5, 7995.

Chiu, Y.-J., Huang, T.-H., Chiu, C.-S., Lu, T.-C., Chen, Y.-W., Peng, W.-H., and Chen, C.-Y. (2012). Analgesic and Anti-inflammatory Activities of the Aqueous Extract from *Plectranthus amboinicus* (Lour.) Spreng. Both *In vitro* and *In vivo*. *Evidence Based Complementary Alternative Medicine* 2012.

Cirino, G., Distrutti, E., and Wallace, J.L. (2006). Nitric oxide and inflammation. *Inflammation and Allergy Drug Targets* 5, 115–119.

Coleman, J.W. (2001). Nitric oxide in immunity and inflammation. *Elsevier International Immunopharmacology* 1, 1397–1406.

Čolović, M.B., Krstić, D.Z., Lazarević-Pašti, T.D., Bondžić, A.M., and Vasić, V.M. (2013). Acetylcholinesterase Inhibitors: Pharmacology and Toxicology. *Current Neuropharmacology* 11, 315–335.

Cook, F.E.M. (1995). Economic Botany Data Collection Standard. Prepared for the International Working Group on Taxonomic Databases for Plant Sciences (TDWG). *Kew: Royal Botanic Gardens*

Coutinho, I., Pereira, G., Simões, M., Côrte-Real, M., Gonçalves, J., and Saraiva, L. (2009). Selective activation of protein kinase C-delta and -epsilon by 6,11,12,14-tetrahydroxy-abieta-5,8,11,13-tetraene-7-one (coleon U). *Biochemical Pharmacology* 78, 449–459.

Cragg, G.M., and Newman, D.J. (2013). Natural products: a continuing source of novel drug leads. *Biochimica et Biophysica Acta* 1830, 3670–3695.

Cragg, G.M., Newman, D.J. (2008). Biodiversity: a continuing source of novel drug leads. *Pure and Applied Chemistry* 77, 7-24.

Crofford, L.J. (2013). Use of NSAIDs in treating patients with arthritis. *Arthritis Research & Therapy* 15, S2.

Dawson, T.M., and Dawson, V.L. (1995). Nitric Oxide: Actions and Pathological Roles. *The Neuroscientist* 1, 7–18.

De la Puerta, R., Martínez-Domínguez, E., and Ruíz-Gutiérrez, V. (2000). Effect of minor components of virgin olive oil on topical anti-inflammatory assays. *Biosciences Zeitschrift für Naturforschung* 55, 814–819.

Dhawan, V. (2014). *Reactive Oxygen and Nitrogen Species: General Considerations. Studies on Respiratory Disorders*. Springer New York, 27–47.

Dias, D.A., Urban, S., and Roessner, U. (2012). A Historical Overview of Natural Products in Drug Discovery. *Metabolites* 2, 303–336.

Doughari, J.H. (2012). *Phytochemicals: Extraction Methods, Basic Structures and Mode of Action as Potential Chemotherapeutic Agents* in *Phytochemicals - A Global Perspective of Their Role in Nutrition and Health*, Venketeshwer Rao 1–32.

Falé, P.L., Amaral, F., Amorim Madeira, P.J., Sousa Silva, M., Florêncio, M.H., Frazão, F.N., and Serralheiro, M.L.M. (2012). Acetylcholinesterase inhibition, antioxidant activity and toxicity of *Peumus boldus* water extracts on HeLa and Caco-2 cell lines. *Food and Chemical Toxicology* 50, 2656–2662.

Falé, P.L.V., Madeira, P.J.A., Florêncio, M.H., Ascensão, L., and Serralheiro, M.L.M. (2011). Function of *Plectranthus barbatus* herbal tea as neuronal acetylcholinesterase inhibitor. *Food & Function* 2, 130–136.

Filho, B., M, J., Medeiros, K.C.P., Diniz, M. de F.F.M., Batista, L.M., Athayde-Filho, P.F., Silva, M.S., Cunha, E.V.L. da, Almeida, J.R.G.S., and Quintans-Júnior, L.J. (2006). Natural products inhibitors of the enzyme acetylcholinesterase. *Revista Brasileira de Farmacognosia* 16, 258–285.

Gabay, O., Sanchez, C., Salvat, C., Chevy, F., Breton, M., Nourissat, G., Wolf, C., Jacques, C., and Berenbaum, F. (2010). Stigmasterol: a phytosterol with potential anti-osteoarthritic properties. *Osteoarthritis and Cartilage* 18, 106–116.

Gabriel, A.B., Juliana, F.F., Silvia, T.E., Eliete, N.S.G., D acirc maris, S., and Luiz, A.S. (2016). Cytotoxic effect of *Plectranthus* neochilus extracts in head and neck carcinoma cell lines. *African Journal of Pharmacy and Pharmacology* 10, 157–163.

García, M.D., Sáenz, M.T., Gómez, M.A., and Fernández, M.A. (1999). Topical anti-inflammatory activity of phytosterols isolated from *Eryngium foetidum* on chronic and acute inflammation models. *Phytotherapy Research* 13, 78–80.

Gaspar-Marques, C., Rijo, P., Simões, M.F., Duarte, M.A., and Rodriguez, B. (2006). Abietanes from *Plectranthus grandidentatus* and *P. hereroensis* against methicillin- and vancomycin-resistant bacteria. *Elsevier Phytomedicine* 13, 267–271.

Ghimeray, A., Jung, U., Lee, H., Kim, Y., Ryu, E., and Chang, M. (2015). *In vitro* antioxidant, collagenase inhibition, and *In vivo* anti-wrinkle effects of combined formulation containing *Punica granatum*, *Ginkgo biloba*, *Ficus carica*, and *Morus alba* fruits extract. *Clinical, Cosmetic and Investigational Dermatology* 389.

Ghodbane, R., Raoult, D., and Drancourt, M. (2014). Dramatic reduction of culture time of *Mycobacterium tuberculosis*. *Nature Scientific reports* 4.

Golüke, N.M.S., van Strien, A.M., Dautzenberg, P.J.L., Jessurun, N., and Keijsers, C.J.P.W. (2014). Skin Lesions after Oral Acetylcholinesterase Inhibitor Therapy: A Case Report. *Journal of American Geriatric Society* 62, 2012–2013.

Grando, S.A., Kawashima, K., Kirkpatrick, C.J., Meurs, H., and Wessler, I. (2012). The non-neuronal cholinergic system: Basic science, therapeutic implications and new perspectives. *Elsevier Life Sciences* 91, 969–972.

Guzik, T.J., Korbut, R., and Adamek-Guzik, T. (2003). Nitric oxide and superoxide in inflammation and immune regulation. *Journal of physiology and pharmacology* 54, 469–487.

Hayashida, N., Chihara, S., Tayama, E., Takaseya, T., Enomoto, N., Kawara, T., and Aoyagi, S. (2001). Anti-inflammatory effects of colforsin daropate hydrochloride, a novel water-soluble forskolin derivative. *The Annals of Thoracic Surgery* 71, 1931–1938.

Hoshino, T., Nakano, C., Ootsuka, T., Shinohara, Y., and Hara, T. (2011). Substrate specificity of Rv3378c, an enzyme from *Mycobacterium tuberculosis*, and the inhibitory activity of the bicyclic diterpenoids against macrophage phagocytosis. *Organic & Biomolecular Chemistry* 9, 2156–2165.

Huss, U., Ringbom, T., Perera, P., Bohlin, L., and Vasänge, M. (2002). Screening of ubiquitous plant constituents for COX-2 inhibition with a scintillation proximity based assay. *Journal of Natural Products* 65, 1517–1521.

Hwang, T.-L., Shen, H.-I., Liu, F.-C., Tsai, H.-I., Wu, Y.-C., Chang, F.-R., and Yu, H.-P. (2014). Ursolic Acid Inhibits Superoxide Production in Activated Neutrophils and Attenuates Trauma-Hemorrhage Shock-Induced Organ Injury in Rats. *PLoS ONE* 9, e111365.

Jin, H. (2014). Acetylcholinesterase and Butyrylcholinesterase Inhibitory Properties of Functionalized Tetrahydroacridines and Related Analogs. *Medicinal Chemistry* 4, 688-696.

Jung, H.-Y., Shin, J.-C., Park, S.-M., Kim, N.-R., Kwak, W., and Choi, B.-H. (2014*). Pinus densiflora* extract protects human skin fibroblasts against UVB-induced photoaging by inhibiting the expression of MMPs and increasing type I procollagen expression. *Toxicology Reports* 1, 658–666.

Ketabforoosh, S.H.M.E., Kheirollahi, A., Safavi, M., Esmati, N., Ardestani, S.K., Emami, S., Firoozpour, L., Shafiee, A., and Foroumadi, A. (2014). Synthesis and Anti-Cancer Activity Evaluation of New Dimethoxylated Chalcone and Flavanone Analogs: Synthesis and Anti-Cancer Activity of New Flavanone Analogs. *Archiv Der Pharmazie* 347, 853–860.

Kirkpatrick, C.J., Bittinger, F., Unger, R.E., Kriegsmann, J., Kilbinger, H., and Wessler, I. (2001). The non-neuronal cholinergic system in the endothelium: evidence and possible pathobiological significance. *Japanese Journal of Pharmacology* 85, 24–28.

Klatz, R., and Goldman, R. (2003). *Stopping the Clock* in *The new anti-aging revolution 3rd edition.* Basic Health Publications

Komatsu, M., and Ichimura, Y. (2010). Physiological significance of selective degradation of p62 by autophagy. *FEBS Letters* 584, 1374–1378.

Kubínová, R., Pořízková, R., Navrátilová, A., Farsa, O., Hanáková, Z., Bačinská, A., Cížek, A., and Valentová, M. (2014). Antimicrobial and enzyme inhibitory activities of the constituents of *Plectranthus madagascariensis* (Pers.) Benth. *Journal of Enzyme Inhibition and Medicinal Chemistry* 29, 749–752.

Kurzen, H., Wessler, I., Kirkpatrick, C.J., Kawashima, K., and Grando, S.A. (2007). The non-neuronal cholinergic system of human skin. *Hormone and Metabolic Research* 39, 125–135.

Layre, E., Lee, H.J., Young, D.C., Martinot, A.J., Buter, J., Minnaard, A.J., Annand, J.W., Fortune, S.M., Snider, B.B., Matsunaga, I. (2014). Molecular profiling of *Mycobacterium tuberculosis* identifies tuberculosinyl nucleoside products of the virulence-associated enzyme Rv3378c. *Proceedings of the National Academy of Sciences* 111, 2978–2983.

Lee, K.K., Cho, J.J., Park, E.J., and Choi, J.D. (2001). Anti-elastase and anti-hyaluronidase of phenolic substance from *Areca catechu* as a new anti-ageing agent. *International Journal of Cosmetic Science* 23, 341–346.

Lee, S.-Y., Kim, H.-J., and Han, J.-S. (2013). Anti-inflammatory Effect of Oyster Shell Extract in LPS-stimulated RAW 264.7 Cells. *Preventive Nutrition and Food Science* 18, 23–29.

Lovkova, M.Y., Buzuk, G.N., Sokolova, S.M., and Kliment'eva, N.I. (2001). Chemical Features of Medicinal Plants. *Applied Biochemistry and Microbiology* 37, 229–237.

Lukhoba, C.W., Simmonds, M.S.J., and Paton, A.J. (2006). *Plectranthus*: A review of ethnobotanical uses. *Journal of Ethnopharmacology* 103, 1–24.

Mann, F.M., and Peters, R.J. (2012). Isotuberculosinol: the unusual case of an immunomodulatory diterpenoid from *Mycobacterium tuberculosis*. *Medicinal Chemical Communications* 3, 899–904.

Maria Fátima Simões, P.R. (2010). Two new diterpenoids from *Plectranthus* species. *Phytochemistry Letters* 3, 221–225.

Mark, M.R., Domino, E.F., Han, S.S., Ortiz, A., Mathews, B.N., and Tait, S.K. (1983). Effect of parasympathetic denervation on acetylcholine levels in the rat parotid gland. Is there an extraneuronal pool of acetylcholine? *Elsevier Life Sciences* 33, 1191–1197.

Marques, C.G., Pedro, M., Simões, M.F.A., Nascimento, M.S.J., Pinto, M.M.M., and Rodríguez, B. (2002). Effect of abietane diterpenes from *Plectranthus grandidentatus* on the growth of human cancer cell lines. *Planta Medica* 68, 839–840.

Martins, I.L., Miranda, J.P., Oliveira, N.G., Fernandes, A.S., Gonçalves, S., and Antunes, A.M.M. (2013). Synthesis and biological activity of 6-selenocaffeine: potential modulator of chemotherapeutic drugs in breast cancer cells. *Molecules* 18, 5251–5264.

Masuda, M., Murata, K., Fukuhama, A., Naruto, S., Fujita, T., Uwaya, A., Isami, F., and Matsuda, H. (2009). Inhibitory effects of constituents of *Morinda citrifolia* seeds on elastase and tyrosinase. *Journal of Natural Medicines* 63, 267–273.

Matu, E.N., and van Staden, J. (2003). Antibacterial and anti-inflammatory activities of some plants used for medicinal purposes in Kenya. *Journal of Ethnopharmacology* 87, 35–41.

Meena, L.S., and Rajni, K. (2010). Survival mechanisms of pathogenic *Mycobacterium tuberculosis* H37Rv. *FEBS Journal* 277, 2416–2427.

Mendonça-Filho, R.R. (2006). *Bioactive Phytocompounds: New Approaches in the Phytosciences* In *Modern Phytomedicine: Turning Medicinal Plants into Drugs*, John Wiley & Sons 1–24.

Mishra, B.B., and Tiwari, V.K. (2011). Natural products: An evolving role in future drug discovery. *European Journal of Medicinal Chemistry* 46, 4769–4807.

Moon, J.-Y., Yim, E.-Y., Song, G., and Lee, N.H. (2010). Screening of elastase and tyrosinase inhibitory activity from Jeju Island plants. *Eurasian Journal of Biosciences* 4, 41–53.

Naghibi, F., Mosaddegh, M., Motamed, S.M., and Ghorbani, A. (2005). *Labiatae* Family in folk Medicine in Iran: from Ethnobotany to Pharmacology. *Iranian Journal of Pharmaceutical Research* 63–79.

Nakano, C., Ootsuka, T., Takayama, K., Mitsui, T., Sato, T., and Hoshino, T. (2011). Characterization of the Rv3378c gene product, a new diterpene synthase for producing tuberculosinol and (13*R*,*S*)-isotuberculosinol (nosyberkol), from the *Mycobacterium tuberculosis* H37Rv genome. *Bioscience, Biotechnology, and Biochemistry* 75, 75–81.

Nashed, B., Yeganeh, B., HayGlass, K.T., and Moghadasian, M.H. (2005). Antiatherogenic effects of dietary plant sterols are associated with inhibition of proinflammatory cytokine production in Apo E-KO mice. *Journal of Nutrition* 135, 2438–2444.

Nsimba, R.Y., Kikuzaki, H., and Konishi, Y. (2008). Ecdysteroids act as inhibitors of calf skin collagenase and oxidative stress. *Journal of Biochemical and Molecular Toxicology* 22, 240–250.

Ong, C.K.S., Lirk, P., Tan, C.H., and Seymour, R.A. (2007). An Evidence-Based Update on Nonsteroidal Anti-Inflammatory Drugs. *Clinical Medicine & Research* 5, 19–34.

Pandey, K.B., and Rizvi, S.I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity* 2, 270–278.

Pathak, S., Awuh, J.A., Leversen, N.A., Flo, T.H., and Åsjø, B. (2012). Counting Mycobacteria in Infected Human Cells and Mouse Tissue: A Comparison between qPCR and CFU. *PLoS ONE* 7, e34931.

Peñuelas-Urquides, K., Villarreal-Treviño, L., Silva-Ramírez, B., Rivadeneyra-Espinoza, L., Said-Fernández, S., and de León, M.B. (2013). Measuring of *Mycobacterium tuberculosis* growth: A correlation of the optical measurements with colony forming units. *Brazilian Journal of Microbiology* 44, 287–289.

Pereira, M., Matias, D., Pereira, F., Reis, C., Simões, M.F., and Rijo, P. (2015). Antimicrobial screening of *Plectranthus madagascariensis* and *P. neochilus* extracts. *Biomedical and Biopharmaceutical Research* 12, 127–138.

Petrovic, N., and Murray, M. (2010). Using *N,N,N',N'*-tetramethyl-p-phenylenediamine (TMPD) to assay cyclooxygenase activity *In vitro*. *Methods in Molecular Biology* 594, 129–140.

Pizzorno, J.E., and Murray, M.T. (2013). *Textbook of Natural Medicine 4th edition*. Elsevier Health Sciences.

Popoola, O.K., Marnewick, J.L., Rautenbach, F., Ameer, F., Iwuoha, E.I., and Hussein, A.A. (2015). Inhibition of Oxidative Stress and Skin Aging-Related Enzymes by Prenylated Chalcones and Other Flavonoids from *Helichrysum teretifolium*. *Molecules* 20, 7143–7155.

Quy Diem D., Angkawijaya A.E., Tran-Nguyen P.L., Huynh L.H., Soetaredjo F.E., Yi-Hsu S.I. (2014). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. *Journal of Food and Drug Analysis* 22, 296–302.

Ray, P.D., Huang, B.-W., and Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Elsevier Cellular Signalling* 24, 981–990.

Rey-Ladino, J., Ross, A.G., Cripps, A.W., McManus, D.P., and Quinn, R. (2011). Natural products and the search for novel vaccine adjuvants. *Elsevier Vaccine* 29, 6464–6471.

Rice, L.J., Brits, G.J., Potgieter, C.J., and Van Staden, J. (2011). *Plectranthus*: A plant for the future? *South African Journal of Botany* 77, 947–959.

Ridnour, L.A., Sim, J.E., Hayward, M.A., Wink, D.A., Martin, S.M., Buettner, G.R., and Spitz, D.R. (2000). A spectrophotometric method for the direct detection and quantitation of nitric oxide, nitrite, and nitrate in cell culture media. *Analytical Biochemistry* 281, 223–229.

Rijo, P.D. de M. (2011). Phytochemical study and biological activities of diterpenes and derivatives from *Plectranthus* species. PhD Thesis. *University of Lisbon, Faculty of Pharmacy.*

Rijo, P., Gaspar-Marques, C., Simões, M.F., Duarte, A., Del Carmen Apreda-Rojas, M., Cano, F.H., and Rodríguez, B. (2002). Neoclerodane and labdane diterpenoids from *Plectranthus ornatus*. *Journal of Natural Products* 65, 1387–1390.

Rijo, P., Simões, M.F., and Rodríguez, B. (2005). Structural and spectral assignment of three forskolin-like diterpenoids isolated from *Plectranthus ornatus*. *Magnetic Resonance in Chemistry* 43, 595–598.

Rijo, P., Simões, M.F., Francisco, A.P., Rojas, R., Gilman, R.H., Vaisberg, A.J., Rodríguez, B., and Moiteiro, C. (2010). Antimycobacterial metabolites from *Plectranthus*: royleanone derivatives against *Mycobacterium tuberculosis* strains. *Chemistry & Biodiversity* 7, 922–932.

Rijo, P., Rodriguez, B., Duarte, A., and Fatima Simoes, M. (2011). Antimicrobial Properties of *Plectranthus ornatus* Extracts, 11-acetoxyhalima-5, 13-dien-15-oic Acid Metabolite and its Derivatives. *The Natural Products Journal* 1, 57–64.

Rijo, P., Batista, M., Matos, M., Rocha, E., Jesus, S., and Simões, M. (2012). Screening of antioxidant and antimicrobial activities on *Plectranthus* spp. extracts. *Biomedical and Biopharmaceutical Research* 9, 225–235.

Rijo, P., Fernandes, A.S., Simões, F., and Pinheiro, L. (2013a). Avaliação de diterpenos isolados de *P. ornatus* como potenciais inibidores da COX-1. *Biomedical and Biopharmaceutical Research* 9, 111-118.

Rijo, P., Faustino, C., and Simões, M.F. (2013b*). Antimicrobial natural products from Plectranthus plants* in *Microbial Pathogens and Strategies for Combating Them*: *Science, Technology and Education*. A. Méndez-Vilas, 2, 922–931.

Rijo, P., Falé, P.L., Serralheiro, M.L., Simões, M.F., Gomes, A., and Reis, C. (2014a). Optimization of medicinal plant extraction methods and their encapsulation through extrusion technology. *Elsevier Measurement* 58, 249–255.

Rijo, P., Duarte, A., Francisco, A.P., Semedo-Lemsaddek, T., and Simões, M.F. (2014b). *In vitro* antimicrobial activity of royleanone derivatives against Gram-positive bacterial pathogens. *Phytotherapy Research* 28, 76–81.

Riley, P.A. (1997). Melanin. *International Journal of Biochemistry and Cell Biology* 29, 1235–1239.

Robbers, J.E., Speedie, M.K., and Tyler, V.E. (1996). *Pharmacognosy and Pharmacobiotechnology.* Baltimore: Williams & Wilkins.

Rodrigues, M.J., Gangadhar, K.N., Vizetto-Duarte, C., Wubshet, S.G., Nyberg, N.T., Barreira, L., Varela, J., and Custódio, L. (2014). Maritime halophyte species from southern Portugal as sources of bioactive molecules. *Marine Drugs* 12, 2228–2244.

Rodrigues, M.J., Neves, V., Martins, A., Rauter, A.P., Neng, N.R., Nogueira, J.M.F., Varela, J., Barreira, L., and Custódio, L. (2016). *In vitro* antioxidant and anti-inflammatory properties of *Limonium algarvense* flowers' infusions and decoctions: A comparison with green tea (*Camellia sinensis*). *Elsevier Food Chemistry Journal* 200, 322–329.

Romagnoli, A., Etna, M.P., Giacomini, E., Pardini, M., Remoli, M.E., Corazzari, M., Falasca, L., Goletti, D., Gafa, V., Simeone, R., et al. (2012). ESX-1 dependent impairment of autophagic flux by *Mycobacterium tuberculosis* in human dendritic cells. *Autophagy* 8, 1357–1370.

Rosa, S., Correia, V., Ribeiro, I., Rijo, P., Simões, F., Saraiva, N., and Fernandes, A. (2015). *In vitro* antioxidant properties of the diterpenes Parvifloron D and 7α-acetoxy-6β-hydroxyroyleanone. *Biomedical and Biopharmaceutical Research* 12, 59-67

Rout, S.P., Choudary, K.A., Kar, D.M., Das, L., and Jain, A. (2009). Plants in traditional medicinal system -future source of new drugs. *International Journal of Pharmacy and Pharmaceutical Sciences* 1, 1–23.

Samuelsson, G., and Bohlin, L. (2010). *Drugs of Natural Origin: A Treatise of Pharmacognosy 6th edition*. Stockholm: Swedish Pharmaceutical Press.

Sandhu, G.K. (2011). Tuberculosis: Current Situation, Challenges and Overview of its Control Programs in India. *Journal of Global Infectious Diseases* 3, 143–150.

Schiller, E. (2004). *Respiratory burst* in *Free radicals and inhalation pathology*. Springer-Verlag Berlin Heidelberg.

Schlereth, T., Birklein, F., an Haack, K., Schiffmann, S., Kilbinger, H., Kirkpatrick, C.J., and Wessler, I. (2006). *In vivo* release of non-neuronal acetylcholine from the human skin as measured by dermal microdialysis: effect of botulinum toxin. *British Journal of Pharmacology* 147, 183–187.

Sergent, T., Piront, N., Meurice, J., Toussaint, O., and Schneider, Y.-J. (2010). Anti-inflammatory effects of dietary phenolic compounds in an *In vitro* model of inflamed human intestinal epithelium. *Chemico-Biological Interactions* 188, 659–667.

Seto, S., Tsujimura, K., Horii, T., and Koide, Y. (2013). Autophagy Adaptor Protein p62/SQSTM1 and Autophagy-Related Gene Atg5 Mediate Autophagosome Formation in Response to *Mycobacterium tuberculosis* Infection in Dendritic Cells. *PLoS One* 8, e86017 .

Shadyro, O.I., Glushonok, G.K., Glushonok, T.G., Edimecheva, I.P., Moroz, A.G., Sosnovskaya, A.A., Yurkova, I.L., and Polozov, G.I. (2002). Quinones as free-radical fragmentation inhibitors in biologically important molecules. *Free Radical Research*. 36, 859–867.

Sharma, J.N., Al-Omran, A., and Parvathy, S.S. (2008). Role of nitric oxide in inflammatory diseases. *Inflammopharmacology* 15, 252–259.

Shi, L., Feng, X.E., Cui, J.R., Fang, L.H., Du, G.H., and Li, Q.S. (2010). Synthesis and biological activity of flavanone derivatives. *Bioorganic & Medicinal Chemistry Letters* 20, 5466–5468.

Sieuwerts, S., De Bok, F., Mols, E., De Vos, W., and Van Hylckama Vlieg, J. (2008). A simple and fast method for determining colony forming units. *Letters in Applied Microbiology* 47, 275–278.

Silva, L., Gomes, A.C., and Rodilla, J.M.L. (2011). Diterpene Lactones with Labdane, Halimane and Clerodane Frameworks. *Natural Product Communications* 6, 497–504.

Simões, M.F., Rijo, P., Duarte, A., Matias, D., and Rodríguez, B. (2010). An easy and stereoselective rearrangement of an abietane diterpenoid into a bioactive microstegiol derivative. *Phytochemistry Letters* 3, 234–237.

Sun, J., Zhang, X., Broderick, M., and Fein, H. (2003). Measurement of Nitric Oxide Production in Biological Systems by Using Griess Reaction Assay. *Sensors* 3, 276–284.

Swain, T. (1972). *Plants in the Development of Modern Medicine*. Harvard University Press Tony Swain.

Thirugnanasampandan, R., Ramya, G., Gogulramnath, M., Jayakumar, R., and M S, K. (2014). Evaluation of cytotoxic, DNA protecting and LPS induced MMP-9 down regulation activities of *Plectranthus amboinicus* (Lour) Spreng. essential oil. *Pharmacognosy Journal* 7, 32–36.

Thring, T.S., Hili, P., and Naughton, D.P. (2011). Antioxidant and potential anti-inflammatory activity of extracts and formulations of white tea, rose, and witch hazel on primary human dermal fibroblast cells. *Journal of Inflammation* 8, 27.

Thring, T.S.A., Hili, P., and Naughton, D.P. (2009). Anti-collagenase, anti-elastase and anti-oxidant activities of extracts from 21 plants. *BMC Complementary and Alternative Medicine* 9, 27.

Tu, P.T.B., and Tawata, S. (2015). Anti-Oxidant, Anti-Aging, and Anti-Melanogenic Properties of the Essential Oils from Two Varieties of *Alpinia zerumbet*. *Molecules* 20, 16723–16740.

Valasani, K.R., Chaney, M.O., Day, V.W., and Shidu Yan, S. (2013). Acetylcholinesterase inhibitors: structure based design, synthesis, pharmacophore modeling, and virtual screening. *Journal of Chemical Information and Modeling* 53, 2033–2046.

Vallisuta, O., Nukoolkarn, V., Mitrevej, A., Sarisuta, N., Leelapornpisid, P., Phrutivorapongkul, A., and Sinchaipanid, N. (2014). *In vitro* studies on the cytotoxicity, and elastase and tyrosinase inhibitory activities of marigold (*Tagetes erecta* L.) flower extracts. *Experimental and Therapeutic Medicine* 7, 246–250.

Van Jaarsveld, E. (2006). *Southern African Plectranthus 11th edition*. Fernwood Press.

Van Wart, H.E., and Steinbrink, D.R. (1981). A continuous spectrophotometric assay for *Clostridium histolyticum* collagenase. *Analytical Biochemistry* 113, 356–365.

Villaseñor, I.M., Angelada, J., Canlas, A.P., and Echegoyen, D. (2002). Bioactivity studies on β-sitosterol and its glucoside. *Phytotherapy Research* 16, 417–421.

Villaseñor, I.M., Canlas, A.P., Faustino, K.M., and Plana, K.G. (2004). Evaluation of the bioactivity of triterpene mixture isolated from *Carmona retusa* (Vahl.) Masam leaves. *Journal Ethnopharmacology* 92, 53–56.

Wagstaff, S.J., Hickerson, L., Spangler, R., Reeves, P.A., and Olmstead, R.G. (1998). Phylogeny in *Labiatae*, inferred from cpDNA sequences. *Plant Systematics and Evolution* 209, 265–274.

Wahab, Norliza Abdul, Rahman, R.A., Ismail, A., and Hashim, S.M. and P. (2014). Assessment of Antioxidant Capacity, Anti-collagenase and Anti-elastase Assays of Malaysian Unfermented Cocoa Bean for Cosmetic Application. *Natural Products Chemistry & Research* 2, 132–137.

Waksmundzka-Hajnos, M., and Sherma, J. (2011). *High performance liquid chromatography in phytochemical analysis 1st edition*. CRC Press.

Wang, H., and Leigh, J. (2006). Effects of nitric oxide synthase inhibitor ω-Nitro-L-Arginine Methyl Ester, on silica-induced inflammatory reaction and apoptosis. *Particle and Fibre Toxicology* 3, 14.

Wang, S.-Y., Wu, J.-H., Shyur, L.-F., Kuo, Y.-H., and Chang, S.-T. (2005). Antioxidant Activity of Abietane-Type Diterpenes from Heartwood of *Taiwania cryptomerioides* Hayata. *Holzforschung* 56, 487–492.

Wellsow, J., Grayer, R.J., Veitch, N.C., Kokubun, T., Lelli, R., Kite, G.C., and Simmonds, M.S.J. (2006). Insect-antifeedant and antibacterial activity of diterpenoids from species of *Plectranthus*. *Phytochemistry* 67, 1818–1825.

Wink, M. (2010). *Biochemistry of plant secondary metabolism 2nd edition* in *Annual Plant Reviews*. Wiley-Blackwell Publishing Ltd.

Wittenauer, J., Mäckle, S., Sußmann D., Schweiggert-Weisz, U., Carle, R. (2015). Inhibitory effects of polyphenols from grape pomace extract on collagenase and elastase activity. *Elsevier Fitoterapia* 101, 179-187.

Yamauchi, K., Mitsunaga, T., and Batubara, I. (2011). Isolation, Identification and Tyrosinase Inhibitory Activities of the Extractives from *Allamanda cathartica*. *Natural Resources* 2, 167–172.

Ying, Q.L., Rinehart, A.R., Simon, S.R., and Cheronis, J.C. (1991). Inhibition of human leucocyte elastase by ursolic acid. Evidence for a binding site for pentacyclic triterpenes. Biochemical Journal 277, 521–526.

APPENDIXES

Appendix I – Cytotoxicity evaluation using the MTT assay: Graphical results for the tested compounds concentrations

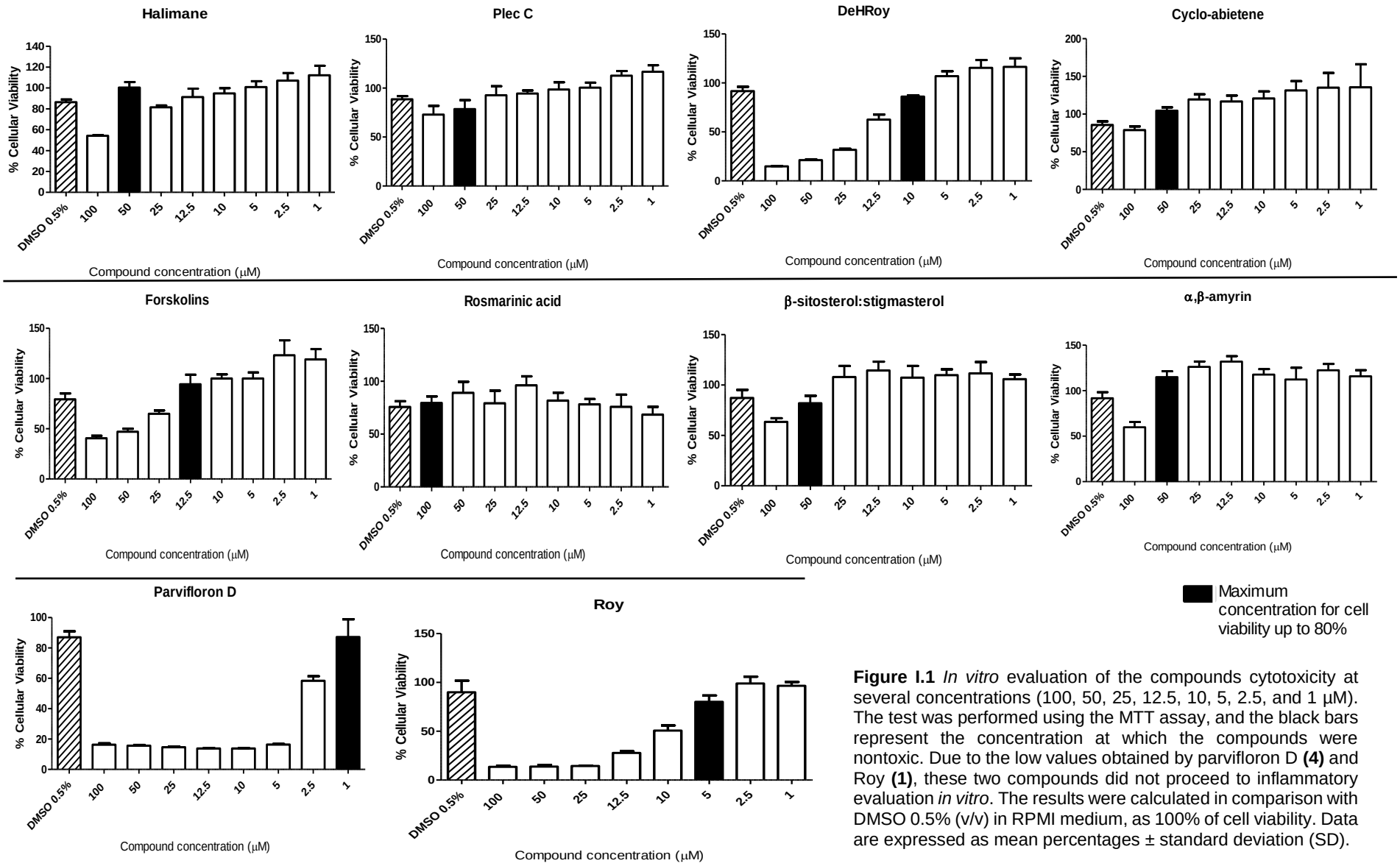


Figure I.1 *In vitro* evaluation of the compounds cytotoxicity at several concentrations (100, 50, 25, 12.5, 10, 5, 2.5, and 1 μM). The test was performed using the MTT assay, and the black bars represent the concentration at which the compounds were nontoxic. Due to the low values obtained by parvifloron D **(4)** and Roy **(1)**, these two compounds did not proceed to inflammatory evaluation *in vitro*. The results were calculated in comparison with DMSO 0.5% (v/v) in RPMI medium, as 100% of cell viability. Data are expressed as mean percentages ± standard deviation (SD).

Appendix II – Anti-inflammatory assay: Cyclooxygenase-2 enzymatic inhibition *in vitro* with TMPD assay

Reagents and materials

N,N,N',N'-tetramethylphenylenediamine (TMPD) 98%, and hematin 97% were purchased from Alfa Aesar. Arachidonic acid 98% was obtained from Cayman Chemical Company. Diclofenac sodium 99.4% was acquired from Fagron. Cyclooxygenase-2 (COX-2) ≥70% (SDS-PAGE), from human recombinant expressed in Sf 21 cells, and 2,6-tert-butyl-4-methylphenol (BHT) were purchased from Sigma-Aldrich.

Enzymatic assay for COX-2 inhibition - Trials

COX-2 activity can be measured in an assay using arachidonic acid as substrate, and TMPD as co-substrate. This works as an artificial electron donor that undergoes co-oxidation by PGG_2 producing a blue product, detected at 590 nm (Petrovic and Murray, 2010).

Although this is an indirect method, the oxidation of TMPD has been shown to accurately reflect the rate of conversion of arachidonic acid to PGH_2 (Petrovic and Murray, 2010).

The assay was performed several times by optimization of COX-2, hematin, TMPD, and arachidonic acid concentrations. The several modifications of concentrations did not improve the colorimetric detection of COX-2 activity. From all the attempts, it was not observed a change in the assay color in each well, therefore it was not possible to pursue to COX-2 inhibition evaluation *in vitro*.

In general, the colorimetric assay should detect the increase of blue color from the co-oxidation of TMPD by PGG_2, which is proportional to COX-2 activity. Therefore, the velocity reaction of control (ΔAbs_{595nm}/min) should be linear, and close to 0.060.

Diclofenac is usually used as the positive control which holds IC_{50} value of 0.63 µM (Blanco *et al*., 1999), whereas the negative controls comprise the respective sample solvent, at same dilution.

From the several attempts, it can be suggested that the assay was not established due to the spontaneous oxidation of TMPD crystals. The co-substrate was prepared in several solutions, including Tris-HCl buffer, ethanol 70% (v/v), absolute ethanol, DMSO, and HCl 1% (v/v). None of the trials decreased the apparent initial blue color of TMPD solution.

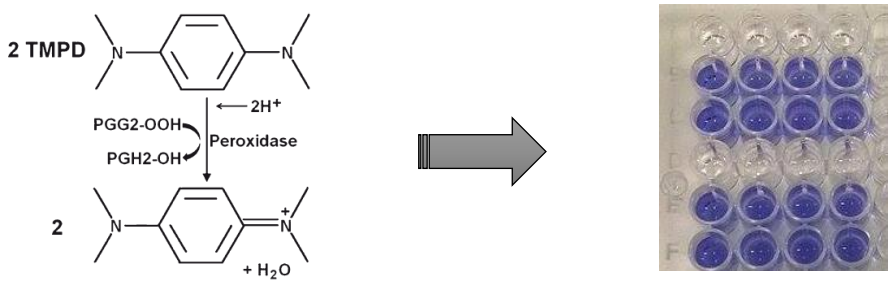


Figure II.1 Detection of COX-2 peroxidase activity with *N,N,N',N'*-tetramethylphenylenediamine (TMPD). The artificial electron donor TMPD undergoes co-oxidation by PGG_2 to a blue product (oxidized TMPD), that is detected at 595 nm. TMPD is a readily oxidizable compound that serves as a reducing co-substrate for heme peroxidases (Petrovic and Murray, 2010).