UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS

Valorisation of underutilized endogenous forest biomass

"Documento Definitivo"

Doutoramento em Biologia
Especialidade de Biotecnologia

Cláudia Sofia dos Santos Tavares

Tese orientada por:
Prof.ª Doutora Ana Cristina Figueiredo
Doutora Luísa Bivar Roseiro
José Adelino Gameiro

Documento especialmente elaborado para a obtenção do grau de doutor

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“O essencial é invísivel para os olhos” em *O Principezinho*

Antoine de Saint-Exupéry, 2001, p. 74

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Abstract

Biomass represents one of the renewable resources with greatest potential of application, being an efficient raw material for the use in many industries. The large amount of wasted biomass, namely forest residues, constitute a dramatic environmental problem. These residues are available in large quantities and poorly used, while boosting forest fires.

The present work intended the valorisation of Portuguese forest residues, namely of *Cupressus lusitanica* Mill and *Cistus ladanifer* L., while contributing to a small-scale biorefinery within regional small and medium-sized enterprises (SMEs) premises to reach zero waste. A steam-distillation procedure was applied to each of these biomasses, resulting four distinct fractions: a) essential oils (EOs), b) hydrolates (Hs), c) distiller condensation waters (DCWs) and d) extracted solid residues (ESRs), which underwent chemical and biological activity characterization while different valorisations were tested.

α-Pinene, limonene, δ-3-carene and sabinene were the main *C. lusitanica* EO constituents, whereas its Hs volatiles were dominated by *cis*-3-hexen-1-ol, camphor, umbellulone, *p*-cymene-8-ol and terpinen-4-ol. *C. ladanifer* EO major constituents were α-pinene and camphene, whereas 2,6,6-trimethyl cyclohexanone and *trans*-pinocarveol dominated the Hs volatiles. DCWs and ESRs showed a high phenolic content, mainly tannins. Catechins were the major compounds found in both species. Gallic acid, quercetin, hydroxycinnamic acid derivatives, salicylic acid, apigenin and syringic acid were only identified in *C. ladanifer*. *C. lusitanica* and *C. ladanifer* ESRs were both rich in lignin. All fractions showed antioxidant activity while Hs showed the highest anti-inflammatory one.

Besides the potential of *C. lusitanica* and *C. ladanifer* biomasses as source of natural bioactive compounds, preliminary assays showed the potential of these biomasses in artisanal soaps and pellets production. A simplified economic viability study showed that the valorisation of these biomasses within the framework of a local small-scale biorefinery may be economically viable for SMEs.

Keywords: *Cistus ladanifer* L., *Cupressus lusitanica* Mill., forest biomass, small-scale biorefinery, valorisation

Resumo

A biomassa vegetal representa uma das fontes de energia renovável com maior potencial para produção de biocombustíveis, energia e produtos químicos, sendo útil como matéria-prima para diversas indústrias. As grandes quantidades de biomassa que são desperdiçadas, nomeadamente sobrantes florestais, constituem um grave problema a nível ambiental, uma vez que, na maioria das vezes, são deixadas ao abandono potenciando incêndios florestais.

Estes sobrantes correspondem à fração de biomassa produzida a partir das operações de exploração florestal, onde se destacam as operações de abate, corte, desbaste, poda e limpeza, das quais resultam muitos resíduos como folhas, ramos, troncos e cascas. De acordo com os últimos dados disponíveis, a produção anual de biomassa florestal em Portugal é cerca de 6,5 milhões de toneladas, onde apenas 2,2 milhões de toneladas são efetivamente utilizadas, sendo essa utilização essencialmente para fins energéticos. Foi a indústria da pasta de papel a pioneira no aproveitamento da biomassa florestal, sendo atualmente também utilizada para aproveitamento energético noutras indústrias como a indústria transformadora de madeira. Os principais consumidores deste tipo de biomassa em Portugal são as centrais termoelétricas a biomassa florestal, dedicadas à produção de eletricidade. Apesar da sua importância, esta biomassa ainda constituiu um recurso energético com um papel pouco relevante para a economia dos países desenvolvidos. A aposta na biomassa florestal tem-se restringido apenas à produção de energia, quer sob a forma de calor quer sob a forma de eletricidade, contudo o seu potencial não se limita apenas à produção de energia, mas poderá igualmente ser utilizada para a obtenção de outros produtos de elevado interesse para uma vasta gama de indústrias.

Nesse sentido e, de forma a aproveitar todo o potencial da biomassa florestal, surgem as indústrias de biorrefinaria. Uma biorrefinaria consiste numa unidade indústrial que pretende utilizar de forma integral e sustentável os recursos endógenos, de forma a obter uma vasta gama de produtos, não só calor e eletricidade, mas também biocombustíveis, materiais e produtos químicos, criando dessa forma novas cadeias de valor em volta da biomassa num conceito de bioeconomia circular. Desta forma, a criação de pequenas biorrefinarias regionais poderão ser assim uma alternativa viável para as pequenas e médias empresas ligadas ao setor das florestas.

Em Portugal, são inúmeras as empresas cuja atividade está diretamente ligada à floresta e que não tiram partido das grandes quantidades de biomassa que resultam das suas atividades. A Silvapor – Ambiente e Inovação, Lda., é uma empresa portuguesa localizada na Quinta da Devesa, concelho de Idanha-a-Nova, distrito de Castelo Branco, que tem como principais atividades a prestação de serviços florestais, agrícolas e outros, nomeadamente trabalhos de silvicultura geral, específica e preventiva. Como resultado das suas atividades, derivam muitos resíduos sem valor aparente e sem qualquer destino, sendo muitas vezes deixados ao abandono no solo ou queimados a céu aberto. A preocupação com a quantidade de resíduos resultantes das suas atividades e a oportunidade de obtenção de um rendimento complementar, levaram a Silvapor a instalar uma pequena destilaria nas suas instalações. Como principal objetivo a

empresa pretendeu valorizar estes resíduos através da obtenção de óleos essenciais, contudo, do processo de extração resultam outras frações que poderão igualmente ser valorizadas. Esta valorização dos resíduos sobrantes provenientes das atividades florestais, num conceito de biorrefinaria e desperdício zero, foi o ponto de partida para todo o trabalho aqui apresentado.

Neste sentido, o presente trabalho teve como principal objetivo a valorização integral da biomassa florestal endógena subaproveitada de *Cupressus lusitanica* Mill e *Cistus ladanifer* L., resultante das atividades de limpeza e manutenção pela empresa Silvapor, no contexto de uma micro-biorrefinaria. Pretendeu-se avaliar o potencial de valorização destas biomassas através da caracterização química e biológica das quatro frações que se obtém por destilação por arrastamento de vapor: i) óleos essenciais (EOs[1]), ii) hidrolatos (Hs), iii) águas de condensação (DCWs) e iv) resíduos sólidos extratados (ESRs) e pela utilização de algumas destas frações, nomeadamente os EOs e os ESRs, na produção de outros produtos como sabonetes e pellets, respetivamente.

A composição química dos EOs e dos compostos voláteis dos Hs foi avaliada por cromatografia gasosa e cromatografia gasosa acoplada a espetrometria de massa. No caso dos hidrolatos foi necessária uma extração prévia líquido-líquido com *n*-pentano para isolamento dos voláteis. As DCWs e os extractos de ESRs foram avaliados quanto ao teor em fenólicos totais, flavonóides e taninos, sendo o perfil fenólico das DCWs e dos extratos obtidos por ultrasons com etanol seguido de acetona a 70 % dos ESRs caracterizado por eletroforese capilar de zona. A atividade antimicrobiana dos EOs e dos Hs, foi testada, usando o método de difusão em agar, contra as bactérias *Escherichia coli* e *Staphylococcus aureus* e contra a levedura *Candida albicans*. A atividade antioxidante dos EOs, Hs, DCWs e ESRs foi determinada utilizando diferentes métodos, nomeadamente: i) captação do radical livre ABTS (sal diamónico do ácido 2,2'-azino-bis(3-etilbenzotiazolina-6-sulfonato)), ii) captação do radical anião superóxido, iii) inibição da xantina oxidase e iv) quelação de iões metálicos. A atividade anti-inflamatória dos Hs, DCWs e ESRs foi também determinada utilizando o método de desnaturação da albumina. Os ESRs foram avaliados em termos da sua composição lenhocelulósica, nomeadamente teor em celulose, hemicelulose, lenhina (Klason e solúvel), proteína e cinzas.

Os EOs de ambas as espécies mostraram ser constituídos maioritariamente por hidrocarbonetos monoterpénicos, enquanto os monoterpenos oxigenados dominaram os voláteis dos Hs. O α-pineno, o limoneno, o δ-3-careno e o sabineno foram os componentes dominantes (≥10 %) encontrados no EO de *C. lusitanica*, enquanto o *cis*-3-hexen-1-ol, a cânfora, a umbelulona, o *p*-cimeno-8-ol e o terpinen-4-ol foram os componentes dominantes (≥10 %) identificados nos voláteis do H. Os compostos maioritários (≥10 %) identificados no EO de *C. ladanifer* foram o α-pineno e a cânfora, enquanto o 2,6,6-trimetil ciclohexanona e o

[1] Para evitar a sobreposição, e/ou confusão, de abreviaturas, optou-se por apresentar na versão em Português do resumo as mesmas abreviaturas utilizadas na versão em Inglês.

trans-pinocarveol dominaram (≥10 %) os voláteis do H desta mesma espécie. As DCWs e os extratos dos ESRs mostraram ser ricos em compostos fenólicos, maioritariamente taninos, mostrando um perfil fenólico complexo, com a predominância de catequinas. Foi também possível identificar compostos como o ácido gálico, quercetina, derivados do ácido hidroxicinâmico, ácido salicílico, apigenina e ácido siríngico apenas nas amostras do *C. ladanifer*. Das diferentes extrações feitas aos ESRs, foi a extração com acetona a 70 % a mais eficiente na remoção dos compostos fenólicos para ambas as espécies. A análise à composição lenhocelulósica dos ESRs mostrou que ambas as biomassas são ricas em lenhina. Das diferentes atividades biológicas testadas, observou-se que os EOs de *C. lusitanica* e *C. ladanifer* apresentaram uma fraca atividade antimicrobiana contra *E. coli*, *S. aureus* e *C. albicans*. Por outro lado, todas as frações apresentaram uma considerável atividade antioxidante, tendo sido os EOs de ambas as espécies os que apresentaram melhores resultados pelo método de inibição da xantina oxidase. Das várias frações testadas, foram os Hs de ambas as espécies que apresentaram elevada atividade anti-inflamatória.

Para além do potencial como fonte de compostos bioativos de elevado interesse para diversas indústrias, ensaios preliminares mostraram também o potencial de algumas das frações, nomeadamente dos EOs e dos ESRs, na formulação de outros produtos como os sabonetes sólidos artesanais e os pellets.

Em conclusão, verificou-se que a valorização da biomassa florestal subaproveitada integrada num conceito de micro-biorrefinaria numa pequena e média empresa local é uma realidade possível e economicamente viável.

Palavras-chave: Biomassa florestal, *Cistus ladanifer* L., *Cupressus lusitanica* Mill., micro-biorrefinaria, valorização

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List of Abbreviations

A	AAE	Ascorbic acid equivalent
	ABTS	2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
	Ace	Acetone
	AE	Allopurinol equivalent
	AIP	Associação Industrial Portuguesa
	AP	Aerial parts
B	B	Bark
	BHI	Brain Heart infusion
	Br	Branches
	BSA	Bovine serum albumin
	Bu	Bulb
	Bud	Buds
C	CFG	Global cash flow
	CGAMC	Costs of good acquired and materials consumed
	Ck	Catkins
	ClaS	*C. ladanifer* soap
	CluS	*C. lusitanica* soap
	CM	Collection moment
	CO_2	Carbon dioxide
	$CO_2^{\bullet-}$	Carbon dioxide radical
	$CO_3^{\bullet-}$	Carbonate radical
	CS	Control soap
	Cu	Copper
	CZE	Capillary zone electrophoresis
D	D.A	Depreciation and amortization
	DCW (DCWs)	Distiller condensation water (s)
	DGADR	Direção Geral de Agricultura e Desenvolvimento Rural
	DLLME	Dispersive liquid-liquid microextraction
	dnp	Data not provided
	DP	Degree of polymerization
	DPPH	1,1-Diphenyl-2-picrylhydrazyl-hydrate
E	EDTAE	EDTA equivalent
	EO (EOs)	Essential oil (s)
	EO AP	Essential oil analysis procedure
	EOC	Portuguese commercial essential oil samples
	EO IP	Essential oil isolation procedure
	EP	Extraction procedure
	ESR (ESRs)	Extracted solid residue (s)
	ESR(Sx)	Soxhlet extract of ESR with different solvents
	ESS	External supplies and services
	EtOH	Ethanol
	€	Euro

F	F	Flowers
	FAx	Floral axis
	FB	Forest biomass
	FC	Financial charges
	Fe	Iron
	FH	Flower heads
	FHe	Flowering herb
	F_L	Flowers and leaves
	F_L_S	Flowers, leaves and stems
	Fr	Fruits
	FRAP	Ferric reducing-antioxidant power
	FT	Flowering tops
G	GAE	Gallic acid equivalents
	GC	Gas chromatography
	GC-MS	Gas chromatography-Mass spectrometry
	GR	Gross result
H	H (Hs)	Hydrolate (s)
	HAS	Human serum albumin
	HCl	Hydrochloric acid
	HD	Hidrodistillation
	HDF	Hidrodiffusion
	He	Herbs
	HIV	Human immunodeficiency virus
	$HO^{\bullet}$	Hydroxyl radical
	$HO_2^{\bullet}$	Hydroperoxyl radical
	HOCl	Hypochlorous acid
	HSE	Headspace extraction
	HS-SPME	Headspace solid-phase microextraction
	HV AP	Hydrolate volatiles analysis procedure
	HV IP	Hydrolate volatiles isolation procedure
	HYDRO/Ben	Hidrodistillation using benzene
	H_2O_2	Hydrogen peroxide
I	IAPMEI	Agência para a Competitividade e Inovação
	ICF	Investing cash flow
	IMPIC	Instituto dos Mercados Públicos, do Imobiliário e da Construção
	IRR	Internal Rate of Return
	ISO	International Organization for Standardization
L	L	Leaves
	LB	Lignocellulosic biomass
	L_B	Leaves and bark
	L_Br	Leaves and branches
	LLE Ben	Liquid-liquid extraction with benzene
	LLE Chl	Liquid-liquid extraction with chloroform
	LLE Cy	Liquid-liquid extraction with cyclohexane

	LLE Dcm	Liquid-liquid extraction with dichloromethane
	LLE DE	Liquid-liquid extraction with diethyl ether
	LLE EAc	Liquid-liquid extraction with ethyl acetate
	LLE Et	Liquid-liquid extraction with ether
	LLE EtOH	Liquid-liquid extraction with ethanol
	LLE MeCl	Liquid-liquid extraction with methylene chloride
	LLE NaCl/Dcm	Liquid-liquid extraction with sodium chloride/dichloromethane
	LLE *n*-Hep	Liquid-liquid extraction with *n*-heptane
	LLE *n*-Hex	Liquid-liquid extraction with *n*-hexane
	LLE *n*-Pen	Liquid-liquid extraction with *n*-pentane
	LLE Peth	Liquid-liquid extraction with petroleum ether
	L_Fr	Leaves and fruits
	L_S	Leaves and seeds
	L_St	Leaves and stems
	LNEG	Laboratório Nacional de Energia e Geologia
M	M	Month
	Max	Maximum
	MIC	Minimum inhibitory concentration
	Min	Minimum
	ML-term	Medium and long-term
	Mn	Manganese
N	n	Number of predicted time periods
	NAD^+	Nicotinamide adenine dinucleotide
	NADH	Nicotinamide adenine dinucleotide phosphate
	NBT	Nitro-blue tetrazolium
	NCF	Net cash flow
	nd	Not determined
	NERCAB	Associação Empresarial da Região de Castelo Branco
	NPV	Net Present Value
O	$O_2^{\bullet-}$	Superoxide radical
	OCF	Operating cash flow
	OH	Hydroxyl group
	OR	Operational result
	ORAC	Oxygen radical absorvance capacity
P	P	Pericarp
	PBP	Pay-Back Period
	PC	Personnel costs
	PCs	Phenolic compounds
	PE	Petals
	PET	Poroplast extraction technique
	PMS	Non-enzymatic phenazine methosulfate
	PPI	Project Profitability Index
	P&T-ATD	Purge-and-trap-automatic thermal desorption

Q	QE	Quercetin equivalents
R	r	Discount rate
	R	Rhizome
	RAT	Result after tax
	RBT	Result before tax
	RM	Raw material without any previous treatment
	RM(Sx)	Soxhlet extract of RM with different solvents
	$RO^{\bullet}$	Alkoxy
	$RO_2^{\bullet}$	Peroxy
	ROS	Reactive oxygen species
	Rt	Roots
	RV	Residual value
S	S	Seeds
	SD	Steam-distillation
	SMEs	Small and medium-sized enterprises
	Sp	Spathe
	St	Steams
	St_B	Stems and bark
T	t	Period of time
	T	Tax
	TEAC	Trolox equivalent antioxidant capacity
	TEP	Total expected profits
	TI	Total investment
	tons	tonnes
	TSA	Tryptic soy agar
U	UAEs	Ultrasound-assisted extracts
	UV	Ultraviolet
V	VAT	Value-added tax
	VP	Vegetative period
W	W	Wood
	WD	Water distillation
X	XO	Xanthine oxidase
Y	v/d.w.	Volume / dry weight
	v/f.w.	Volume / fresh weight
	YMB	Yeast malt broth

List of Publications

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Cláudia S. Tavares, Alice Martins, M. Leonor Faleiro, M. Graça Miguel, Luís C. Duarte, José A. Gameiro, Luísa B. Roseiro, A. Cristina Figueiredo, 2020. Bioproducts from forest biomass: Essential oils and hydrolates from wastes of *Cupressus lusitanica* Mill. and *Cistus ladanifer* L. *Industrial Crops and Products*, 144: 112034. DOI: doi.org/10.1016/j.indcrop.2019.112034. WOS: 000510521700035.

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Context and Motivation

Forest biomass production accounts for around 6.5 million tons/year in Portugal, with only 2.2 million tons/year effectively used for energy production (CAM, 2013). Diverse maintenance and logging operations result in different types of residues, such as bark, leaves, branches and wood parts. This biomass is usually underused, left in the ground or sent for energy recovery. However, in an integrated biorefinery concept, this biomass can be used as a raw material for the simultaneous production of fuels, energy, chemicals and other value-added products (Carvalheiro *et al*., 2008; Moure *et al*., 2001; SIADEB, 2013). Within this group of products, bioactive compounds can be highlighted, such as polyphenols and compounds that occur in essential oils, such as terpenoids. These compounds are widely valued due to their potential biological activities as antioxidant, antimicrobial and anti-inflammatory, among others, being thus of high interest to pharmaceutical, food and cosmetic industries (Albano *et al*., 2012; Raut and Karuppayil, 2014; Roseiro *et al*., 2013). Obtaining tradable products from underutilized endogenous resources, such as forest wastes, is highly demanded. However, this is an issue without an easy solution since it involves not only know-how on the technical-scientific aspects that lead to economically viable processes, but also on the logistical and regional aspects that impose major constraints on the economic viability of many of the processes currently available. Indeed, most of these processes are characterized by focusing on the production of only a single product from these wastes and, in most cases, from a single waste or a small part of it. Thus, the strategy could then involve the full valorisation of the available biomass, within a biorefinery concept, producing a broader set of products towards zero waste. This recovery will bring many benefits, thus reducing the ground waste available fuel in the event of a fire, production of new value-added products, greater economic power and job creation.

This work intended to contribute to the valorisation of some Portuguese forest residues, resulting from cleaning and maintenance activities of a national company – Silvapor - Ambiente e Inovação, Lda. As such, the company may use the residues resulting from its activities into added-value products within a local small-scale biorefinery approach, thus reducing their accumulation in the environment, boosting local use of biomass and creating new products with greater national and international economic potential.

Objectives

The large amount of biomass that is wasted is currently a serious environmental problem. Forest biomass is one example of such residues, due to the large quantities available while poorly used, being thus a boost for forest fire events. Therefore, the reutilisation of these residues, mainly from maintenance and logging operations of small/medium enterprises, must

be considered.

Bearing this in mind, the main goal of the present work was the full valorisation of two Portuguese forest species residues, *Cupressus lusitanica* Mill. and *Cistus ladanifer* L., within an integrated biorefinery concept. For that purpose, these two biomasses were subject to steam-distillation at an industrial scale in Silvapor premises, a SME company dedicated to forest maintenance, in order to obtain essential oils and hydrolates as immediate added-value products thereof. The remaining by-products of this process, namely, distilled condensation waters and extracted solid residues, were also studied and characterised, aiming to further valorization within a local small-scale biorefinery concept and towards zero waste.

The specific goals were:

i) Chemical characterization of *C. lusitanica* and *C. ladanifer* essential oils and hydrolates obtained by steam-distillation of its biomasses, and comparison with the same products obtained by hydrodistillation at a lab scale;

ii) Chemical characterization of the extracted solid residues of *C. lusitanica* and *C. ladanifer* in terms of cellulose, hemicellulose, Klason lignin, soluble lignin, extractives, protein and ash;

iii) Investigation for the presence of value-added compounds, namely phenolics, in distilled condensation waters and in extracted solid residues;

iv) Evaluation of some biological activities, (namely, antimicrobial, antioxidant and anti-inflammatory activities), of essential oils, hydrolates, distilled condensation waters and extracted solid residues;

v) Valorisation of the four different fractions obtained by steam-distillation and production of new value-added products using some of the previous fractions, namely, incorporation of essential oils in hygiene products, such as soaps, and use of the residual extracted solid residue to produce solid fuels, such as pellets;

vi) Simplified financial and economic analysis associated to the steam-distillation process and valorisation of *C. lusitanica* and *C. ladanifer* biomasses.

Thesis outline

The PhD thesis was organised in 5 chapters, 2 of these chapters consisting of peer review scientific works published in International Crops and Products journal. These 2 chapters contain abstract, introduction, methodology, results, discussion, conclusions, and independent bibliography, according to the guide for authors of the referred journal.

Chapter I consists in an introduction to the topics covered in this thesis. Being biomass the raw material of this work, this chapter intends to define this subject, namely on lenhocelulosic biomass, as well as its relationship with the concepts of biorefinery and circular bioeconomy.

While presenting Silvapor - Ambiente e Inovação Lda., the biomass under study, *Cupressus lusitanica* Mill. e *Cistus ladanifer* L resulting from the cleaning and maintenance work carried out by Silvapor is addressed. This chapter also defines the four distinct fractions that result from the distillation process of both biomasses, carried out in Silvapor: i) essential oils, ii) hydrolates, iii) distiller condensation waters and iv) extracted solid residues. Transversally to all fractions, different biological activities were assessed in this work, thus this topic is also referred in this chapter, namely the antioxidant, anti-inflammatory and antimicrobial activities.

Chapter II discusses the work developed with two of the fractions, namely the essential oils and hydrolates, resulting from the steam-distillation process of *C. lusitanica* and *C. ladanifer* biomasses. The composition of the corresponding essential oils and hydrolates volatiles was presented, as well as the results of the biological activities determined, namely the antimicrobial, antioxidant and anti-inflammatory ones.

Chapter III reports the work developed with the other two fractions, distiller condensation waters and extracted solid residues. The potential of both fractions was evaluated for total phenolic content and antioxidant and anti-inflammatory activities. The extracted solid residues were also chemically characterized in terms of its lenhocelulosic composition.

Chapter IV addresses the possible valorisations that may be given to the *C. lusitanica* and *C. ladanifer* considering the literature and the results obtained in the present study, giving special relevance to the production of soaps and pellets. In this chapter, a simplified economic analysis is also made on the viability of the biomass valorisation process for Silvapor.

Final considerations are described in **Chapter V**, where global conclusions and future work is presented.

Chapter I

Introduction

1. Biomass and Biorefinery

According to the latest data, the portuguese forest occupies about 36 % of the mainland (ICNF, 2015). Forest maintenance, such as thinning and cleaning of trees, can lead to considerable amounts of residues, including shrubs, trees and their trunks, barks, branches, leaves, flowers and even fruits (PCM, 2017). The availability of biomass associated with its composition is the basis to produce other types of products, such as biofuels, bioenergy and other bioproducts, that are increasingly replacing products derived from fossil fuels. The expansion and utilization of fossil fuels was crucial to modern industrial growth and human society innovation. However, its use leads to severe environmental problems. The most serious is the large amount of carbon dioxide (CO_2) produced during combustion of fossil fuels. Carbon dioxide is one of the greenhouse gases that contrinute to global warming (Kiang, 2018). Other problems is related to the fact that fossil fuels resources are not renewable and finite (Basu, 2013). In this way, biomass could be a strong alternative to the use of non-renewable resources, because it's a CO_2-neutral fuel, renewable and infinite (Basu, 2013; Kiang, 2018).

In a biorefinery concept, the full valorisation of biomass to obtain other products is becoming crucial to a better environment, economy and society (Han *et al*., 2018; Kehili *et al*., 2016). Given the need for an integrated forest policy that promotes the sustainability of the forest and its management, as well as the prevention of forest fires, it is intended that the development of biorefineries using endogenous resources can create new and sustainable value chains around the biomass in the so-called circular economy and bioeconomy (PCM, 2017, Zeller *et al*., 2018).

1.1. Biomass

According to the Directive 2009/28/EC, biomass is defined as "biodegradable fraction of products, waste and residues from biological origin from agriculture (including vegetal and animal substances), forestry and related industries including fisheries and aquaculture, as well as the biodegradable fraction of industrial and municipal waste".

Among all renewable energies, biomass is the only one that can be simultaneously converted into energy, fuels, materials and chemical products, being efficient as a raw material for industrial processes, such as biorefinery (COM, 2005/628; Kamm and Kamm, 2004). However, there are large amounts of biomass that are wasted, namely industrial and agro-industrial wastes of lignocellulosic nature (Carvalheiro *et al*., 2008), which are increasingly an environmental issue. One example is the forest biomass, in particular forest leftovers, which can constitute a serious problem due to the large quantities available, thus boosting forest fires. Forest biomass (FB) is identified as lignocellulosic materials from forest exploitation activities, under-cover forests and uncultivated areas (CAM, 2013). It is estimated that the annual production of FB in the Europe is 18 600 million tons / year, being around 48 % used for energy production (Camia *et al*., 2018). According to the latest data, in Portugal, the annual production of FB is around

6.5 million tons, while the availability of biomass with potential of being used is only 2.2 million tons. It should also be noted that, in practice, all the valorisation given to this biomass is for energy purposes (CAM, 2013).

1.1.1. Lignocellulosic biomass

The lignocellulosic biomass (LB) corresponds to different types of plant biomass, constituted mainly by polysaccharides (cellulose and hemicelluloses) and lignin (Verma *et al*., 2011), with an estimated global production of 10-50 billion tons/year (Salanti *et al*., 2010). Cellulose, hemicellulose and lignin are the main constituents of the cell walls of plant biomass, being these polymers responsible for the support structure of plant cell walls. The primary structure of the cell walls is composed of rigid fibers, called cellulose microfibrils enclosed by hemicellulose molecules, with lignin impregnated in hemicellulose molecules (Figure 1.1) (Pereira *et al*., 2003; Santos *et al*., 2012).

In the secondary structure of the cell, lignin is, many times, the major component. Contrary to the first layer, the second one presents a greater rigidity, forming a complex vascular system, resistant to the penetration of molecules of high molar mass, like enzymes, and to microbiological attacks (Pereira *et al*., 2003). Thus, the degradation of lignocellulosic materials is difficult and can only be achieved by chemical or mechanical processing (Aguiar and Ferraz, 2011).

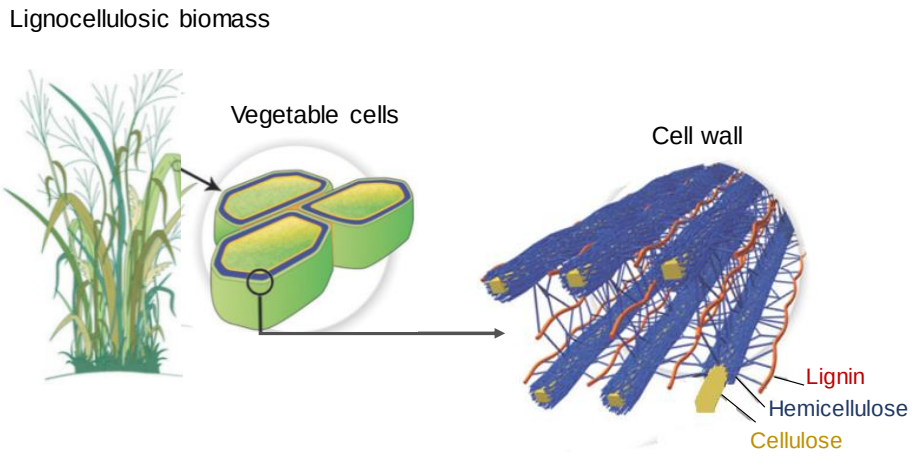

Figure 1.1. Structure of lignocellulosic biomass (Adapted from Santos *et al*., 2012).

The major structural components of LB are cellulose (40-50 %), hemicellulose (20-30 %) and lignin (20-30 %) (Lee *et al*., 2014; Isikgor and Becer, 2015) (Table 1.1). These individual parts give the LB a very strong polymer structure making it difficult to use. Thus, it is usually necessary to use pre-treatments involving physical, chemical and/or biological processes in order to obtain a selective and efficient separation of these fractions (Silveira *et al.*, 2015).

Table 1.1. Types of lignocellulosic biomass and their relative proportion of cellulose, hemicellulose and lignin (Adapted from Isikgor and Becer, 2015).

Lignocellulosic biomass		Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood	Eucalyptus	54.1	18.4	21.5
	Oak	40.4	35.9	24.1
	Poplar	50.8-53.3	26.2-28.7	15.5-16.3
Softwood	Douglas fir	44.0	11.0	27.0
	Pine	42.0-50.0	24.0-27.0	20.0
	Spruce	45.5	22.9	27.9
Agricultural and agro-industrial materials	Barley Hull	34.0	36.0	13.8-19.0
	Barley Straw	36.0-43.0	24.0-33.0	6.3-9.8
	Corn Cobs	33.7-41.2	31.9-36.0	6.1-15.9
	Wheat Straw	35.0-39.0	23.0-30.0	12.0-16.0
	Oat Straw	31.0-35.0	20.0-26.0	10.0-15.0
	Rice Straw	29.2-34.7	23.0-25.9	17.0-19.0
	Sugarcane Bagasse	25.0-45.0	28.0-32.0	15.0-25.0
	Miscanthus	36.2-39.9	21.9-28.9	9.5-18.6

In addition to the structural components, LB consist of proteins, pectins, starch, inorganic compounds, such as ash, and other groups of compounds called extractives (Kuila and Sharma, 2017). The extractives correspond to a large variety of compounds that can be divided into three groups: i) phenolic compounds, such as, flavonoids, phenolic acids, lignans and tannins; ii) terpenes, terpenoids and their derivatives; iii) aliphatic compounds such as alkanes, alkanols, waxes and free fatty acids. Furthermore, they are part of extractive inorganic compounds that comprise the whole fraction of ash, namely compounds like potassium, magnesium and silicon (Alén, 2000; Pereira *et al*., 2003). Unlike cellulose, hemicellulose and lignin, the extractives are easily extracted using suitable solvents. Separating and obtaining different components of LB is possible and thus result into value-added products like biofuels, materials and chemicals.

Cellulose

Cellulose is a linear homopolysaccharide made of repeating units of cellobiose linked to each other by β-1,4 glycosidic bonds. Cellobiose is formed by two glucose molecules bounded to each other through hydrogen bonds which the oxygen atom establishes with the 3-OH bond of the next molecule (Figure 1.2) (Rebouillat and Pla, 2013). The degree of polymerization (DP) of cellulose ranges from 500-15000 monomeric units according to the type of material. Agricultural residues, such as wheat straw and sugarcane bagasse, are composed of cellulose chains with lower DP (about 1000), while hardwood and softwood materials have a higher DP (about 5000) (Hallac and Ragauskas, 2011). Therefore, a high DP corresponds to a more rigid cellulosic material. Linear cellulose chains organize in parallel forming elementary fibrils that

are bound together by strong hydrogen bonds. The aggregation of elementary fibrils leads to the formation of microfibrils, consisting of highly ordered crystalline regions and amorphous sections (Ruel *et al*., 2012, Kirk and Farrel, 1987). Due to its crystalline structure and hydrogen bonds, cellulose has a high chemical resistance and is insoluble in most solvents (Fenger and Wegener, 1989; Sjöström, 1981). In the context of a biorefinery, it is expected that the pulp plays an important role as a raw material to produce various products, such as biofuels, nanocomposites and biofilms.

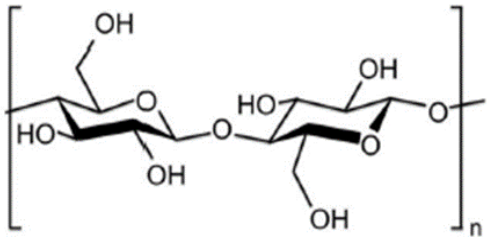

Figure 1.2. Molecular structure of a cellulose polymer chain (Adapted from Rebouillat and Pla, 2013).

Hemicellulose

Hemicellulose is an amorphous heteropolysaccharide, composed of several heteropolymers including xylan, galactomannan, glucuronoxylan, arabinoxylan, glucomannan and xyloglucan (Isikgor and Becer, 2015, Rose, 2003). The heteropolymers are composed by a wide range of 5- and 6- carbon monosaccharide units: pentoses (e.g. xylose and arabinose), hexoses units (e.g. glucose, mannose and galactose) and associated uronic acids (e.g. 4-O-methyl-glucuronic and galacturonic acids) (Figure 1.3) (Isikgor and Becer, 2015; Pereira *et al*., 2003; Puls, 1997). Unlike cellulose, hemicelluloses have low DP (between 80-200) (Waldron, 2010). This low DP associated with the lack of crystalline regions in the hemicellulose structure allows the hemicelluloses to be quite soluble in acids or alkaline solutions, being able to be hydrolysed in their monomeric components (Alén, 2000; Pereira, 2003). Depending on the lignocellulosic biomass, hemicelluloses differ in composition. Hardwood hemicelluloses contain mostly xylans, while softwood contain mostly glucomannans (Isikgor and Becer, 2015). The diverse macromolecular composition of hemicellulose offers a wide range of possibilities for the use of this fraction of lignocellulosic biomass. For example, furfural is one of the major chemicals resulting from the dehydration of pentoses derived from hemicellulose (Binder *et al*., 2010). Also, xylitol and arabinol are examples of products which can be obtained from hydrolysates of hemicellulose (Dietrich *et al*., 2017). Like cellulose, hemicellulose has also an important role in the context of a biorefinery, since it serves as a raw material to produce other products, such as thickeners, adhesives, emulsifiers and biofuels.

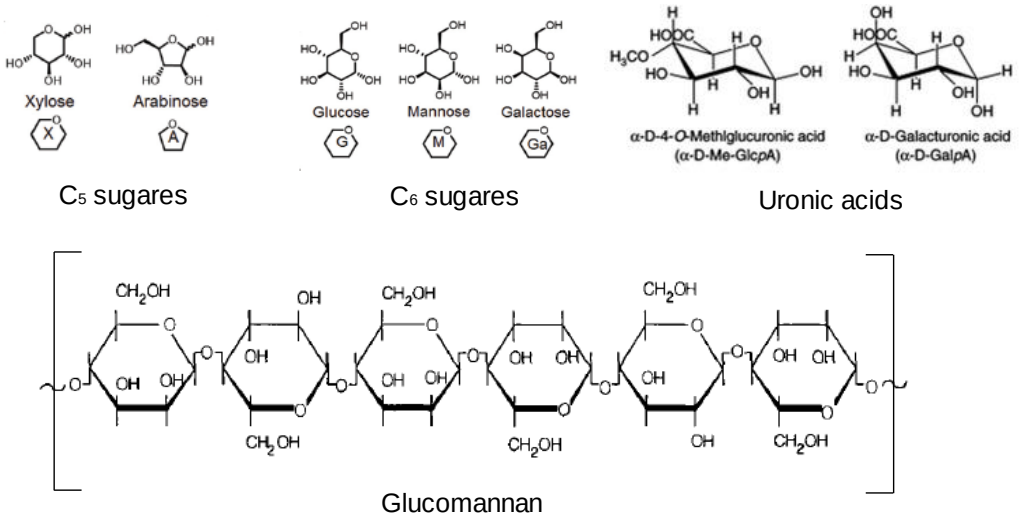

Figure 1.3. Chemical structure of the main monosaccharide units in hemicelluloses and an example of structure of glucomannan (Isikgor and Becer, 2015; Pereira *et al*., 2003; Puls, 1997).

Lignin

Lignin is a branched heteropolymer of polyphenolic and amorphous nature consisting of basic phenylpropane units linked together by carbon-carbon bonds and ether linkages. This aromatic macromolecule is formed by the dehydrogenation polymerization in the presence of three precursors and an enzyme, a process known as lignification. These precursors are the coniferilic, *p*-coumarilic and synapylic alcohols, which give rise to phenylpropane units guaiacyl, *p*-hydroxyphenyl and seringyl, respectively (Figure 1.4) (Brandt, 2013). Its origin can vary depending on whether it is hardwood or softwood. Hardwood lignin is generally composed of coniferyl and synapyl alcohols, while softwood lignin is mainly derived from coniferyl alcohol (Jönsson, 2016). In general, lignin has a complex three-dimensional structure, hard to degrade, giving the plants an important physical support (Fenger and Wegener, 1989). Due to its chemical nature, lignin is little affected by acids, bases or enzymes, being one of the most resistant natural polymers. However, and compared with polysaccharides, it is more sensitive to oxidation reactions or the action of organic solvents (Weng *et al*., 2008). Lignin is also a by-product of the pulp and paper industry, presenting several applications as a binder, dispersant and emulsifier (Chakar and Ragauskas, 2004). Within a biorefinery, lignin has shown to be a promising source of aromatic chemicals. For instance, the production of polyurethane and polyesters from lignin for bioplastic production can be highlighted (Bonini *et al*., 2005; Pandey and Kim, 2011).

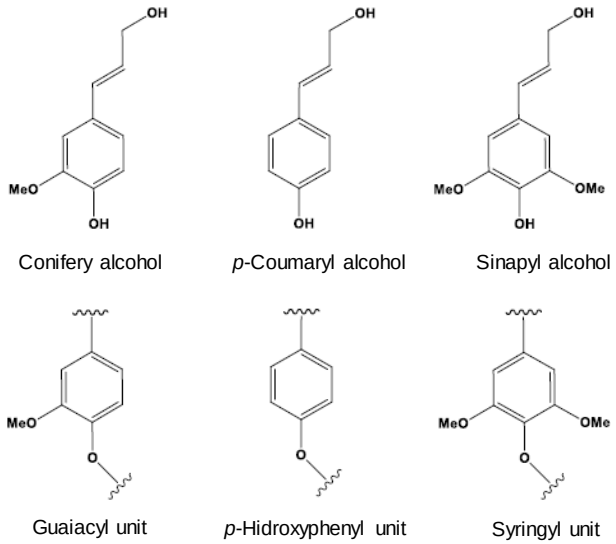

Figure 1.4. Chemical structure of three precursors and the respective phenylpropanoid units composing lignin macromolecule (Adapted from Brandt, 2013).

Other compounds

In addition to the structural components, cellulose, hemicellulose and lignin, lignocellulosic materials also have in their constitution other compounds, such as proteins, pectins, ashes and extractives (Fenger and Wegener, 1989; Kuila and Sharma, 2017). Focusing on the extractives, they comprise a variety of generally low molecular weight chemical compounds, being non-structural components contained in plant cells without being chemically attached to the cell wall (Pereira *et al*., 2003). Most of the extractives are secondary metabolites that play other roles in the plant besides the ones involved in the growth and cell development. This includes the protection of the plants against pathogens (Barnett and Jeronimidis, 2003). Extractives comprise very different classes of compounds and can be classified into the following groups: phenolics, terpenoids, fatty acid esters (fats and waxes) and alkaloids (Alén, 2000; Fenger and Wegener, 1989). In contrast to cellulose, hemicellulose and lignin, the extractives are extractable using solvents of suitable polarity without changes in the structural characteristics of the cell (Pereira *et al*., 2003). Although they constitute a small fraction of LB (under 10 %), they are currently of interest to the food, cosmetic, and pharmaceutical industries (Barnett and Jeronimidis, 2003).

1.2. Biorefinery

The search for clean and economically viable alternatives, such as renewable resources, with the aim of replacing fossil fuels, has sharply increased. Also, the concern with reuse and sustainable development has been growing over the last years, leading to the concept of biorefinery. By analogy to oil refineries, the concept of biorefinery emerged, consisting of an

industrial unit that integrates several processes of biomass conversion in a sustainable way for the simultaneous production of a wide range of value-added products, such as, energy, fuels, chemicals and materials (Figure 1.5) (Carvalheiro *et al*., 2008; SIADEB, 2013).

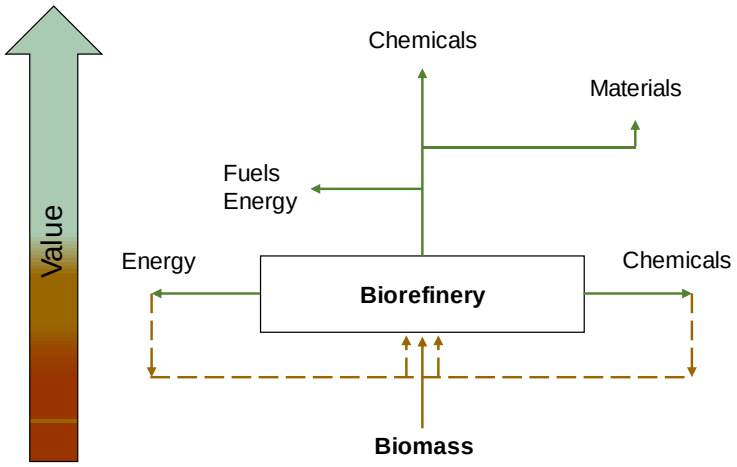

Figure 1.5. Concept of biorefinery (Adapted from SIADEB, 2013).

Despite the advantages of the use of biomass in relation to other resources of fossil origin, the process associated with the transformation of the biomass into other products is not easy, because it consists of several fractions, with very variable chemical composition. Thus, it requires advanced studies and processes to enable them to be used efficiently (Kamm and Kamm, 2004; Kamm, 2007; Ragauskas *et al*., 2003). Biorefineries can be classified according to the raw material used, the technological process and/or process technology. There are several technological processes that may be involved in the processing of biomass in order to obtain a greater number of added value products, such as separation processes, chemical/biochemical conversions and thermochemical conversions. Thus, biorefineries can be classified according to the type of technological process involved, and defined in different platforms: biochemistry, thermochemistry, biodiesel and biogas (Kamm *et al*., 2006; Kamm, 2007). The biochemical platform involves physical, physical-chemical, chemical or biological pre-treatment processes in order to convert the biomass into simple sugars and subsequent fermentation, with a view of, for example, producing liquid biofuels. The thermochemical platform involves the decomposition of the biomass by gasification and/or pyrolysis, using heat and catalysts (Kamm, 2007; Kamm *et al*., 2006). Both platforms are the most important and the use of each of them will depend on the type of installation, the technology involved and the products that are intended to be obtained. A higher operation efficiency of these facilities requires the improvement of the biochemical and thermochemical processes, as well as the development of new equipment. This will lead to a low amount of generated waste and consequently improved yield (Bozell and Petersen, 2010).

1.2.1. Small-scale Biorefinery

Biorefineries are needed to supply the market in the next years with all types of products, including, fuels, chemicals, materials and energy. However, the development of large-scale biorefineries will be always facing issues related to logistics and transports, especially due to limitations in terms of raw material availability and its accessibility (Loaiza *et al*., 2017).

A promising approach to speed up the implementation of integrated biorefineries is the stimulation and sponsoring of small and regional biorefineries. Small-scale biorefinery are useful ecologically, economically, and socially (Visser and Ree, 2016). Small-scale biorefineries have many advantages, namely: i) could be located on rural zones; ii) it requires relatively low initial investments; iii) it can integrate simple and inexpensive process technologies; iv) it results in the reduction of transportation costs; v) it may involve local stakeholders; vi) it increases rural employment, and, most importantly, vii) it makes full use of available local resources (Bruins and Sanders, 2012).

Small-scale biorefineries is a subject that has not been much studied in literature. Although they are very important for the rural development, this concept is still not really applied by Small and Medium-sized Enterprises (SMEs). One of the aims of this work it to show that it is possible to create a small-scale biorefinery by a National SME.

1.3. Circular economy, Bioeconomy and Circular Bioeconomy

The current fossil-based economy has been assigned as non-renewable, unsustainable, environmentally dangerous, incapable to manage waste and preserve resources. In addition, environmental concerns, such as climate change issues, have increased dramatically (Jong and Jungmeier, 2015). Alternatively, the European Union has been looking for solutions to these problems without affecting the economic growth. In response, two concepts have been proposed: circular economy and bioeconomic (COM, 2012/60; COM, 2015/614; McCormick and Kautto, 2013). The circular economy represents the ideology of preserving the economic value of products, materials, and resources for as long as possible, thus minimizing the generation of waste (COM, 2015/614). On the other hand, the use of biomass resources as raw materials to produce food, bio-based materials and bioenergy is the basis of a bioeconomy (COM, 2012/60; McCormick and Kautto, 2013). Both concepts share some of the targets like a more sustainable and resource efficient world with a low carbon footprint, reduction of fossil carbon and waste valorisation (Carus and Dammer, 2018). The crossover between circular economy and bioeconomy corresponds to the new concept of circular bioeconomy. Sharing, reusing and recycling, all associated with the usage of biomass resources for production of new materials and bioenergy, are the main principles of a circular bioeconomy (Carus and Dammer, 2018). The merging of these concepts will result in the production, utilization, consumption, storage, recycling and reuse of biological and renewable resources towards: i) rational

management of resources; ii) sustainable value chain creation in industry; iii) zero or reduced impact in the existing and future environmental issues and iv) sustainable growth of population worldwide (COM, 2012/60; COM, 2015/614; McCormick and Kautto, 2013).

2. Silvapor – Ambiente e Inovação, Lda.

With 30 years of history, Silvapor – Ambiente e Inovação, Lda., is a company located at Quinta da Devesa, municipality of Idanha-a-Nova, district of Castelo Branco. Its activity started in 1989, initially as Silvapor - Agricultura e Silvicultura Lda. From 2015, its name changed to Silvapor - Ambiente e Inovação Lda., designation which is currently used (Silvapor, 2019).

Silvapor is characterized by its entrepreneurial and agile structure, supported by a dynamic team. In addition to the team located at Idanha-a-Nova, other mobile teams are distributed throughout different regions of the country. Each team is committed to the sustainable development of the forest, maintaining the necessary environmental concern that results from its activities. Using its know-how, creativity and equipment, Silvapor is prepared to respond to the needs of the customers.

Forest related, agricultural and other services, namely general, specific and preventive works, as well as the production and commercialization of forest plants are the main activities of Silvapor. With the objective of enriching the range of skills, Silvapor is also engaged in other type of works, such as dune work, installation and maintenance of gardens, elimination of spontaneous vegetation along riverside areas, dams and river basins, berms and roadways slopes.

"The difference is in the quality" is the slogan of Silvapor and based on this saying, the company participated, between 2007 and 2008, in the Small and Medium-sized Enterprises (SMEs) Project "Business Excellence" (*Excelência Empresarial*) promoted by the Portuguese industrial association AIP (*Associação Industrial Portuguesa*) and by the business association of the region of Castelo Branco NERCAB (*Associação Empresarial da Região de Castelo Branco*). As a result, Silvapor attained in 2008, the certification no. 2008 / CEP.3335 on quality management systems according to the Portuguese standard *NP EN ISO 9001:2008 Sistemas de Gestão da Qualidade*. In addition, Silvapor has the construction permit IMPIC no. 60,795, from IMPIC (*Instituto dos Mercados Públicos, do Imobiliário e da Construção*), the Portuguese entity that regulates and supervises the construction and real estate sector.

In 2012, Silvapor obtained the licence 010-AT, issued by the Portuguese department of agriculture and rural development DGADR (*Direção Geral de Agricultura e Desenvolvimento Rural*), which allows supplying services of land-based application of plant protection products. In the same year, Silvapor has been awarded by the Portuguese agency for competitiveness and innovation IAPMEI (*Agência para a Competitividade e Inovação*) with the Leader SME Status (*SME Líder*) that distinguishes national SMEs with the best economic-financial performance and risk levels.

Due to the current and future requirements, innovation in companies is fundamental. As such, Silvapor has also dedicated the last years to innovation, research and development. With the project INFORMAT – Intelligent Forest Management Technologies, Silvapor won the 5th edition of the Entrepreneurship and Innovation Award 2018 (*Prémio Empreendedorismo e Inovação 2018*) in the Rural Development category, awarded by the credit institution *Crédito Agrícola*.

Internationally, Silvapor has the exclusive representation of "Pepiniéres NAUDET", the largest company in France in the production of forest plants and services (Silvapor, 2019).

The concern with the amount of waste that results from the company activities and the opportunity to obtain a complementary income, led the SME Silvapor to install a small distillery in its facilities, in order to study the possibility of obtaining essential oils thereof. This valorisation of residual waste from forest activities, in a concept of zero waste within a small-scale biorefinery approach to this SME, was the cause for all the study presented in this dissertation.

3. Biomass under study

Cupressus lusitanica Mill. and *Cistus ladanifer* L. were the species studied in the present work. These were selected, since large amounts of these species biomass result from landscaping works of Silvapor, Ambiente e Inovação Lda. *C. lusitanica* is an ornamental tree, abundant in the region, thus requiring constant maintenance. *C. ladanifer*, is a species that, due to its low height, constitutes a problem in the case of a fire, since it helps spreading it closer to the ground. For this reason, soil maintenance is very important, keeping it free from biomass which could be a problem. Generally, the waste resulting from these activities is not used for any purpose other than for domestic burning and is often left discarded on the ground. Thus, this results in an environmental problem, because its accumulation on the fields constitutes a fuel for fires.

3.1. *Cupressus lusitanica* Mill

Cypress trees are Cupressaceae native to the temperate climate and are specifically located in Mediterranean regions, North America and Asia. Cypresses are beautiful trees popular for landscaping. They can grow extremely tall, up to 45 m in height. It is estimated that more than two dozen types of trees are found in different regions all over the world. Different types of cypress have distinct shapes. The foliage of cypress is known to have different shades of green, from dark green to lighter bluish green depending the type of plants. These plant species produce fruits (cones) of different sizes, the largest size around 5 cm. Each cone has approximately 30 seeds. All the cypress varieties have one thing in common, they demonstrate to be heaven for all types of wildlife, namely birds that like cypress trees due to their strong

branches, excellent for building nests (Shaheen *et al*., 2020).

Wood of cypress is also very famous for firewood, and commonly used in manufacturing tables, boats, cabinets and boxes. Essential oil of cypress species is abundantly used in hair shampoo and various beauty products and when is topically applied, it reduces inflammation because it possesses potential to decrease swollen blood vessels, as a result of which it helps to recover from oedema. In aromatherapy, it is used to transmit a sense of emotional tranquillity. The EO of cypress species has several health benefits, as it significantly contributes to a number of pharmaceutical products due to its antiseptic, antispasmodic, astringent, deodorant, diuretic, haemostatic, hepatic, sedative and sudorific properties, along with the capabilities to treat the respiratory disorders. Alcoholic extracts of cypress branches are known for their strong antibacterial potentials against gram-positive and gram-negative bacteria. Aqueous and alcoholic extracts of these plants have also shown antioxidant and anti-inflammatory potentials. *In vitro* studies proved that extracts of some Cupressaceae species have anticancer potential and ability to inhibit human immunodeficiency virus (HIV) (Shaheen *et al*., 2020).

Cupressus lusitanica Mill. is a cypress tree (Figure 1.6), commonly known as cedar of Goa, Mexican cedar and Portuguese cedar (Kuiate *et al*., 2006). It is a native tree from Central America (Costa Rica, Guatemala and Mexico), growing nowadays in many countries, including Brazil, California, France, Italy, New Zealand, Portugal and Spain (Adams *et al*., 1997; Kuiate *et al*., 2006). In Portugal, its presence is mainly in Mata do Buçaco, Parque da Pena, Monserrate and Serra da Gardunha, mostly in central and northern parts of the country, but it is used in all country as an ornament tree (Araújo, 1966). *C. lusitanica* is a fast-growing tree that can reach 25 to 30 m in height (Figure 1.6 A). It has a dense pyramidal crown and a trunk surrounded by a brown bark (Figure 1.6 B). It is also distinguished from other cypresses by the blue-green leaves with free pointed tips in spreading sprays (Figure 1.6 C) and yellow flowers, between February and March (Figure 1.6 D). The fruits are small cones 1 to 1.5 cm diameter, gray-blue when young, but with age, have a brown and bright hue (Figure 1.6 E). Although *C. lusitanica* can adapt to different climates, it prefers humid ones (Kuiate *et al*., 2006; Serralves, 2019).

C. lusitanica is essentially used as an ornamental tree and in commercial forest plantations. The wood is also used in furniture manufacture due to its lightness, low density, fine texture and high dimensional stability (Kuiate *et al*., 2006). The leaves are used traditionally to protect stored grains from insect infestation and also to treat skin diseases caused by dermatophytes (Bett *et al*., 2016; Kuiate *et al*., 2006; Teke *et al*., 2013). The EO from the leaves is commonly used to treat haemorrhoids, rheumatism, whooping cough and styptic problems (Teke *et al*., 2013). The EO is also used in aromatherapy and massage to restore tranquillity, soothe anger, improve blood circulation and treat coughs and bronchitis (Bett *et al*., 2016).

There is still little information on the chemical composition of *C. lusitanica*, being only possible to find information on the composition of EOs extracted from this species (referred in Chapter II, Table 2.3).

Figure 1.6. *Cupressus lusitanica* Mill. tree (A), bark (B), leaves (C), flowers (D) and fruits (cones) (E) (Adapted from Serralves, 2019).

Some biological properties have been attributed to *C. lusitanica* EO. Hassanzadeh *et al*. (2010) reported antibacterial activity of *C. lusitanica* EO against *Bacillus cereus* and antifungal activity against *Aspergillus niger*. Teke *et al*. (2013) reported the use of the EO as a natural antimicrobial agent for human infectious diseases, namely those caused by *Enterococcus faecalis*, *Proteus mirabilis* and *Candida albicans*. Bett *et al*. (2016) reported that *C. lusitanica* EO is a promising insecticide and repellent to be used against insect pests of stored food grains. Also, Guimarães *et al*. (2010) reported the antioxidant activity of *C. lusitanica* EO using different antioxidant activity assays, namely 1,1-diphenyl-2-picrylhydrazyl-hydrate (DPPH) free radical scavenging, reducing power and lipid peroxidation inhibition.

3.2. *Cistus ladanifer* L.

Cistus plants are Cistaceae Mediterranean native shrubs (Barrajón-Catalan *et al*., 2016). *Cistus* genus is disseminated over different areas of the Mediterranean region, namely Canary Islands, Greece, Italy, Portugal, Spain, Turkey and north-west Africa (Barrajón-Catalan *et al*.,

2010). Most *Cistus* species are very fragrant with sweet-smelling, being much appreciated in the perfume industry and for ornamental proposes (Barrajón-Catalan *et al*., 2016). *Cistus* plants are perennial shrubs with evergreen, opposite, usually slightly rough-surfaced, 2-8 cm long leaves. The flowers are hermaphroditic and actinomorphic, with three or five sepals opposite to petals. The colours of petals ranging from white to purple and dark pink depending on the subgenus, with a conspicuous dark red spot at the base of each petal in a few species, like *Cistus ladanifer* (Barrajón-Catalan *et al*., 2016). In some species, the leaves are coated with a highly aromatic resin called labdanum. Labdanum cannot be considered an EO, although it contains a high percentage of volatile compounds, most of them in common with the corresponding EOs. EOs and resins produced by plants have several vital functions. They can contribute to pollination, act out as insect repellent agents, protect against bacterial and fungal attack, as well as prevent against ultraviolet damage (Greche *et al*., 2009).

Traditional folk medicine has used extracts and EOs of *Cistus* species for a large variety of purposes such as antioxidant, anti-inflammatory, antiulcerogenic, cytotoxic and vasodilator remedies. Aqueous extracts of leaves have also been used as an antidiarrheic and antispasmodic (Zidane *et al*., 2013). *Cistus* EOs and extracts have demonstrated antimicrobial activity which has been reported against several Gram-positive and Gram-negative bacteria and against some fungal infections (Barrajón-Catalan *et al*., 2010; Greche *et al*., 2009; Tomás-Menor *et al*., 2013). *Cistus* EOs have been only marginally used as a flavouring agent for food (Barrajón-Catalan *et al*., 2016).

C. ladanifer is a shrub (Figure 1.7) with the common English name of rockrose, and "esteva" in Portuguese (Dentinho *et al*., 2014; Frazão *et al*., 2018). *C. ladanifer* is one of the most significant natural shrubs in the Mediterranean, mainly distributed in countries such as Algeria, Greece, Italy, Morocco, Portugal and Spain (Alves-Ferreira, 2019a). In Portugal, it extends throughout the country, especially in the centre and south regions, being present in native forest and uncultivated lands (Dentinho *et al*., 2014; Ferreira *et al*., 2009; Alves-Ferreira, 2019a). *C. ladanifer* is recognizable by the typical white flowers with crimson spots at the base of petals (Figure 1.7 D). It is an odorous shrub up to 2 m high (Costa *et al*., 2009; Mariotti *et al*., 1997). It occurs in acidic soils, poorly developed and with nutritional deficiency (Rossini-Oliva *et al*., 2016). It grows spontaneously, and their overgrowth may lead to environmental problems. This shrub colonizes degraded areas and restricts or inhibits the growth of other plants, and/or the germination of their seeds (Barrajón-Catalan *et al*., 2010; Dentinho *et al*., 2014). Showing high proliferation, it occupies abandoned or unmanaged agricultural areas, representing a fire hazard. It is known that, if not properly controlled, this species is one of the major responsible for fire spreading in the Mediterranean forest (Alves-Ferreira *et al*., 2019a). On the other hand, *C. ladanifer* plays an important role in the recovery of forest areas after fire, providing available mycorrhizal inoculum to colonize tree roots as new zones are developed (Hernández-Rodríguez *et al*., 2013). *C. ladanifer* ecosystem also provides high production of edible mushroom species

(Hernández-Rodríguez *et al*., 2015). In addition, *C. ladanifer* is considered promising for animal feed, for phytoremediation and revegetation programs of contaminated soil and as raw material to produce bioproducts (e.g. bioethanol) (Rossini-Oliva *et al*., 2016; Santos *et al*., 2016).

Figure 1.7. *Cistus ladanifer* L. shrubs (A), leaves (B), flower bud (C) and flower (D).

C. ladanifer leaves are coated with labdanum, which is much appreciated in the perfume industry due to its excellent fixative properties. Labdanum is also used as an additive to aromatize some types of tobacco, and to treat catarrh, diarrhoea, dysentery and menstruation discomfort (Barrajón-Catalan *et al*., 2010; Barrajón-Catalan *et al*., 2016). *C. ladanifer* is particularly interesting for the extraction of EOs (Gomes *et al*., 2005; Mariotti *et al*., 1997; Teixeira *et al*., 2007).

EO of *C. ladanifer* is extremely complex, with, in some cases, up to 300 compounds having been detected (Mariotti *et al*., 1997). Chemical composition of *C. ladanifer* EO has been thoroughly studied (referred in Chapter II, Table 2.7) Although *C. ladanifer* shows a low

essential oil yield, (around 0.1 %), it has a potential use in cosmetic, food and pharmaceutical industries (Frazão *et al*., 2018).

C. ladanifer EO has shown significant antioxidant and antimicrobial properties (Greche *et al*., 2009; Zidane *et al*., 2013). In the same way, several biological activities have been attributed to *C. ladanifer* solvent extracts, such as, antioxidant (Andrade *et al*., 2009; Barrajón-Catalán *et al*., 2010; Zidane *et al*., 2013), antibacterial (Greche *et al*., 2009; Tomás-Menor *et al*., 2013), antifungal (Chaves *et al*., 2001; Greche *et al*., 2009), cytotoxic (Barrajón-Catalan *et al*., 2010), allelopathic (Chaves *et al*., 2001; Herranz *et al*., 2006) and hypoglycemic (El Kabbaoui *et al*., 2016).

Some works reported the composition and potential of *C. ladanifer* lignocellulosic biomass (referred in Chapter III, Table 3.2). In general, extractives, lignin and cellulose were the major fractions (Alves-Ferreira *et al*., 2017; Alves-Ferreira *et al*., 2019a; Alves-Ferreira *et al*., 2019b, Carrión-Prieto *et al*., 2017; Fernandes *et al*., 2018; Ferro *et al*., 2015), reporting potential as a source of phenolic compounds and an interesting feedstock option for production of biofuels (Alves-Ferreira *et al*., 2017; Ferro *et al*., 2015).

4. Biomass fractions of interest

Taking advantage of the small distillery installed at the SME Silvapor facilities, this was used to obtained essential oils (EOs), and their hydrolates (Hs), distiller condensation waters (DCWs) and extracted solid residues (ESRs) as distillation products and/or by-products from the forest biomass, aiming at its valorisation towards zero waste. From all mentioned fractions, the EOs are currently the most valorised, although hydrolates are also commercialized and the extracted solid residues used for heat production. However, the study and demand for Hs has increased over the years, as well as the use of biomass solid residues to produce biofuels such as pellets. Among these fractions, the least exploited, and consequently valued, are the DCWs. However, it is also a fraction with strong potential, particularly as a possible source of bioactive compounds.

In the present work, all four fractions from steam-distillation of *C. lusitanica* and *C. ladanifer* biomass were obtained and studied with the aim of getting added-value products thereof.

4.1. Biomass Distillation

Throughout the present work, the extraction methods used were the steam-distillation at the industrial scale and hydrodistillation at the laboratory scale. Steam-distillation was the extraction method used in Silvapor. Besides the equipment that is already owned by the company, this method is also used at the industrial scale since it allows the distillation of large quantities of biomass and under controlled conditions. Additionally, the hydrodistillation

method was used to compare the composition of some fractions (EOs and Hs) obtained by different methods, at different scales.

4.1.1. Steam-distillation

In the steam-distillation process, water vapor is produced in a boiler and then injected, under controlled pressure, into the barrel (Figure 1.8 A) where the plant material is located (Figueiredo *et al*., 2014). Water vapor passes through the material, and drags the volatile compounds, which is followed by a step of condensation in the so-called condenser. It is in the collector that the separation between the aqueous phase (hydrolate) and the EO occurs, resulting from the condensation process of the vapor mixture, with the EO being, usually, collected from the upper part and the aqueous phase from the lower part of the collector (Axtell and Fairman, 1992).

In this type of extraction, as the plant material is not in direct contact with water and the distiller does not overheat, the degradation of the EO constituents is lower (Axtell and Fairman, 1992). The whole process is a less energy consuming, and faster extraction method compared to hydrodistillation. Industrially, this is the most used method because, and besides the large amounts of biomass that can be distilled, it allows obtaining better quality EOs due to the operating conditions (Figueiredo *et al*., 2014).

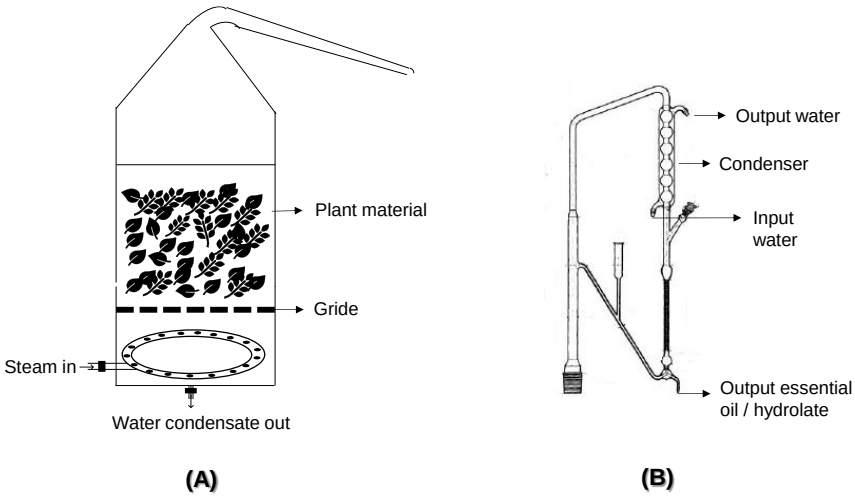

Figure 1.8. Steam-distillation (A) (Adapted from Axtell and Fairman, 1992) and Clevenger (B) (Adapted from Lourenço, 2007) apparatus.

4.1.2. Hydrodistillation

In the hydrodistillation process, the plant material is immersed in water which is then boiled by heating. The steam generated and containing the volatile compounds passes through a condenser, where it cools and condenses. As a result, an aqueous phase and an EO is obtained (Lourenço, 2007). According to the Portuguese Pharmacopeia VIII (2005), the Clevenger

apparatus (Figure 1.8 B) is the distiller used in this type of distillation.

Hydrodistillation is a simple and versatile process, however, it can present some problems such as direct heating that can be difficult to control and stabilize and thus lead to variations in distillation yield. In the case of overheating, the plant material can be burned, reducing the quality of the product or even making it impossible to recover (Proença da Cunha *et al*., 2012). The fact that plant material is in direct contact with water is favourable to hydrolysis, oxidation and other reactions that may lead to the formation of artefacts. On the other hand, some more water-soluble EO compounds may remain in the water and thus not be recovered. It is usually a process that takes time and consequently there is a higher energy consumption (Figueiredo *et al*., 2014).

As a result of this same extraction, different sub-products are obtained, namely i) essential oils, ii) hydrolates, iii) distilled condensation waters and iv) extracted solid residues.

4.2. Essential oils

The EOs are defined by the International Organization for Standardization (ISO) as a "product obtained from natural raw material of plant origin, by steam-distillation, by mechanical processes from the epicarp of citrus fruits, or by dry distillation, after separation of the aqueous phase, if any, by physical processes" (ISO 9235, 2013). Essential oils are a complex mixture of secondary metabolites of low molecular weight, which are produced and stored in the secretory organs of aromatic plants (Proença da Cunha *et al*, 2012). Chemically, EOs consist mainly in terpenes (mono-, sesqui- and diterpenes), along with phenolic compounds, such as phenylpropanoids, and other groups of compounds that can also appear in relevant amounts (Figueiredo *et al*., 2007).

Various processes may be used to extract natural products from plants, but only two are used to obtain EOs: by a mechanical process (e.g. in the case of the *Citrus* genus) or by distillation (Figueiredo *et al*., 2014). The expression is the mechanical process used in fruits of *Citrus* genus, such as orange, lemon and tangerine, whereby pressing the epicarp it's possible to obtain the EO. This is subsequently separated from the water by centrifugation or decantation (Proença da Cunha *et al*., 2012). The distillation process uses water and/or steam to extract volatile compounds. Water in the form of steam passes through the plant material and drags these compounds, the essential oils, which after condensation constitute the supernatant phase floating on the surface of an aqueous phase (also known as hydrolate or hydrosol) (Axtell and Fairman, 1992). Three types of distillation are considered: i) hydrodistillation, ii) steam-distillation and iii) distillation by water/steam (FAO, 1992).

The composition of an EO may be affected by different factors: i) physiological variations such as plant organ (i.e. leaf, flower, fruit) development, type of plant material and seasonal variation, ii) type of material harvested (flowers, leaves, stems, fruits, among others), iii) geographic variation, iv) environmental conditions such as climate, storage and soil quality and

v) genetic factors are aspects that may affect the composition of EO (Figueiredo *et al*., 2008).

EOs are of a particular economic interest because they can be used in several types of industries, such as food, perfumery, and pharmaceutical (Proença da Cunha *et al*., 2007). This interest is due to the various biological activities attributed to them, such as antioxidant, anti-viral, antibacterial, fungicidal, insecticidal, herbicidal, among others (Dadalioglu, 2004; Isman, 2000; Isman, 2006; Milhau *et al*., 1997). In general, the biological activities of the EOs result from the combined effect of several compounds and not from a single one (Faria, 2015). For commercialization purposes, the general rules for labelling and marking of containers of essential oils (ISO/TS 211, 2014), should be followed, that is, the label should include information on the common and the scientific name of the species, the part of the plant used, the cultivation type and extraction method, the country of origin, among others.

4.2.1. Essential oils composition

Terpenoids are one of the major and most diverse classes of natural products. Some classes of terpenoids are composed by substances that generally give plants their fragrance, being the important and most dominant constituents of essential oils (Jan and Abbas, 2018). Terpenoids are biosynthesized from isopentenyl pyrophosphate by mevalonate acid pathway (Ruchica *et al*., 2019). Typical structures contain carbon skeletons represented by $(C_5)_n$ and depending on the number of isoprene units, they are classified as hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}) and tetraterpenes (C_{40}) (Dewick, 2009; Ruchica *et al*., 2019). Hemiterpenes contain a single unit of isoprene, being isoprene the only one. However, oxygen-containing derivatives of isoprene, such as, isovaleric acid and prenol are also included in this category (Jan and Abbas, 2018). Monoterpenes are the major classes of terpenoids, followed by sesquiterpenes and diterpenes.

Monoterpenes

Monoterpenes contain two isoprene units. They are typical volatile components of plants and are widely used in the fragrance industry (Boysen and Hearn, 2010). They also have large pharmaceutical properties, including antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, anticancer, antihistaminic, antispasmodic and local anaesthetic (Kozioł *et al*., 2014). Monoterpenes can be grouped into distinct classes, such as acyclic (e.g., β-myrcene, β-ocimene, citral, citronellal, citronellol, linalool, geraniol, among others), cyclic (e.g., α-terpineol, carvacrol, carvone, eugenol, limonene, *p*-cymene, terpinen-4-ol, thymol, 1,8-cineole, among others) and bicyclic (e.g., α-pinene, β-pinene, *cis*-verbenol, isoborneol, among others) (Figure 1.9) (Kozioł *et al*., 2014). It is common to find oxygenated compounds such as alcohols, aldehydes and acetones, associated with these hydrocarbons (Proença da Cunha, 2005).

Sesquiterpenes

Sesquiterpenes are composed by three isoprene units. Although they are a small group of terpenoids compared to monoterpenes, their sources are extensive, having been isolated from higher plants, terrestrial fungi, marine organisms and insects (Awouafack *et al*., 2013; Boysen and Hearn, 2010). Some sesquiterpenes have spicy flavours as found in ginger (zingiberene) (Boysen and Hearn, 2010). They occur in nature as hydrocarbons or in oxygenated forms including alcohols, aldehydes, acetones and acids (Awouafack *et al*., 2013). Farnesol, farnesene and germacrene-D are some examples of compounds belonging to this group (Figure 1.9). Sesquiterpenes have been reported to possesses several pharmacological activities, such as, antibacterial, antifungal, antiviral, anti-inflammatory, antimalarial, antitumoral, cytotoxic, among others (Awouafack *et al*., 2013).

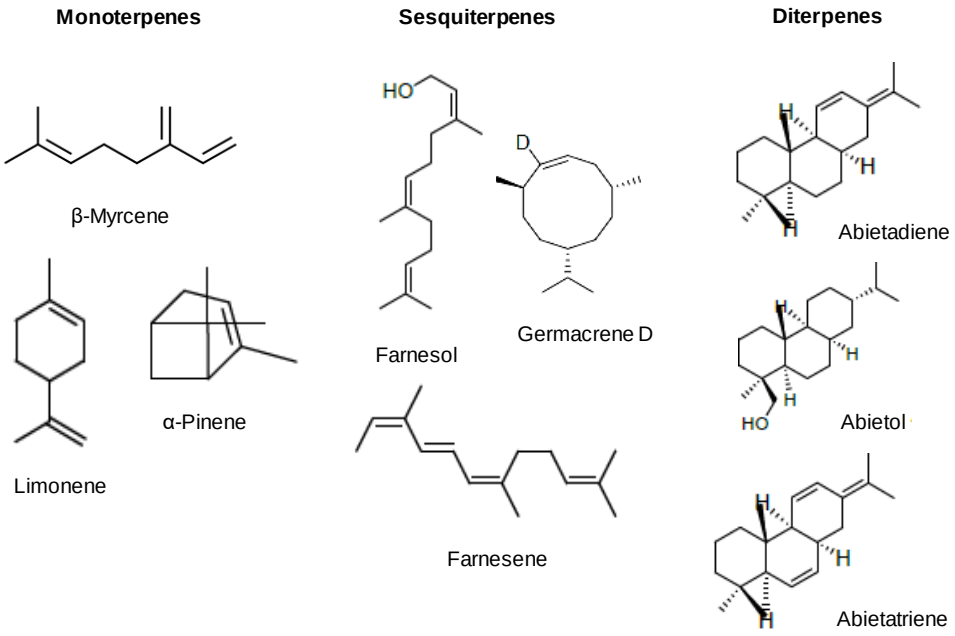

Figure 1.9. Examples of monoterpenes, sesquiterpenes and diterpenes (Adapted from Figueiredo, 2017).

Diterpenes

Diterpenes contain four isoprene units and are classified into several categories, namely abietanes, labdanes, phytanes, clerodanes, pimaranes, among others (Pasdaran and Hamedi, 2017). Some examples are abietadiene, abietol and abietatriene (Figure 1.9). The various biological activities from this class of compounds have increased interest. An example is taxol, a diterpene with potential anticancer properties (Boysen and Hearn, 2010; Pasdaran and Hamedi, 2017).

In general, terpenoids play an important role in plant defence, being the phytoalexins an example (Jan and Abbas, 2018). These natural compounds also show antibacterial, antiviral,

anti-inflammatory, anticancer, antimalarial, antiulcer, antihepaticidal and diuretic properties (Jan and Abbas, 2018). Some pain-relieving properties of cannabis have been attributed to the terpene-phenols cannabinoids present in this plant. Others terpenoids present in cannabis, such as β-myrcene and carvacrol, exhibit a potent anti-inflammatory effect (Hazekamp *et al*., 2010).

4.3. Hydrolates

Hydrolate (H), also referred as hydrosol or floral water, corresponds to the distilled water that remains after the hydro- or steam-distillation, being usually rich in EO water-soluble components (ISO 9235, 2013). Hydrolates are constituted predominantly by hydrophilic compounds (Lobiuc *et al*., 2014), being the volatiles composition dominated by oxygen-containing compounds (Aazza *et al*., 2011). Although highly diluted, the Hs keep the strong smell, but still smother than the corresponding EO (Śmigielski *et al*., 2013). Like the EOs, also the Hs should be carefully stored. Hs should be collected in aseptic containers and stored in a cool place. After storage, lifetime is about 1 year. After this period, the smell and composition tend to change (Jeannot 2003). When commercialized, should include information on the common and the scientific name of the species, chemical composition, the part of the plant used, the cultivation type and extraction method, the country of origin and the certificate of purity. There is nowadays a growing interested in the use of Hs and, for this reason, the information on Hs volatile composition is very important, not only for the producer, but also for the consumer.

4.3.1. Extraction and analysis of the hydrolate volatiles

The analysis of the H volatiles fraction requires a previous extraction, usually using a liquid-liquid extraction (LLE) procedure. There are several solvents used for this extraction procedure, being the most common *n*-pentane, *n*-hexane, ethyl acetate, diethyl ether and dichloromethane (Zatla *et al*., 2017). Of these, *n*-pentane has been shown to be the most efficient for the recovery of volatile compounds from H (Śmigielski *et al*., 2013). Solid phase microextraction (SPME), solid phase extraction and purge-and-trap-automatic thermal desorption (P&T-ATD) are other procedures used for extraction and simultaneous analysis of volatile fraction from Hs (Paolini *et al*., 2008). Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) techniques, are then used to make the volatile component analysis. Despite its importance, there are still few studies on the volatile composition of Hs when compared with EOs. However, more and more Hs and corresponding volatiles composition have raised the interest from the scientific community.

An upadated literature survey, (Table A.1 in Annex A), showed that 282 hydrolates, obtained from 162 species from 39 families, have been studied in terms of the volatile composition. The hydrolates main volatile components (≥ 5 % or ≥ 5 mg/mL or ≥ 1 mg/kg) were detailed in Table

A.1, and whenever reported, the information on the corresponding essential oil main components was also detailed. Whereas in most cases the Hs and EOs share similar main compounds, in some cases the composition of the Hs does not coincide with the EO. For instance, the studies of Edris (2009) with *Cymbopogon citratus*, Matos *et al*. (2015) with *Campomanesia viatoris* and Sutour *et al*. (2008) with *Mentha suaveolens*, for example, showed a major qualitative resemblance between Hs and the corresponding EOs, differing only in quantitative terms. On the other hand, the volatile composition of *Daucus cartora* and *Abis alba* hydrolate, for example, was completely different from the corresponding EO (Zatla *et al*., 2017; Wajs-Bonikowska *et al*., 2015).

Inouye *et al*. (2008) compared the volatile composition from fourty four Hs and that of the corresponding EOs and concluded that in 42 % of the cases the Hs and EO showed different compositions. Moreover, the Hs major components were found in lower concentration when compared to EOs (Inouye *et al*., 2008).

The oxygen-containing compounds are usually dominant in the Hs volaties and, among these, borneol, camphor, carvacrol, terpinen-4-ol, thymol are frequent main Hs volatiles compounds. Generally, the percentage of this type of compounds in this fraction is relatively high and dominant (Lei *et al*., 2018). It is also known that the distribution of oxygenated compounds in Hs differ with type of species and regional conditions (Acheampong *et al*., 2015).

4.3.2. Properties and applications of hydrolates

Several properties have been attributed to Hs, namely biological and organoleptic properties (Aazza *et al*., 2011). Of the various biological properties, the most mentioned have been antimicrobial (antibacterial and antifungal) and antioxidant activities (Hay *et al*., 2018; Lei *et al*., 2020). However, other studies have been developed and more properties have been attributed to Hs, such as, allelopathic (Souza *et al*., 2017), antifeedant (Soto-Armenta *et al*., 2019), ecotoxic (Pino-Otín *et al*., 2019), insecticide (Traka *et al*., 2018), larvidicide (Carvalho *et al*., 2003), nematicide (Andrés *et al*., 2018) and sedative/anaesthetic (Zekri *et al*., 2016).

Due to the various properties attributed to Hs, several works report their potential to be used in the food and cosmetic industries (Ndiaye *et al*., 2017; Śmigielski *et al*., 2013), or in organic farming, in particular to eliminate mushrooms, mould and insects, as well as for soil fertilization (Zatla *et al*., 2017; Paolini *et al*., 2008). An increasing number of studies report their potential to be used in agriculture as biocontrollers (Soto-Armenta *et al*., 2019), bioherbicides (Souza *et al*., 2017), bio-insecticides (Rebolledo *et al*., 2012), bionematicides (Sainz *et al*., 2019) and biopesticie (Zekri *et al*., 2016). Other studies yet reported its potential applications in aquaculture management as sedative for fish transport (Silva *et al*., 2018).

4.4. Distilled condensation waters

The DCWs correspond to one of the sub-products resulting from the steam-distillation process. In general, this fraction is always discarded during the process and is not at any time considered as a potential valued product. Being zero waste the one of the objectives of biorefinery, the valorisation of this fraction, in the same way as the previously described fractions (EOs and Hs) are here considered. In this sense, and in the case of an aqueous extract from vegetable material, the presence of bioactive compounds, namely phenolic compounds, is expected. In such case, one possible valorisation of this sub-product may be its use as a natural antioxidants source by different industries.

4.4.1. Phenolic compounds

Phenolic compounds (PCs) are often considered one of the most important groups of secondary metabolites of plants and constitute nonessential human diet components (Ballard and Junior, 2019; Maestri *et al*., 2006). From the chemical point of view, the PCs have a variable structure, with at least one phenol unit, which means with one hydroxyl group (OH) substituent bonded to an aromatic ring (Ballard and Junior, 2019; Gan *et al*., 2019; Maestri *et al*., 2006; Proença da Cunha, 2005; Saranraj *et al*., 2019). There are several methods used for extraction of this type of compounds, the maceration, infusion and decoction are the simplest and ultrassound extraction, Soxhlet and steam-distillation some of the most complex and those who need the specific equipment.

PCs can be divided into different subgroups based on their chemical structures. Flavonoids, tannins, phenolic acids and phenylpropanoids are the main subgroups of PCs (Figure 1.10), while coumarins, lignans, quinones, stilbenes and curcuminoids are the other subgroups (Aldred *et al*., 2009; Figueiredo, 2017; Li *et al*., 2018; Panche *et al*., 2016). Being one of the major sub-groups of phenolic compounds and due the many biological properties attributed, the flavonoids and tannins were explored in this work.

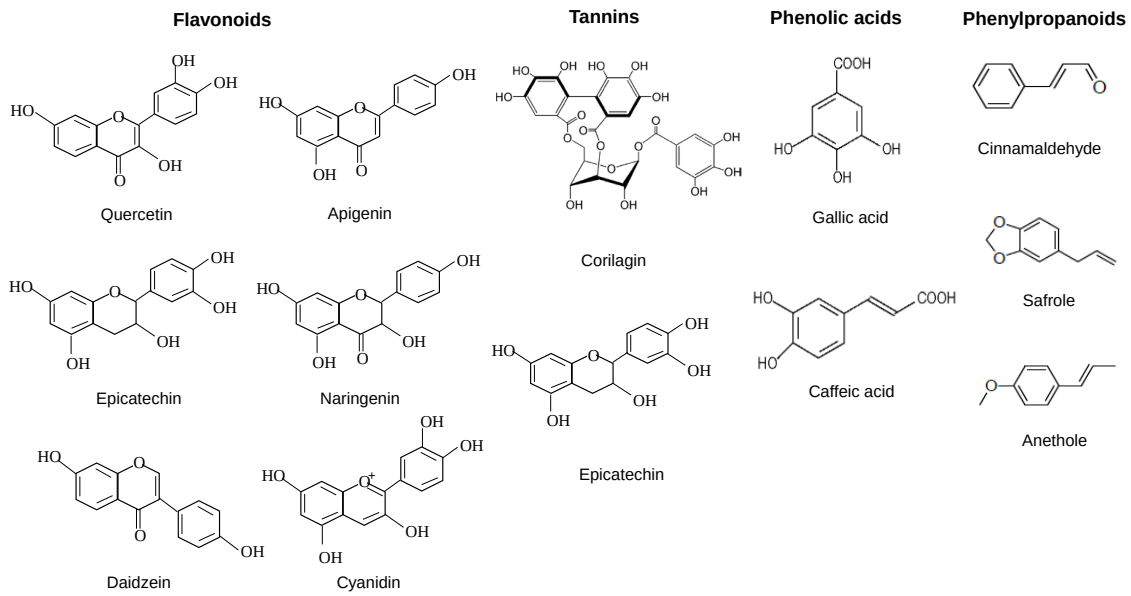

Figure 1.10. Examples of phenolic compounds (Adapted from Aldred *et al*., 2009; Figueiredo, 2017; Li *et al*., 2018; Panche *et al*., 2016).

Flavonoids

Flavonoids are the most important group of phenolic compounds in the human diet and are widely present in plants. They represent one of the most predominant classes of compounds found in vegetables, fruits, nuts and plant-derived drinks, including wine and tea (Babu and Liu, 2009; Ballard and Junior, 2019; González-Gallego *et al*., 2014; Horn *et al*., 2001; Trugo *et al*., 2003). The basic structure of flavonoids is the flavan nucleus with 15 carbon atoms organized in three rings: an aromatic A-ring fused to a heterocyclic C-ring that is attached through a single carbon-carbon bond to an aromatic B-ring (Babu and Liu, 2009; Ballard and Junior, 2019). Normally, they are classified into six subgroups based on their chemical structure: flavanols, flavonols, flavones, isoflavones, flavanones and anthocyanins (Babu and Liu, 2009; Ballard and Junior, 2019; Gan *et al*., 2019). This classification is carried out according to the replacement in the arrangements of hydroxyl, methoxy, prenyl and glycosidic side groups and in the conjugation of rings (Ballard and Junior, 2019).

All these subgroups of flavonoids can be found in food and beverages in the human diet (Table 1.2). Flavonoids are synthesized in plants and are responsible for protection against ultraviolet radiation, pathogens and herbivores (Babu and Liu, 2009; Proença da Cunha, 2005). The beneficial properties of flavonoids may be derived from their antioxidant characteristics as free-radical neutralizers (Trugo *et al*., 2003). Besides antioxidant activity, others have been reported to flavonoids in preventing common diseases, such as inflammation, cancer, cardiovascular disease, gastrointestinal disorders and metabolic syndrome (e.g. diabetes, obesity) (Ballard and Junior, 2019; González-Gallego *et al*., 2014; Horn *et al*., 2001; Neilson *et al*., 2017; Trugo *et al*., 2003).

Table 1.2. Flavonoid subgroups, representative compounds and common food sources (adapted from Babu *et al*., 2009, Ballard *et al*., 2019, Neilson *et al*., 2017).

Subgroups	Compounds	Sources
Flavanol	Catechin, epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate	Apples, legumes, cocoa, chocolate, green tea and red wine
Flavonol	Quercetin, myricetin and rutin	Apple, grapefruit, broccoli, onion, tomato, black tea and red wine
Flavone	Apigenin, chrysin, luteolin	Citrus, parsley, peppers, tomato skin, vegetables, herbs, cereals, red wine
Isoflavone	Daidzein, genistein and puerarin	Legumes (e.g., soybean) and herbs
Flavonone	Eriodictyol, hesperetin and naringin	Citrus fruits, peppermint and tomatoes
Anthocyanin	Cyanidin, delphinidin and malvidin	Berries, cherry, raspberry, strawberry and red wine

Due to the properties shown by these phenolic compounds, their moderate consumption in diet is important to reduce the risk of having several diseases (Babu and Liu, 2009; Ballard and Junior, 2019; Heldt and Piechulla, 2011; Neilson *et al*., 2017).

Tannins

Tannins are the second most abundant phenolic compounds present in plants. Its main role is to protect plants against pests and extreme effects such as drought, heat and UV radiation (Sussela, 2019). With molecular weight between 500 and 3000 Da, these phenolic compounds give a characteristic astringent taste when present in foods (Horwath., 2015; Ogwuru and Adamczeski, 2000). Tannins are classified as hydrolysable, complex and condensed tannins, being both hydrolysable and condensed tannins two major classes that can be found in plants (Horwath, 2015). Hydrolysable tannins are produced by angiosperms and may be classified into gallotannins and ellagitannins. From the chemical point of view, it consists in a central glucose molecule linked to molecules of gallic acid (gallotannins) or hexahydroxydiphenic acid (ellagitannins) (Heldt and Piechulla, 2011; Pietta *et al*., 2003). Agrimoniin, corilagin, geranin, pentagalloylglucose and tannic acid are some examples of hydrolyzed tannins (Pietta *et al*., 2003). Hydrolysable tannins can be hydrolyzed by weak acids or weak bases to produce carbohydrates and phenolic acids (Izawa *et al*., 2010). Usually, these compounds are stored in leaves, fruit pods and galls (Zaprometov and Timiriazev, 1998).

Condensed tannins, also called proanthocyanidins, are produced by angiosperms and gymnosperms and formed by oxidative condensation of flavonoids (Horwath., 2015; Sussela, 2019; Zaprometov and Timiriazev, 1998). They are polymers of 2-50 flavanols units, with three

rings linked by C-C bonds that are not susceptible to hydrolysis (Heldt and Piechulla, 2011; Horwath., 2015). Epicatechin and epigallocatechin are examples of these compounds (Pietta *et al*., 2003). The condensed tannins are responsible for the astringent tastes of many fruits and wines (Stewart and Stewart, 2008) and are usually stored in heartwood and bark (Zaprometov and Timiriazev, 1998).

Extracts of plants rich in tannins are many used in medicinal applications. Some examples include, leaves of *Geranium thunbergii*, that contain gerannin, and are indicated to treat intestinal disorders (Okuda and Ito, 2011). Roots of *Potentilla erecta*, with epigallocatechin and catechins in their composition, are indicated to treat inflammations, bleeding, diarrhoea, bacterial, fungal and viral infections, as well as antiseptic for the mouth and throat (Tomczyk and Latte, 2009). The bark of *Syzygium cumini*, with corilagin, is indicated for bronchitis, asthma and ulcers (Ayyanar and Subash-Babu, 2012). Beyond the already mentioned bioactivities, tannins also show antidiabetic, anti-obesity, cytotoxic, cardioprotective properties (Sieniawska and Baj, 2017). As flavonoids, also tannins have shown health benefits when carefully consumed in the human diet (Izawa *et al*., 2010; Okuda and Ito, 2011; Sieniawska and Baj, 2017).

In general, phenolic compounds have several demonstrated properties, not only for the plant, but also for the human body. In plants, they can protect against UV radiation, pathogens, oxidative stress and harsh climatic conditions. For the human body, they have diverse biological effects such as antioxidants, anti-inflammatory, antibacterial, antifungal, anti-diabetic, anticancer, cardioprotective and others. All these properties show that PCs have the potential to be used in treatment for various diseases. However, it is important to take into account the dose at which they are taken since the toxicity of these concentrated compounds is still unknown.

4.5. Extracted solid residues

In addition to the EOs, Hs and DCWs, other considerable fraction results from the steam-distillation process, the ESRs, which are still a major volume of residue from the original biomass. In general, these residues are used only for for production of energy in the form of heat, an application of low economic value (Alves-Ferreira *et al*., 2017). However, and considering that this type of extraction is relatively soft, solid residue remains practically unchanged at the structural level, thus presenting the potential to be used in the production of other products with a higher economic value. An exemple is the use of this type of resiudes to produce bioethanol from the fermentation of sugars. These residues are lignocellulosic material, rich in cellulose and hemicellulose, that are commonly hydrolyzed by treatment with acids and then fermented by microorganisms for bioethanol production (Balat, 2011). In addition to cellulose and hemicellulose, lignin is another major fraction os this type of residues, which can be extracted and recovered through appropriate fractionation processes (Ferreira *et al*., 2009).

The lignin can be integrated into polymeric materials, including conductive polymers, polyurethanes, and thermoplastic (Varanasi *et al*., 2012). They can also be used to produce adhesives, binders and phenolic resins (Stewart, 2008).

These residues are also a source of extractives that can potentially be valued as bioactive products, namely phenolic compounds (Alves-Ferreira *et al*., 2019b). Despite the large quantities and the bioactive potential of these extracted solid residues, information on the chemical characterization and biological properties of the active compounds is still scarce in literature. Sánchez-Vioque *et al*. (2013) have detected phenolic compounds with antioxidant activity in *Cistus ladanifer*, *Lavandula* x *intermedia*, *Santolina rosmarinifolia* and *Thymus mastichina* and Torras-Claveria *et al*. (2007) have reported flavonoids with antioxidant activity in *Lavandula* x *intermedia*.

In addition, the valorisation of lignocellulosic fraction and extractives, there are a still solid residue that can be valued. This residue could be still transformed into a solid biofuel, such as pellets. This valorisation allows reaching the final goal, in the sense that it shows that all fractions resulting from the distillation processes can be valorised, and thus obtain zero waste.

5. Biological activities

Transversally to all fractions resulting from the steam-distillation process are the biological properties exhibited by some of the compounds present in their composition. Active compounds produced during secondary metabolism of plants are usually responsible for the biological properties of some species (Silva and Júnior, 2010). Among the several biological properties attributed to these compounds, the antibacterial, anticancer, anti-inflammatory, antioxidant and antiviral activities can be highlighted (Ortega and Campos, 2019).

The continuous search for natural antioxidants and anti-inflammatory drugs, as well as new alternatives to combat microorganisms responsible for numerous infections, lead to the choice of antioxidant, anti-inflammatory and antimicrobial activities to be the biological properties under study in this dissertation.

5.1. Antioxidant activity

Antioxidants are defined as substances that inhibit or delay the oxidation of biologically relevant molecules either by quenching free radicals or by chelation of redox metals. They are important because they act as a protection mechanism against reactive oxygen species (ROS) (Flora *et al*., 2015).

ROS are produced in all aerobic cells, normally as by-products of respiratory metabolism and resulting from oxygen reduction. ROS include free radicals' such as superoxide ($O_2^{\bullet-}$), hydroxyl ($HO^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$), carbonate ($CO_3^{\bullet-}$), peroxy ($RO_2^{\bullet}$), alkoxy ($RO^{\bullet}$) and carbon dioxide ($CO_2^{\bullet-}$) and non-radical molecules with high reactivity, such as hydrogen

peroxide (H_2O_2), ozone (O_3), hypochlorous acid (HOCl), among others (Bertout *et al*., 2004). The high reactivity of these species is related to their electron configurations.

ROS are responsible for causing irreparable damage to biological systems, attacking and damaging almost all molecules due to their very short half-life, being thus extremely reactive. The production of ROS does not only have metabolic origin. Foods, air pollution, UV radiation, among other factors are also responsible for the imbalance between ROS production and its elimination, the as-called oxidative stress (Miguel, 2007).

Antioxidants are substances who protect cells against ROS (Bertout *et al*., 2004). The equilibrium between oxidation and antioxidation is critical in maintaining healthy biological systems. Antioxidants can be classified as primary or secondary, according to their mode of action. Firstly, antioxidants react directly with free radicals making them more stable. Secondly, antioxidants do not react directly with free radicals, but with substances that interact with them. The last ones can retard the rate of radical initiation by elimination of initiators. This occurs due the ability of these antioxidants scavenging of oxygen, chelating metal that catalyzes free radical reaction or inhibition of enzymes (e.g. xanthine oxidase) (Mishra and Bisht, 2011).

Antioxidant activity should not be concluded based on a sigle antioxidant method. Several *in vitro* techniques are carried out for evaluating antioxidant activity (Alam *et al*., 2013). Some of the most used used are DPPH scavenging, ABTS radical cation decolorization, hydrogen peroxide scavenging, ferric reducing-antioxidant power (FRAP), inhibition of superoxide anion radical formation and oxygen radical absorvance capacity (ORAC) (Pisoschi and Negulescu, 2011). ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) cation radical decolourisation method, inhibition of superoxide anion radical formation, inhibition of xanthine oxidase and chelating metal ions was used in present study to evaluate the antioxidant activity.

5.1.1. ABTS cation radical decolourisation method

The ABTS method, also known as Trolox equivalent antioxidant capacity (TEAC) assay, measures the antioxidants relative ability to scavenge the generated ABTS radical, as compared with the standard Trolox. The ABTS radical is generated by the reaction of ABTS salt with a strong oxidizing agent (e.g., potassium persulfate). This is a method that relies on the transfer of an electron, where an oxidant (ABTS radical) accepts an electron from the antioxidant, leading to a colour change. The reduction of the green blue ABTS radical by antioxidants is measured by spectrophotometric methods (Singh and Singh, 2008).

The ABTS method was used because it is a simple, rapid method and has been widely used for screening and routine determinations. On the other hand, it can be used in both water and organic solvents, which enables the antioxidant capacity of both hydrophilic and lipophilic compounds (Opitz *et al*., 2014).

5.1.2. Inhibition of superoxide anion radical formation method

Superoxide anion is a highly reactive species produced when oxygen is reduced by a single electron or through a hypoxanthine-xanthine or a non-enzymatic system (Miguel, 2007). Xanthine oxidase exists in tissues in the form of dehydrogenase xanthine that transfers electrons to nicotinamide adenine dinucleotide (NAD^+), reducing it to NADH and oxidizes xanthine or hypoxanthine to uric acid. Under stress conditions, dehydrogenase xanthine is converted into xanthine oxidase that produces superoxide. However, electron transfer is not to NAD^+ but to molecular oxygen that is transformed into superoxide anion (Gulcin, 2020). In a non-enzymatic system, superoxide anion is generated through the reaction of phenazine methosulphate in the presence of NADH and molecular oxygen. In both cases, superoxide anion reduces nitro-blue tetrazolium (NBT) into formazan. Formazan formation is evaluated by spectrophotometric methods (Whaley-Connell *et al*., 2012).

Unlike the ABTS radical, the superoxide anion is a highly reactive species produced in the human body and xanthine oxidase is a major source of ROS *in vivo*. In this sense, inhibition of superoxide anion radical formation and inhibition of xanthine oxidase were also used.

5.1.3. Inhibition of xanthine oxidase method

Xanthine oxidase (XO) is an enzyme that generates ROS such as superoxide radicals ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). These ROS are formed when XO catalyzes the oxidation of hypoxanthine to xanthine (Reaction 1) which can further catalyze the oxidation of xanthine to uric acid (Reaction 2) (Figure 1.11) (Kostić *et al*., 2015).

$$\text{Hipoxanthine} + O_2 + H_2O \rightarrow \text{Xanthine} + H_2O_2 \qquad \text{(Reaction 1)}$$

$$\text{Xanthine} + 2O_2 + H_2O \rightarrow \text{Uric acid} + 2O_2^{\bullet-} + 2H^+ \qquad \text{(Reaction 2)}$$

Figure 1.11. Production of ROS from xanthine oxidase (Adapted from Kostić *et al*., 2015).

An excess of uric acid in body induces a medical condition known as gout (Rome and Frecklington, 2015; Santi *et al*., 2018). Allopurinol is used as the first-line treatment of this pathology, but it has been reported that its prolonged use may cause serious problems, such as hepatitis, hypersensitivity, dermatological eruption, diarrhoea and others (Page, 2008). Alternative natural compounds to XO inhibitors are needed, and some flavonoids, curcumin, and other compounds have been reported as a possible option (Santi *et al*., 2018). Xanthine oxidase activity can be determined by measuring the formation of uric acid from xanthine. The most frequently used method consists in a pre-incubation of assay mixture of the test sample with xanthine oxidase enzyme and buffer solution (pH 7.4), where the most commonly used buffer is a carbonate or a phosphate. The reaction is then initiated by the addition of the substrate

(xanthine solution). The assay mixture is incubated again during a period of 15 to 30 min. The reaction is stopped by the addition of hydrochloric acid. Spectrophotometric determination of the XO activity is based on the measurement of the uric acid production from xanthine substrate (Kostić *et al*., 2015).

5.1.4. Chelating metal ions method

The antioxidant action may be due the different mechanisms. Chelating of transition metals is one of the possible mechanisms of the antioxidative action and was studied in present study. Transition metals, such as iron (Fe), copper (Cu) and manganese (Mn), which have unpaired electrons in the valence orbital, accept and give electrons that promote the transfer of an electron to an oxygen and result in the formation of ROS (Miguel, 2010). An example of the role of transition metals in the production of ROS is in the Fenton reaction, where the conversion of hydrogen peroxide (H_2O_2) to hydroxyl radical ($HO^{\bullet}$) is promoted by Fe^{2+} (Pisoschi and Negulescu, 2011). A way of neutralizing the effect of transition metals is by using chelating agents. Chelating agents are chemical compounds that bond to metals in two or more sites, as chelating molecules possess the electrons required to form bonds with positively charged metal ions. A chelating agent forms a stable complex with toxic metals, protecting biological targets. It has been suggested that an ideal chelator should have high solubility in water, be resistant to biotransformation, have the ability to reach the sites of metal storage, retain chelating capability at pH of body fluids and form metal complexes that present lower toxicity than the free metal ions (Flora *et al*., 2015).

The chelating method that uses ferrozine, which can result in the formation of complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is reduced, which leads to a diminished red colour of the complex ferrozine-Fe^{2+}. Thus, the rate of colour reduction allows assessing the chelating activity of the chelator (Alam *et al*., 2013).

5.2. Anti-inflammatory activity

When human cells are damaged by microbes and by physical and chemical agents, the injury is in the form of stress. Inflammation is thus a complex protective process response of the body to stress and against dangerous agents, such as microorganisms or damaged cells. It is a vital immune response by the host that enables the elimination of harmful stimuli as well as the healing of damaged tissue (Ahmed, 2011).

The inflammatory response can be divided into acute and chronic phases (Köher-Forsberg and Benros, 2018). The acute one is the initial response, which is characterized by resident cell activation, with movement of plasma and leukocytes (especially granulocytes) from de blood into the injured tissues. This inflammatory response usually lasts only a few days and it is manifested through redness, heat, swelling, pain and loss of function (Leelaprakash and Dass,

2011). If the cause for the inflammatory response is not eliminated, there will be a progression towards a chronic inflammatory response. On the other hand, chronic inflammation is a prolonged response characterized by a gradual change in the cellular composition of the inflammation area with lymphocytes, macrophages and plasma cells replacing neutrophils, which, over time, cause permanent damage in the affected zone (Eschenbacher, 2007). In both types of inflammation, there is an increasing local blood flow, vasodilation, fluid extravasation, liberation of proinflammatory mediators, increase of protein denaturation and others (Barboza *et al*., 2018).

Inflammation is associated with many neurodegenerative diseases such as Alzheimer, Parkinson and multiple sclerosis. Over the last decade, it turned out that inflammation plays a critical role in promoting cancer (Ahmed, 2011). Thus, the continued demand for efficient and natural anti-inflammatory drugs is increasing. Anti-inflammatory studies using natural products, namely terpenoids, showed potent anti-inflammatory activity (Sultana and Saify, 2012). *In vitro* anti-inflammatory activity can be evaluated by different methods. One of the most widely used *in vitro* method was the inhibition of albumin denaturation assay, being the method used in present study.

5.2.1. Inhibition albumin denaturation method

Human serum albumin (HAS) is the most-abundant protein in plasma and is also a major component of most extracellular fluids (Ezra *et al*., 2017). HAS has been related to Alzheimer's disease, the most common progressive chronic neurodegenerative disorder and one of the leading causes of dementia in people who are over 65 years old (Cvetković-Dožić *et al*., 2001). To date, there is no treatment available that can stop the progressive deterioration of cognitive functions in Alzheimer's disease patients. However, there are treatments focussing on pathologies associated with Alzheimer's disease, such as problems associated to the functioning of human serum albumin (HAS), which demonstrated to have the potential to treat this disease. The additional relationship with the Alzheimer´s disease is that levels of these protein decrease with aging (Ezra *et al*., 2017). Most biological proteins lose their biological function when denature, which means that they lose their structure by the application of external stress, compounds or heat. Denaturation of proteins has been widely reported because of its connection to inflammation (Leelaprakash and Dass, 2011). Using the inhibition albumin denaturation method, it is possible to evaluate the anti-inflammatory activity of extracts. It consists of a simple method, where a bovine serum albumin (BSA) is generally used. BSA function well for a protein standard because it is usually available in high purity and an inexpensive cost. Under the action of heat, the protein denaturation is promoted and is observed through turbidity. The samples turbidity is also measured with spectrophotometric methods.

5.3. Antimicrobial activity

Infectious disease remains a major cause of death and illness, being the second most common cause of mortality. Although the development of antimicrobials has significantly reduced death and morbidity from infectious diseases, at least in the developed world, the appearance of resistance to antimicrobials endangers these advances (Song, 2003). Because of available antimicrobials failure to treat infectious diseases, the need to find new antimicrobial agents is of utmost importance (Manandhar *et al*., 2019). Therefore, many researchers have focused on the investigation of natural products as source of new bioactive molecules (Valgas *et al*., 2007). In the field of antimicrobial activity, antibacterial and antifungal activity are the most studied, because de large number of infections that bacteria and fungi microorganisms cause.

Gram-negative, such as *Escherichia coli*, and Gram-positive, such as *Staphylococcus aureus* bacteria and the yeast, such as *Candida albicans* have been the most studied microorganisms in virtue of the significant number of infections leaded by them. For these reasons were the microorganisms studied in present dissertation.

E. coli is one of the most important pathogens (Allocati *et al*., 2013). Some strains cause serious illness, which can result in severe diarrhoea and even kidney damage (Rowe and Sprigg, 2014). In the same way, *S. aureus* is an opportunistic pathogen who can cause a wide range of diseases. In addition to food poisoning, *S. aureus* is responsible for other diseases such as skin infections, internal organ infections and poisoning (e.g. toxic shock syndrome) (Langsrud, 2009). Antibiotics are also essential for the control and treatment of *E. coli* and *S. aureus* infections in humans and animals. However, antimicrobial resistance is associated to the quantity of antibiotic consumption (Allocati *et al*., 2013). Hence, the incorrect use of antimicrobials increased the resistance in pathogens increasing (Kadlec *et al*., 2015) and the need for new antibacterial agents is essential.

Some species of *Candida* can be pathogens, being *C. albicans* the most common. Candidiasis infections either superficial, affecting the skin and mucous membranes, or invasive, affecting the gastrointestinal (oropharyngeal to oesophageal), respiratory and urinary (bladder and kidney) systems. Several drugs are available for the treatment of *Candida* infections, however, in all cases, microbial resistance is an increasing problem (Dowd, 2007). The great resistance of traditional antifungals has encouraged the search for new alternatives among natural products (Oliva *et al*., 2013).

A variety of laboratory methods can be used to evaluate the potential antimicrobial activity of a test sample. The most known and first line of screening are the diffusion (agar diffusion method) and dilution (broth or agar dilution) methods (Balouiri *et al*., 2016).

5.3.1. Agar diffusion method

In the present work, the agar diffusion method was used for the evaluation of the

antimicrobial activity. The main reasons for using it is because this method is reproducible, easy to perform, requires a small amount of sample, is of low-cost and allows testing huge numbers of microorganisms and antimicrobial agents (Balouiri *et al*., 2016).

The agar diffusion method, in which discs or holes are employed, is the most widely used. The reservoirs used consist of filters made from paper discs (about 6 mm in diameter) or holes that are directly made in the medium itself. The effectiveness of the sample is revealed by the size of the growth inhibition zone of the microorganism that is used, while the degree of activity is expressed as the diameter of the mentioned zone (Faleiro, 2007). Depending on the inhibition demonstrated by the sample in the first step, the next one is usually to determine the minimum inhibitory concentration (MIC) of the same sample, that is, the lowest concentration that completely inhibits the growth of a microorganism (Valgas *et al*., 2007). However, the agar diffusion method is not appropriate to determine the MIC, because it is impossible to quantify the amount of the antimicrobial agent diffused into the agar medium (Balouiri *et al*., 2016).

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Chapter II

Bioproducts from forest biomass: Essential oils and hydrolates from wastes of *Cupressus lusitanica* Mill. and *Cistus ladanifer* L.

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1. Abstract

Unattended forest wastes are, among others, a potential source of wildfires, as well as a growth media for forest pests. As a way of lowering the detrimental effect of these wastes, it is important to convert these under-valued resources into a value-generating market forest wastes use. Essential oils (EOs) and hydrolates (Hs) from *Cupressus lusitanica* and *Cistus ladanifer* waste products, resulting from forest landscaping in Portugal, were evaluated for chemical composition and biological activity. Essential oils and Hs were obtained by steam-distillation (SD) and hydrodistillation (HD). Essential oils and Hs volatiles were analysed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The antimicrobial activity of EOs was studied by disk agar diffusion method against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. Antioxidant activity of EOs and Hs was evaluated by 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) free radical, superoxide anion radical formation, xanthine oxidase and chelating metal ions assays. Anti-inflammatory activity of Hs was assessed by albumin denaturation assay. Monoterpene hydrocarbons and oxygen-containing monoterpenes dominated *C. lusitanica* EO (SD, 82-86 %, HD, 80-85 %) and Hs volatiles (SD, 93-94 %; HD 64-81 %), respectively. α-Pinene (14-36 %), limonene (8-21 %), δ-3-carene (8-19 %) and sabinene (6-18 %) were the main EO constituents. Hydrolates volatiles were dominated by *cis*-3-hexen-1-ol (0.1-13 %), camphor (1-11 %), umbellulone (traces-48 %), *p*-cymene-8-ol (11-16 %) and terpinen-4-ol (21-31 %). *C. ladanifer* EOs were dominated by monoterpene hydrocarbons (SD, 48-80 % and HD, 29 %) and Hs by oxygen-containing monoterpenes (SD, 38-43 %, HD, 39 %). The EO major constituents were α-pinene (13-28 %) and camphene (5-25 %), whereas 2,6,6-trimethyl cyclohexanone (2-12 %) and *trans*-pinocarveol (5-13 %) dominated the Hs volatiles. This study reports for the first time the chemical composition of the hydrolate volatiles of these two species and their anti-inflammatory properties. Among the studied biological activities, the EOs showed the best antioxidant properties while Hs demonstrated higher anti-inflammatory activity.

2. Introduction

The Portuguese forest occupies approximately 35 % of the mainland, therefore, there is a considerable potential for waste coming from the forest, namely, shrubs, diseased or fire-killed trees, as well as their roots, trunks and branches, removed during forest thinning, or their bark, needles, leaves and even fruits (PCM, 2017). This biomass availability is the basis for making renewable bioenergy, biofuels and other bioproducts that are increasingly replacing fossil-fuel based products, thus, it should be recovered with environmental, economic and social benefits, as an alternative to open burning (Han *et al*., 2018). The concept of biorefinery is defined as an

approach for the generation of value-added products such as chemical products, biofuels, heat and electricity from renewable energy sources such as forest biomass (Kehili *et al*., 2016). There is a need for an integrated forest policy that promotes the sustainability of the forest and its management, and also the prevention of forest fires for defending the physical integrity of the populations and the preservation of their means of subsistence and patrimonial assets. To this aim, is intended that the development of advanced biorefineries using endogenous resources sustainably, generate new value chains around biomass in the so-called bioeconomy and circular economy (PCM, 2017). These concepts are intended to achieve more waste prevention and better resource management (Zeller *et al*., 2018).

Essential oils (EOs) are complex mixtures, internationally defined as the product obtained by hydro-, steam- or dry-distillation of a plant or any of its parts, or by a mechanical process without heating from the epicarp of *Citrus* fruits (Council of Europe, 2010; ISO, 2013). During EO isolation procedure, in addition to EO, also hydrolates can be obtained. An hydrolate, sometimes also referred as hydrosol or floral water, corresponds to the distilled water that remains after the hydro- or steam-distillation and the separation from the corresponding EO, being usually rich in EO water-soluble components (Aazza *et al*., 2011; Hamdi *et al*., 2017; ISO, 2013).

Essential oils, and by-products such as hydrolates, can be obtained within the biorefinery concept from forest biomass as they are high value, low volume commodities, extremely attractive for the perfume, cosmetic and flavour industries (Belabbes *et al*., 2017; Machale *et al*., 1997) and are also known to possess different pharmacological activities. In fact, antimicrobial, antioxidant, anti-inflammatory, antispasmodic, and relaxing properties, among others, have been described for essential oils and hydrolates, both in animals and humans (Belabbes *et al*., 2017; Tognolini *et al*., 2006).

The aim of the present work was to evaluate the potential for obtaining essential oils and hydrolates from the underutilized residues of *Cupressus lusitanica* Mill. (Cupressaceae) and *Cistus ladanifer* L. (Cistaceae). Both species are frequent in Portuguese landscape, and large amounts of their waste products are obtained from forest management.

Cupressus lusitanica ("cipreste-do-Buçaco" in Portuguese), also known as Cedar-of-Goa or Mexican cypress, was introduced in Portugal more than 300 years ago and is still widely used as an ornamental plant and for reforestation (Proença da Cunha *et al*., 2012). *Cistus ladanifer* ("esteva"), also known as rockrose, is a Mediterranean shrub widely distributed in the Iberian Peninsula and with high importance in the perfumery industry due to a particular extract, the labdanum, used as fixative. Both species are referred as having several biological activities (Bett *et al*., 2016; Zidane *et al*., 2013).

Although some studies have addressed the composition of Portuguese *C. lusitanica* and *C. ladanifer* EOs, no previous works evaluated their hydrolate volatiles. The present study, detailed in the flowchart of Figure 2.1, aimed at evaluating *C. lusitanica* and *C. ladanifer*

landscaping wastes EOs and hydrolates volatiles composition, as well as their antioxidant, antimicrobial and anti-inflammatory activities.

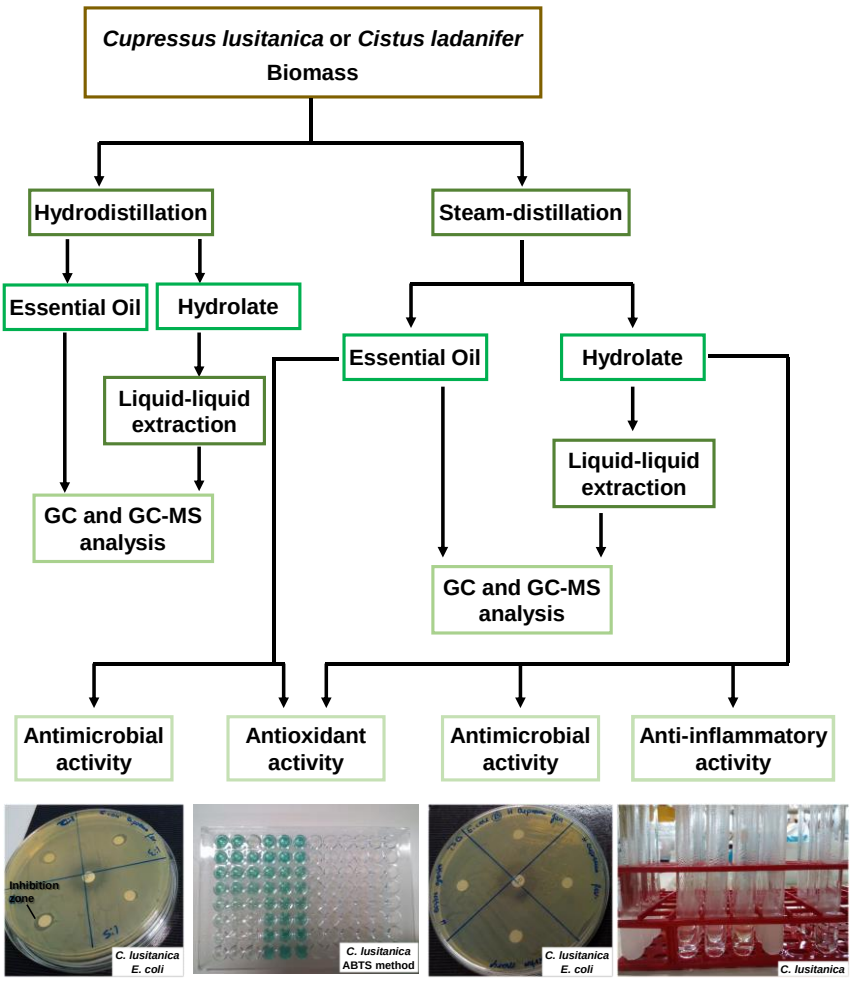

Figure 2.1. Flowchart of the extraction, chemical composition and biological assessments, including representative images of the results obtained with some of the biological activities evaluated.

3. Materials and methods

3.1. Plant material

Waste samples of *Cupressus lusitanica* Mill. and *Cistus ladanifer* L. aerial parts, resulting from forest landscaping, were collected at Beira-Baixa, Portugal, in 2017 and 2018 by Silvapor - Ambiente e Inovação Lda. After collection, the plant material was either extracted fresh or after drying, under open air conditions (Table 2.1). For *C. ladanifer* essential oil, the analysis of five Portuguese commercial samples, obtained in different years from three local producers, was included, only in the chemical study, for comparison purposes. The plant material was identified at the Herbarium of the Museum, Laboratory and Botanical Garden of Lisbon and kept under voucher number LISU262295 for *C. lusitanica* and LISU266731 for *C. ladanifer*.

Table 2.1. *Cupressus lusitanica* and *Cistus ladanifer* collection sites and sampling year, plant part used for extraction, extraction procedure and type of extract with corresponding code.

Plant species	Collection place in Portugal	Collection date	Plant condition	Type of extract	Extraction method	Code
Cupressus lusitanica	Alcains	January 2017	Dry*	EO	SD	17 January
				H		17 January
		February 2017	Dry**	EO	SD	17 February
				H		17 February
	Idanha-a-Nova	May 2018	Dry**	EO	HD	18a May
						18b May
						18c May
				H		18a May
						18b May
						18c May
Cistus ladanifer	Medelim	March 2017	Fresh	EO	SD	17 March
				H		17 March
		August 2017	Fresh	EO	SD	17 August
				H		17 August
		August 2017	Fresh	EO	HD	17 August
				H		17 August

* For a month; ** For a week; EO: Essential oil; H: Hydrolate; SD: Steam-distillation; HD: Hidrodistillation. a – c: replicate samples.

3.2. Essential oil isolation and hydrolates volatiles extraction

The EOs were obtained by hydrodistillation (HD) and steam-distillation (SD). Hydrodistillation was run for 3 h, using a Clevenger type apparatus according to the European Pharmacopoeia (Council of Europe 2010), with a distillation rate of 3 mL/min. Approximately 420 g of *C. lusitanica* and 270 g of *C. ladanifer*, were used for hydrodistillation. Steam-distillation was performed at Silvapor®, using approximately 100 kg of each species in a stainless-steel distiller (1100 L, Vieirinox®, Aveiro, Portugal), during 1 h:30 min at 0.5 bar.

Volatiles from hydrolates were obtained by a liquid-liquid extraction, using in-lab distilled *n*-pentane, in a ratio of 3 volumes of *n*-pentane per volume of hydrolate (x 3). Pentane extracts were concentrated, at room temperature under reduced pressure on a rotary evaporator (Rotary Evaporator RE-51). Each extract was then collected in a vial and concentrated to a minimum volume (100 µL), at room temperature, under nitrogen flux, using a blow-down evaporator system. Essential oils and hydrolate volatiles were stored at -20 °C, until analysis.

3.3. Essential oil and hydrolates volatiles composition analysis

The EOs and the hydrolates volatiles were analysed by gas chromatography-mass

spectrometry (GC-MS) for component identification, and by gas chromatography (GC) for components quantification.

3.3.1. Gas chromatography (GC) analysis

Essential oils and hydrolates volatiles were analysed using a Perkin Elmer Autosystem XL gas chromatograph equipped with two flame ionization detectors (FIDs), a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (100 % polydimethylsiloxane, 30 m x 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica column ((50 % phenyl)-methylpolysiloxane, 30 m x 0.25 mm i.d., film thickness 0.15 μm; J & W Scientific Inc.). Oven temperature was programmed, 45-175 °C, at 3 °C/min, subsequently at 15 °C/min up to 300 °C, and then held isothermal for 10 min; injector and detector temperatures, 290 °C and 280 °C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm/s. The percentage composition of the volatiles was computed by the normalization method from the GC peak areas, without the use of correction factors, calculated as mean values of two injections from each sample.

3.3.2. Gas chromatography-mass spectrometry (GC-MS) analysis

Gas chromatography-mass spectrometry analysis were run on a Perkin Elmer Clarus 600T gas chromatograph, equipped with DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific, Inc.), and interfaced with a Perkin-Elmer Clarus 600T mass spectrometer (software version 5.4.2.1617, PerkinElmer, Shelton, CT, USA). Injector and oven temperatures were as above; transfer line temperature, 280 °C; ion source temperature, 220 °C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; scan range, 40-300 *m/z*; scan time, 1 s. The identity of the components was assigned by comparison of their retention indices, relative to C_8-C_{24} *n*-alkane indices and GC-MS spectra from a laboratory-made library, constructed based on the analyses of reference oils, laboratory-synthesized components and commercially available standards.

3.4. Essential oils and hydrolates bioactivities analysis

Essential oils and hydrolates obtained by steam-distillation were used in the bioactivities assays as depicted in the flowchart, Figure 2.1.

The antimicrobial activity was tested against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* using disk agar diffusion method. Two separate assays were performed and each with two replicates.

The antioxidant activity of was determined using different methodologies, namely the i) capacity for scavenging the free radicals 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and superoxide anion, ii) xanthine oxidase inhibiting activity and iii) chelating metal ions capacity. Each assay had four replicates.

The anti-inflammatory activity of hydrolates obtained by steam-distillation was evaluated through the albumin denaturation assay. Each assay had four replicates.

3.4.1. Antimicrobial activity determination

The antimicrobial activity of the EOs and hydrolates was determined by disk agar diffusion method, according to Faleiro *et al*. (2003). The tested microorganisms were *Escherichia coli* DSM 1077, *Staphylococcus aureus* ATCC 6538 and *Candida albicans* 1098. The bacterial strains were maintained in tryptic soy agar (TSA) at 4 °C. Original cultures are maintained at -70 °C in glass beads. Tryptic soy broth was used to cultivate bacteria, except *S. aureus* which was grown on brain heart infusion (BHI, Biokar) at 37 °C for 18 h and *C. albicans* which was grown on yeast malt broth (YMB) and incubated at 37 °C. A volume of 0.1 mL of the culture was used to inoculate the TSA plates. Sterile filter paper discs (6 mm Ø), containing 3 µL of the samples (essential oil dilute 1:2 with *n*-propanol or hydrolate), were distributed on the agar surface. Chloramphenicol was used as the positive control for *E. coli* and *S. aureus*, and amphotericin B (10 µg/mL) for *C. albicans*. Inhibition zones were determined after an incubation period of 24 h at 37 °C.

3.4.2. Antioxidant activities

3.4.2.1. ABTS cation radical decolourisation assay

The experiments were carried out using an improved ABTS decolourisation assay (Re *et al*., 1999) which is applicable for both lipophilic and hydrophilic compounds, adapted to a microplate format using spectrophotometric detection and microtiter 96-well plates. The ABTS radical cation ($ABTS^{+\bullet}$) was generated by oxidation of ABTS with potassium persulphate. The $ABTS^{+\bullet}$ solution was prepared by mixing 7 mM ABTS (5 mL) and 140 mM potassium persulphate (88 µL) leading to a 2.45 mM final concentration, and the mixture was incubated in the dark at room temperature for 16 h. The $ABTS^{+\bullet}$ solution was then diluted with 80 % (v/v) ethanol to obtain an absorbance of 0.700±0.005 at 734 nm. Aliquots of the essential oil or hydrolate (30 µL) were added to the radical solution (3 mL) and 200 µL of each were placed in each well. Trolox (*(±)*-6-Hydroxyl-2,5,7,8-tetramethylchromane-2-carboxylic-acid) was used as standard. The decrease in the initial absorbance was recorded at zero and after 6 min on a microplate reader (Multiscan GO, ThermoFischer Sc.). The percent absorbance reduction was determined as follows: % $ABTS^{+\bullet}$ inhibition = $((Abs_b - Abs_f)/Abs_b) \times 100$, where Abs_b is the

absorption of blank sample (t = 0 min) and Abs_f is the absorption of tested solution (t = 6 min).

3.4.2.2. Inhibition of superoxide anion radical formation

Scavenging ability of superoxide anion radical was evaluated as previously reported by Soares (1996) with some modifications. Superoxide anions were generated in a non-enzymatic phenazine methosulfate (PMS) - nicotinamide adenine dinucleotide phosphate (NADH) (PMS-NADH) system by oxidation of NADH and assayed by reduction of nitroblue tetrazolium (NBT). The superoxide anion was generated in 240 µL of phosphate buffer (19 mM, pH 7.4) containing 60 µL NBT (43 µM) solution, 180 µL NADH (166 µM) solution and 60 µL of the oil or hydrolate samples. The reaction was started with the addition of 60 µL PMS solution (2.7 µM) to the mixture. The reaction mixture was incubated at room temperature for 10 min and the absorbance reading was performed at 560 nm in a UV/VIS spectrophotometer. Ascorbic acid was used as standard. The percentage of inhibition was calculated using the equation: % inhibition = $((A_0-A_1)/A_0) \times 100$, where A_0 is the absorbance of the control (without sample) and A_1 is the absorbance in the presence of the sample.

3.4.2.3. Inhibition of xanthine oxidase

Xanthine oxidase activity was determined by measuring the formation of uric acid from xanthine. Xanthine oxidase inhibiting activity was measured with a modified assay according to Umamaheswari *et al*. (2013). The assay mixture consisted of 50 µL of the EO or hydrolate samples, 175 µL of phosphate buffer (50 mM, pH 7.5) and 22.5 µL of xanthine oxidase enzyme solution (0.4 units / mL in phosphate buffer, pH 7.5), which was prepared immediately before use. After pre-incubation at room temperature for 15 min, the reaction was initiated by the addition of 165 µL of 0.150 mM xanthine solution (substrate). The assay mixture was incubated for 30 min. The reaction was stopped by adding 50 µL of 1 M HCl, and the absorbance was measured at 290 nm using UV/VIS spectrophotometer. Allopurinol was used as standard. The percentage of inhibition was determined according to the following formula: % inhibition = $((A_0 - A_1)/A_0) \times 100$, where A_0 is the absorbance of the control (without sample) and A_1 is the absorbance in the presence of the sample.

3.4.2.4. Chelating metal ions

The degree of chelating of ferrous ions by EO or hydrolate samples was evaluated by the method described by Wang *et al*. (2004). Samples (200 µL of EO or hydrolate) were incubated with 100 µL of $FeCl_2.4H_2O$ (2 mM). The reaction was initiated by adding 400 µL of ferrozine (5 mM), then after 10 min, the absorbance was measured at 562 nm. An untreated sample served as the control. EDTA (ethylenediaminetetraacetic acid) was used as standard. The

percentage of chelating ability was determined according to the following formula: % inhibition = (($A_0 – A_1$)/ A_0) x 100, where A_0 is the absorbance without sample and A_1 is the absorbance in the presence of the sample.

3.4.3. Assessment of in vitro anti-inflammatory activity of hydrolates

In vitro anti-inflammatory activity of hydrolate samples was evaluated by using the albumin denaturation assay, according to Leelaprakash and Dass (2011) with minor modifications. The reaction mixture consisted of 1 mL hydrolate and 1 mL of 0.2 % aqueous solution of bovine serum albumin (BSA) fraction V. The samples were incubated at 37 °C for 20 min and then heated to 72 °C for 5 min. After cooling, the turbidity was measured spectrophotometrically at 660 nm. Water was used as blank, and acetylsalicylic acid as a standard anti-inflammatory drug. Percent inhibition of protein denaturation was calculated as follows: % inhibition = (($Abs_{control}$ – Abs_{sample}) / $Abs_{control}$) X 100, where $A_{control}$ is the absorbance without sample and A_{sample} is the absorbance in the presence of the sample.

3.5. Statistical analysis

Results are presented as mean values with their corresponding standard deviations. All analytical determinations were carried out in quadruplicate. The mean value was considered for each analysis.

4. Results and discussion

4.1. *Cupressus lusitanica* Mill.

4.1.1. Essential oil

Cupressus lusitanica essential oils yields ranged between 0.12 and 0.26 % (v/d.w.) for steam-distillation (SD) and 0.1 % for hydrodistillation (HD), Table 2.2. In total, seventy-eight compounds were identified by SD and eighty-six by HD, accounting for 94-98 % and 95-97 % of the total composition, respectively (Table 2.2).

Monoterpene hydrocarbons were the major constituents obtained both by steam-distillation (SD, 82-86 %) and by hydrodistillation (HD, 80-85 %).

Independently of the time of the year of plant collection and of the distillation procedure, α-pinene (SD, 14-36 %; HD 24-32 %), sabinene (SD, 13-18 %; HD 6-11 %), δ-3-carene (SD, 8-17 %; HD 13-19 %), limonene (SD, 8-20 %; HD 15-21 %) and terpinen-4-ol (SD, 2-3 %; HD 3-6%), were the main components (≥ 5 %) of *C. lusitanica* essential oils (Table 2.2).

Previous studies on the EOs from *C. lusitanica* grown in Portugal, and in Cameroon and Kenya, have also shown, in general, α-pinene, sabinene, δ-3-carene, limonene and terpinen-4-

ol as main components (Table 2.3). β-Pinene, γ-terpinene, umbellulone, abietadiene and linalool were also reported in some *C. lusitanica* essential oils, although always in a percentage higher than that found in the present study (Table 2.3). Conversely, germacrene D, *epi*-zonarene, *trans*-totarol, *cis*-calamenene and *o*-cymene were also reported in these species essential oils but were not identified in the present study. The chemical composition of EO depends on several factors, namely the part of plant used and collection time, in addition to other geographical, physiological, environmental, genetic and socio-political factors (Figueiredo *et al*., 2008), which may justify the differences observed in this study compared with what was previously described.

4.1.2. Hydrolate volatiles

Twenty-seven and fifty-two compounds were identified in *C. lusitanica* hydrolate volatiles obtained by steam-distillation (SD) and by hydrodistillation (HD), attaining 93-94 % and 78-88 %, of the total, respectively (Table 2.2). The hydrolate volatiles obtained by both methods were dominated by the oxygen-containing monoterpenes (SD, 93-94 %; HD 64-81 %). Nevertheless, although the hydrolate volatiles obtained by SD were qualitative and quantitatively similar at the two collection moments, they differed from those obtained by HD. Whereas umbellulone (48 %) dominated SD hydrolate volatiles, this compound was present in traces in HD hydrolate volatiles (Table 2.2). Although umbellulone is considered more soluble in ethanol and DMSO, the SD pressure conditions may facilitate the extraction of this compound. *p*-Cymen-8-ol (11-16 %) was one of the dominant compounds of the HD hydrolate volatiles but was not detected in SD hydrolate volatiles. This difference may be related to the extraction method used and/or the harvesting year. In order to understand whether the difference in the chemical composition is related either to the extraction method or the harvesting time, further distillations will be carried out at different harvesting moments and with both SD and HD.

Although no previous study addressed *C. lusitanica* hydrolate volatiles, Nakagawa *et al*. (2016), also evaluated the EO and the hydrolate (hydrosol) from another Cupressaceae, *Cryptomeria japonica*. The hydrolate volatiles were rich in oxygenated monoterpenes compared with the essential oils, in agreement with that found in the present study.

Table 2.2. Percentage composition of the essential oils, isolated by steam-distillation and hydrodistillation, and hydrolate volatiles isolated from *Cupressus lusitanica* aerial parts (for codes *vide* Table 2.1).

Components	**RI**	***Cupressus lusitanica***							
		EO				**H**			
		SD		**HD**		**SD**		**HD**	
		17 January	**17 February**	**18a-c May**		**17 January**	**17 February**	**18a-c May**	
				Min	**Max**			**Min**	**Max**
***cis*-3-Hexen-1-ol**	868			t	0.1	0.2	0.1	**2.2**	**13.2**
n-Hexanol	882					0.1	0.1	0.2	0.9
Isobutyl isobutyrate	909	0.4	0.1	t	t	0.1	0.1	t	t
Tricyclene	921	0.2	0.3	0.1	0.7				
α-Thujene	924	0.7	1.6	0.5	0.9				
Benzaldehyde	927					0.1	0.1	0.1	0.2
α-Pinene	930	**13.8**	**35.7**	**24.2**	**32.1**				
α-Fenchene	938	t	0.4	0.6	0.7				
Camphene	938	1.0	0.5	0.6	0.7				
Thuja-2,4(10)-diene *	940	0.5	t	t	0.1				
Sabinene	958	**17.7**	**13.4**	**5.5**	**10.9**				
β-Pinene	963	0.5	0.9	0.9	1.4				
Dehydro 1,8-cineole	973	t	t	t	t				
β-Myrcene	975	2.9	4.4	3.0	3.5				
α-Phellandrene	995	0.1	0.3	0.1	0.1			0.2	0.2
Isopentyl isobutyrate	995	0.1	t	t	0.1				
δ-3-Carene	1000	**16.8**	**7.6**	**13.2**	**19.0**				
α-Terpinene	1002	1.3	2.0	1.1	2.1				
Benzene acetaldehyde	1002							0.1	0.2
p-Cymene	1003	0.4	0.9	0.2	0.3				
β-Phellandrene	1005	1.9	4.6	0.7	0.8			t	0.2
1,8-Cineole	1005					2.8	2.4		
Limonene	1009	**19.5**	**7.7**	**15.1**	**20.9**				
Acetophenone	1017							t	t
cis-β-Ocimene	1017	0.2	0.1	t	0.1				
trans-β-Ocimene	1027	t	0.5	0.3	0.5				
γ-Terpinene	1035	2.0	2.3	1.6	3.2				
trans-Sabinene hydrate	1037	0.1	0.1	t	0.1			0.1	0.2
cis-Linalool oxide	1045					0.1	0.1	0.2	0.4
Fenchone	1050	0.4	t	t	t	0.1	0.1	t	0.1
2-Nonanone	1058	0.2	0.1	0.1	0.4	0.1	0.1	0.1	0.4
trans-Linalool oxide	1059					0.1	0.1	0.5	1.3
6-Methyl-3,5-heptadien-2-one	1064							t	0.1
Terpinolene	1064	2.0	2.5	1.9	2.1				
cis-Sabinene hydrate	1066	0.1	t	t	0.1			0.2	0.5
n-Nonanal	1073	t	t	0.1	0.1				
Linalool	1074	t	0.3	0.3	0.6	1.5	1.5	1.1	2.1
2-Methyl butyric acid	1074			t	0.2				
trans-Thujone	1081	0.5	0.1	t	0.1	0.1	0.2	t	0.2
endo-Fenchol	1085			t	0.1			t	0.1

Components	RI	*Cupressus lusitanica*							
		EO				H			
		SD		HD		SD		HD	
		17 January	17 February	18a-c May		17 January	17 February	18a-c May	
				Min	Max			Min	Max
α-Campholenal	1092	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.2
cis-Limonene oxide	1095							0.1	0.2
trans-*p*-2-Menthen-1-ol	1099	t	0.2	0.1	0.2	1.2	1.0	1.2	1.9
Camphor	1102	0.3	0.5	0.1	3.6	**5.4**	4.4	**1.0**	**11.1**
trans-Pinocarveol	1106	1.3	0.5	t	t			0.2	0.5
cis-Verbenol	1113	t	t	0.1	0.1				
cis-*p*-2-Menthen-1-ol	1114	0.1	0.2	0.1	0.1	1.1	1.0	1.6	1.8
trans-Verbenol	1114			0.1	0.2				
Camphene hydrate*	1114			0.1	0.2				
Isopulegol	1116							1.8	2.5
Citronellal	1121	t	t						
trans-Pinocamphone	1121	t	t						
cis-Pinocamphone	1122			t	0.1				
Pinocarvone	1123	t	t	0.2	0.2				
Umbellulone*	1128	0.7	3.2			**47.5**	**48.2**	t	0.1
Borneol	1134			0.1	0.2			1.0	1.6
Lavandulol	1142			t	t			t	0.1
***p*-Cymen-8-ol**	1148							**10.5**	**15.7**
Terpinen-4-ol	1148	2.3	2.9	**3.4**	**5.9**	**23.5**	**24.0**	**21.0**	**31.4**
α-Terpineol	1159	0.1	t	0.2	0.6	3.7	4.2	**5.9**	**9.7**
4-*cis*-Decenal	1163	0.1	t						
Verbenone	1164			t	0.1	1.1	1.0	2.5	3.9
Myrtenol	1168			t	0.1				
2-Decanone	1166	0.2	0.3						
cis-Piperitol	1182	t	t						
trans-Carveol	1189			0.1	0.1	0.1	0.1	0.3	0.7
trans-Piperitol*	1189	t	t	0.1	0.2			0.5	0.8
α-Fenchyl acetate	1200	t	t	t	t				
cis-Carveol	1202							0.1	0.4
Citronellol	1207	t	t	1.2	1.5	0.4	0.5	**3.4**	**6.1**
Thymol methyl ether	1210	0.3	0.1						
trans-Ocimenone	1211			t	t	0.3	0.3	t	0.5
Piperitone	1211	t	t	0.1	0.2	0.5	0.5	0.2	0.4
Carvacrol methyl ether	1224	t	t	t	0.1				
Geraniol	1236			t	0.1				
Isoamyl hexanoate	1240			t	0.1				
cis-Chrysanthenol acetate	1242							0.2	0.4
Linalyl acetate	1245	0.1	t	t	0.1				
n-Decanol	1259			t	0.1				
Nonanoic acid	1263							t	t
Bornyl acetate	1265	0.1	0.2	t	0.2	0.1	0.1		
p-Cymen-7-ol	1265	t	0.1						
trans-Verbenyl acetate	1267	0.1	t						

Components	RI	*Cupressus lusitanica*							
		EO				H			
		SD		HD		SD		HD	
		17 January	17 February	18a-c May		17 January	17 February	18a-c May	
				Min	Max			Min	Max
2-Undecanone	1275			t	t				
Thymol	1275					3.2	3.6	t	0.1
trans-Pinocarvyl acetate	1278			t	0.1				
Carvacrol	1286			t	t	0.1	0.1	t	0.1
Myrtenyl acetate	1290							t	0.1
Terpinen-4-ol acetate*	1297	0.1	0.5						
trans-Carvyl acetate	1303	t	t	t	t				
α-Terpenyl acetate	1334	0.1	0.4	t	t	0.3	0.3		
Citronellyl acetate	1343	0.7	t	t	0.1				
α-Cubebene	1345	t	t						
cis-Carvyl acetate	1346			t	t				
Borneol propinoate	1361	t	t						
Geranyl acetate	1370			t	t				
α-Copaene	1375	t	t						
β-Bourbonene	1379	0.1	t						
Longifolene	1399	t	t						
β-Caryophyllene	1414	t	t	0.1	0.1				
cis-Thujopsene	1423	1.1	0.1	t	t				
α-Humulene	1447	t	t	t	0.1				
Cadina-3,5-diene*	1458	2.3	1.4	0.3	0.5				
γ-Muurolene	1469	t	t					t	0.2
α-Muurolene	1494			t	0.1				
trans,trans-α-Farnesene	1500			t	t				
γ-Cadinene	1500	0.2	0.1	t	t				
trans-Calamenene	1505	0.3	0.1	t	t				
δ-Cadinene	1505	0.2	0.2	t	t			t	0.1
β-Caryophyllene oxide	1561			t	0.1			0.1	0.1
Humulene epoxide II*	1580			t	0.1			t	t
1-*epi*-Cubenol	1600			t	t			t	t
epi-α-Cadinol	1616							t	0.1
α- Cadinol	1626	0.1	0.1	t	0.1			0.1	0.4
Isopimara-9(11),15-diene	1821	t	t	t	t				
Sandaracopimara-8(14),15-diene	1956	t	0.1	t	t			t	t
Abietatriene	2027	t	t	0.1	0.2			t	0.1
Abietadiene	2060	0.1	0.1	0.7	1.1			0.1	1.1
% Identification		94.4	97.8	94.8	96.9	93.9	94.3	77.5	88.4
Grouped components									
Monoterpene hydrocarbons		81.5	85.7	79.5	85.3			0.2	0.4
Oxygen-containing monoterpenes		7.5	9.4	7.4	13.5	93.3	93.8	63.7	81.5
Sesquiterpene hydrocarbons		4.2	1.9	0.4	0.8			t	0.3

Components	RI	*Cupressus lusitanica*							
		EO				H			
		SD		HD		SD		HD	
		17 January	17 February	18a-c May		17 January	17 February	18a-c May	
				Min	Max			Min	Max
Oxygen-containing sesquiterpenes		0.1	0.1	0.1	0.2			0.2	0.6
Diterpene hydrocarbons		0.1	0.2	0.8	1.3			0.1	1.2
Others		1.0	0.5	0.4	0.8	0.6	0.5	3.1	15.0
Yield (%, v/d.w.)		0.26	0.12	0.1	0.1				

RI: In-lab calculated retention index relative to C_8-C_{21} *n*-alkanes on the DB-1 column; EO: Essential oil; H: Hydrolate; SD: Steam-distillation; HD: Hidrodistillation; Min: Minimum; Max: Maximum; t: traces (< 0.05 %); * Identification based on mass spectra only; Values ≥ 5 % in bold; v/d.w.: Volume / dry weight.

Table 2.3. Data on previous studies on *Cupressus lusitanica* essential oils obtained by hydrodistillation.

Country of Origin	Collection time	Plant Part	Essential oil yield (%, v/f.w.)	Main components (≥ 5 %)	Reference
Cameroon	June 2003	Leaves	0.33	α-Pinene (7 %), umbellulone (18 %), germacrene D (8 %), *epi*-zonarene (5 %)	Kuiate *et al*., 2006
Cameroon	March 2004	Fruits	0.50	α-Pinene (64 %), myrcene (6 %), δ - 3- carene (7 %)	Kuiate *et al*., 2006
Cameroon	August 2010	Leaves	0.32	Linalool (6 %), umbellulone (6 %), terpinen-4-ol (6 %), germacrene D (19 %), *epi*-zonarene (8 %), *cis*-calamenene (8 %)	Teke *et al*., 2013
Kenya	August 2012	Leaves	0.35	α-Pinene (10 %), sabinene (8 %), δ- 3- carene (7 %), o-cymene (6 %), limonene (8 %), umbellulone (18 %), terpinen-4-ol (6 %)	Bett *et al*., 2016
Portugal		Leaves and Branchlets	0.05-0.30	α-Pinene (18 %), β-pinene (7 %), sabinene (7 %), limonene (6 %), γ-terpinene (6 %)	Carmo and Frazão, 1989
Portugal		Leaves	0.34	α-Pinene (6-17 %), sabinene (7-10 %), δ- 3- carene (1-8 %), umbellulone (traces-6 %), abietadiene (11-24 %), *trans*-totarol (5-7 %)	Adams *et al*., 1997

v/f.w..: Volume/ fresh weight; traces: <0.05 %.

4.1.3. Biological activities

4.1.3.1. Antimicrobial activity

Escherichia coli DSM 1077 and *Staphylococcus aureus* ATCC 6538 were used in these assays to determine the activity of *C. lusitanica* EOs against Gram-negative and Gram-positive bacteria, respectively. The determination of the susceptibility of *C. albicans* was done since this

microorganism besides being a commensal yeast is also an opportunistic pathogen that can cause infections of the oral or vaginal mucosa of healthy individuals (Gow and Yadav, 2017).

The essential oils isolated from *C. lusitanica* showed weak antimicrobial activity for different microorganisms (2 bacteria and 1yeast) (Table 2.4). The antibiotic (chloramphenicol) and antifungal (amphotericin B) used demonstrated a significantly higher activity (30-40 ± 0.0 mm and 13-15 ± 0.0 mm, respectively) than the tested EOs. The essential oil from *C. lusitanica* obtained in January and February had the highest activity against *S. aureus* (9.5 ± 0.0 and 9.3 ± 0.0 mm, respectively). The results also showed that the antimicrobial activity of essential oils obtained at different times (17 January and 17 February) were similar. Teke *et al*. (2013), tested the essential oil of *C. lusitanica* obtained by HD against *E. coli*, *S. aureus* and *C. albicans* using agar disc diffusion technique and the results obtained were in the same order of magnitude as those obtained in the present study.

Table 2.4. Antimicrobial activity of the essential oils of *Cupressus lusitanica* and *Cistus ladanifer*.

Microorganism	Diameter of the inhibition zone (mm)*			
	***Cupressus lusitanica* EO**		***Cistus ladanifer* EO**	
	17 January	**17 February**	**17 March**	**17 August**
Escherichia coli DSM 1077	7.2 ± 0.1	8.8 ± 0.1	9.3 ± 0.1	12.0 ± 0.0
Staphylococcus aureus ATCC 6538	8.5 ± 0.0	9.3 ± 0.0	8.5 ± 0.1	9.8 ± 0.0
Candida albicans 1098	7.5 ± 0.0	8.5 ± 0.0	8.8 ± 0.0	9.0 ± 0.0

* Including disc diameter, 6 mm Ø; EO: Essential oil; The values are the mean of four experiments ± standard deviation.

4.1.3.2. Antioxidant activity

4.1.3.2.1. Essential oil

The antioxidant activity of essential oils is of great interest as they can be used in food for preserving it from oxidation processes, thus prolonging its shelf-life without losing nutritional quality attributes. They can also be used in health for scavenging free radicals, preventing some diseases in which cellular damage are caused by these radicals (Miguel, 2010). The possible toxicity associated with synthetic antioxidants, as well as consumer preference for natural foods, has led to a growing demand for natural antioxidants (Augustyniak *et al*., 2010).

In the present study, different antioxidant activity evaluation methods showed diverse results, for the different volumes of essential oils assessed (Table 2.5). All the positive controls evaluated showed 100 % inhibition capacity. By ABTS method the EOs showed a moderate inhibition capacity > 50 % (62-63 %) and by xanthine method showed a strong inhibition capacity > 90 % (92-94 %), using 30 µL (9.9 µL/mL) and 50 µL (108 µL/mL) of EO, respectively (Table 2.5). On the other hand, the results showed that the antioxidant activity was similar at the two collection moments (Table 2.5).

Some studies describe the antioxidant activity of EOs from Cupressaceae species (Emami *et al*., 2010; Fayed 2015; Guimarães *et al*., 2010). Guimarães *et al*. (2010) reported the antioxidant activity of *C. lusitanica* EO obtained by HD, using different antioxidant activity assays, namely 1,1-diphenyl-2-picrylhydrazyl-hydrate (DPPH) free radical scavenging, reducing power and lipid peroxidation inhibition, with EC_{50} values of 53.5, 4.4 and 0.7 mg/mL, respectively.

4.1.3.2.2. Hydrolate

Independently of the method used, the hydrolates exhibited lower antioxidant activity than the respective essential oils (< 50 %) (Table 2.5). In this case too, the antioxidant activity was similar at the two collection moments. Inhibition of xanthine oxidase (18-19 %) and chelating metal ions (33-35 %) were the methods with which higher antioxidant activity was observed.

Table 2.5. Antioxidant activities of essential oils and hydrolates from *Cupressus lusitanica* and *Cistus ladanifer* evaluated by different methodologies (for sample codes *vide* Table 2.1).

		Antioxidant activities (%)*			
	Assay	**ABTS**	**Superoxide**	**Xanthine**	**Chelating**
Plant	**Sample Volume (µL)**	30	60	50	200
Cupressus	EO 17 January	62.7 ± 0.8	nd	92.4 ± 0.1	nd
lusitanica	EO 17 February	61.8 ± 0.2	nd	93.7 ± 0.1	nd
	H 17 January	3.3 ± 0.1	7.5 ± 0.2	18.1 ± 0.2	33.0 ± 0.3
	H 17 February	3.1 ± 0.2	8.4 ± 0.1	18.8 ± 1.0	34.6 ± 0.7
Cistus	EO 17 March	68.1 ± 0.1	nd	96.9 ± 0.2	nd
ladanifer	EO 17 August	69.7 ± 1.2	nd	98.5 ± 0.2	nd
	H 17 March	8.2 ± 0.2	14.3 ± 0.1	25.3 ± 0.5	24.1 ± 0.3
	H 17 August	8.1 ± 0.1	13.4 ± 0.5	25.7 ± 0.3	25.1 ± 1.0

* The values are the mean of four experiments ± standard deviation; ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; EO: Essential oil; H: Hydrolate; nd: Not determined.

Depending on the method of evaluation of the antioxidant activity, the EOs and hydrolates showed different results, as expected. Oxidation is a complex process that occurs in several steps and the capacity of a compound for acting in every step is also different (Miguel *et al*., 2010). Therefore, there is not a universal antioxidant able to act in all steps of oxidation mechanisms. Sometimes, two or more antioxidants are used in combination because they possess synergistic effect, not only by acting at different steps of the oxidation but also one antioxidant can also be able to regenerate the other (Cho and Min, 2009). Lower performance or absence activity obtained in a test does not mean that a positive result is not obtained with another test to evaluate the same biological property (Figueiredo, 2017).

4.1.3.3. Anti-inflammatory activity of hydrolates

There were no differences in inflammatory inhibition capacity for hydrolate samples (1 mL) harvested at different moments, 93 % (17 January) and 94 % (17 February). Acetylsalicylic acid used as positive control showed 95 % of inhibition at 0.1 mg/mL. Although no evaluation was performed with *C. lusitanica* hydrolate volatiles individual components, studies have shown that some of the detected compounds, namely terpinen-4-ol, linalool, thymol and 1,8-cineole, showed anti-inflammatory activity (Kamatou *et al*., 2006; Miguel, 2010; Wei and Shibamoto, 2010). The observed anti-inflammatory activity of these compounds still needs to be confirmed with further experiments, viewing to understand the traditional use of the genus *Cupressus* in the treatment of inflammations (Harraz *et al*., 2018). In line with the present results, investigation of new and effective anti-inflammatory agents from natural resources such as forestry biomass, with less undesirable effects than the commercially available ones, is of utmost importance.

4.2. *Cistus ladanifer* L.

4.2.1. Essential oil

In *C. ladanifer* EO, obtained by steam-distillation (SD) and hydrodistillation (HD), sixty-nine and seventy-seven compounds were identified, representing 87-96 % and 73 % of the total, respectively (Table 2.6). The EO yields were respectively 0.01-0.04 and 0.15 % (v/w). Although monoterpenes were dominant in all essential oils, namely the monoterpene hydrocarbons fraction (SD, 48-80 % and HD, 29 %), there were some differences in the EOs composition according to the moment of collection and type of distillation. Whereas α-pinene was the major component in all EOs (SD, 17 March, 28 % and 17 August, 25 %; HD, 17 August, 13 %), the relative amount of the second main component, camphene, was lower at warmer months, both when isolated by SD and HD (SD, 17 March, 25 % and 17 August, 13 %; HD, 17 August, 5 %) (Table 2.6). Interestingly, the relative amount of the oxygen-containing sesquiterpene fraction increased in the same samples (SD, 17 March, 3 % and 17 August, 10 %; HD, 17 August, 9 %) (Table 2.6).

For comparison purposes, five commercial Portuguese *C. ladanifer* EO samples, obtained in different years, by SD, from different producers, were used. All these samples showed similar qualitative and quantitative profile to that of the samples under study (Table 2.6).

Several studies addressed the volatile composition of *C. ladanider*, either by evaluating the plant parts solvent (Oller-López *et al*., 2005, Ramalho *et al*., 1999) or supercritical extracts (Ricón *et al*., 2000), the enantiomeric ratio of the plant essential oils (Costa *et al*., 2009), or the oleoresin essential oil (Weyerstahl *et al*., 1998). Table 2.7 gathers only the data from the essential oils obtained from *C. ladanider* aerial parts, either by HD or SD, the same procedures

and type of plant material used in the present study. Comparison of the literature data (Table 2.7), from plant material collected in different countries showed, in some cases, variable chemical composition, diverse from that herewith reported. If this reflects natural chemical variability of these species essential oils, or if it results from different collection places, physiological stage of the material, existence of different varieties or subspecies, or other distillation related factors, remains elusive. Torcato (2015) studied the effect of *C. ladanifer* leaf age on the essential oil yield and composition. Whereas the EO yield from young leaves ranged between 0.2 and 0.3 % (v/f.w.), it was not higher than 0.1 % in mature leaves. Although α-pinene dominated in the young leaf EO (40-44 %), its relative amount ranged from 9 to 22 % in mature leaf. Conversely, viridiflorol ranged from 1 to 2 % in young leaf and 5 to 22 % in mature leaf (Table 2.7). This stresses the importance of *C. ladanifer* physiological stage in EO composition and yield.

Table 2.6. Percentage composition of the essential oils, isolated by steam-distillation and hydrodistillation, and hydrolate volatiles isolated from *Cistus ladanifer* aerial parts (for codes vide Table 2.1). For comparison purposes, the minimum and maximum percentage range of the components identified in five Portuguese commercial essential oil samples, was included.

Components	**RI**	***Cistus ladanifer***							
		EO			**H**			**EOC**	
		SD		**HD**	**SD**		**HD**		
		17 March	**17 August**	**17 August**	**17 March**	**17 August**	**17 August**	**Min**	**Max**
1,2,4,4-Tetramethyl cyclopentene*	851	1.3	1.3	1.0					1.2
cis-3-Hexen-1-ol	868				1.3	0.5	0.3		t
2-*trans*,4-*trans*-Hexadienal	870						0.1		t
2-Acetylfuran	897						0.2		t
1-Methyl cycloheptanol*	909				2.8	**5.2**	4.6		1.7
Tricyclene	921	4.8	2.3	0.6					2.7
α-Thujene	924	0.2	0.4	0.5					0.4
Benzaldehyde	927				1.8	1.1	0.6		0.8
α-Pinene	930	**27.9**	**24.7**	**13.2**				**29.8**	**59.5**
Camphene	938	**25.3**	**12.6**	**5.0**	0.3	0.1	0.2	**2.6**	**14.7**
Thuja-2,4(10)-diene *	940	0.5	0.5	1.6				1.0	1.3
Sabinene	958	3.2	0.2	t				t	0.3
β-Pinene	963	0.9	0.7	0.4				0.5	0.9
6-Methyl-5-hepten-2-one	968			0.1	0.2	0.5	0.3		0.1
Dehydro-1,8-cineole	973			0.5					
2-Penthyl furan	973	t	0.1	t					
β-Myrcene	975	0.8	0.1	0.2				0.1	0.3
α-Phellandrene	995	0.1	0.3	0.2				0.2	0.4
δ-3-Carene	1000	4.2	t	0.3					0.6
α-Terpinene	1002	0.5	0.4	0.4				0.2	0.9
Benzene acetaldehyde	1002						0.4		
p-Cymene	1003	2.3	2.9	4.3				1.1	3.4

Components	RI	*Cistus ladanifer*							
		EO			H			EOC	
		SD		HD	SD		HD		
		17 March	17 August	17 August	17 March	17 August	17 August	Min	Max
2,6,6-Trimethyl cyclohexanone	1003	2.3	2.9	4.3	**12.4**	**9.1**	2.2	0.4	3.4
1,8-Cineole	1005			0.3	0.4	0.5	0.2		4.3
β-Phellandrene	1005	0.4	0.4	0.3				0.0	1.3
Limonene	1009	**6.6**	1.0	0.1				0.8	1.7
Acetophenone	1017			0.7	2.3	1.9	3.1	0.1	1.9
γ-Terpinene	1035	0.9	0.7	0.3				0.6	1.3
cis-Linalool oxide	1045				0.3	0.3	1.6		0.2
Fenchone	1050	t	0.3	0.7					
trans-Linalool oxide	1059				0.4	0.4	1.0		
2,5-Dimethyl styrene	1059	0.2	0.2	0.8				t	2.7
Terpinolene	1064	0.8	0.3	0.6				t	0.5
6-Methyl-3,5-heptadien-2-one	1064				1.2	1.2	0.9		0.2
n-Nonanal	1073	0.1	0.2					t	0.6
Linalool	1074	0.1	0.1	t				0.3	1.9
cis-Rose oxide	1083	0.1	0.2	t				0.2	0.6
α-Campholenal	1092	0.5	0.6	1.2	1.7	1.6	1.7	0.4	1.8
trans-Rose oxide	1100	t	t						0.2
Camphor	1102	0.2	0.4	0.2	1.8	1.8	1.1		0.5
***trans*-Pinocarveol**	1106	0.4	2.1	**5.3**	**5.0**	**12.6**	**7.8**	**1.8**	**5.9**
cis-*p*-2-Menthen-1-ol	1114	0.1	0.2	1.2					
trans-Verbenol	1114				1.1	0.5	1.2		
Pinocarvone	1121	0.3	1.0	2.1	1.8	3.0	1.7	t	1.5
Borneol	1134	0.7	1.3	3.8	**8.5**	**7.2**	3.1	0.6	2.1
p-Methyl acetophenone	1143				0.5	0.6	0.8		t
Terpinen-4-ol	1148	0.6	0.5	1.0	**6.2**	2.0	1.5	0.6	1.8
p-Cymen-8-ol	1148						2.9		
Myrtenal	1153	0.1	0.4	0.2	1.1	1.0	0.7	0.3	1.3
α-Terpineol	1159	0.1	t	0.7	1.1	0.7	1.1	t	0.7
Verbenone	1164			1.1	4.9	1.4	**7.8**		3.1
Myrtenol	1168	t	0.2	t					0.8
n-Decanal	1180	t	0.2						
trans-Carveol	1189			0.9	1.1	0.9	2.3	0.1	0.7
α-Fenchyl acetate	1200			t					t
Bornyl formate	1200	t	0.2						0.1
Cumin aldehyde	1200			0.4					0.3
Citronellol	1207	0.1	0.1						
cis-Ocimenone	1210			0.4					1.9
Carvone	1210			0.3	0.3	0.3	0.3	t	0.2
trans-Ocimenone	1211			0.3			0.2		0.7
Piperitone	1211						0.1		
β-Fenchyl acetate	1212	t	0.3	0.2					
Geraniol	1236				0.5	0.3			0.2
cis-Chrysanthenyl acetate	1241						t		
p-Cymen-7-ol	1265						0.2		

Components	RI	*Cistus ladanifer*							
		EO			H			EOC	
		SD		HD	SD		HD		
		17 March	17 August	17 August	17 March	17 August	17 August	Min	Max
Bornyl acetate	1265	3.7	**6.3**	4.2	3.2	1.6	1.0	**2.1**	**6.1**
trans-Pinocarvyl acetate	1278	t	0.3	0.2					0.3
Carvacrol	1286			t	3.2	1.6	1.0		0.3
Myrtenyl acetate	1290	0.1	0.2	0.4					0.7
trans-Carvyl acetate	1305	t	0.2	t				0.1	0.3
trans-2-Undecenal	1323			0.1					
Eugenol	1327			0.1					
cis-Myrcenyl acetate	1334	t	0.1						
α-Terpenyl acetate	1334	0.3	t						
Citronellyl acetate	1343	t	t						0.2
α-Cubebene	1345	0.1	0.2	0.1					0.1
Cyclosativene	1363	t	0.1	0.2				t	0.8
α-Copaene	1375	0.2	1.1	0.3				0.1	0.8
β-Caryophyllene	1414	0.1	0.2	0.1					0.2
Aromadendrene	1428			0.1					
allo-Aromadendrene	1456	1.0	2.0	0.3				0.2	1.6
trans-Cadina-1(16)-4-diene	1469			0.5					t
γ-Muurolene	1469	0.1	0.4						
Germacrene D	1474	t	t						0.1
β-Selinene	1476	t	0.2						0.1
Eremophyllene	1477	t	0.5						t
1,1,5,6-Tetramethyl -1,2-dihydronaphthalene*	1480			t					
Viridiflorene	1487	0.1	1.6	0.5			0.1	0.3	4.5
α-Muurolene	1494	t	0.4	0.1					0.1
1,1,5,6-Tetramethyl -1,2,3,4-tetrahydronaphthalene*	1495			0.2				t	0.1
γ-Cadinene	1500	0.1	0.3						
trans-Calamenene	1505	0.1	0.5	0.2					0.4
δ-Cadinene	1505	0.5	1.4	0.3				0.2	0.5
α-Calacorene	1525	t	0.3	t					0.2
Spathulenol	1551	0.1	0.4	0.4	0.1	0.2	0.1		0.1
β-Caryophyllene oxide	1561	0.1	0.2	0.6					0.1
Viridiflorol	1569	1.7	**6.2**	4.6	0.7	0.5	0.9	0.8	1.9
Ledol	1580	0.7	3.0	2.6	0.5	0.5	0.6		0.8
1-*epi*-Cubenol	1600			0.2			0.1		
α-Muurolol	1616	0.2	t	0.3					
α- Cadinol	1626	0.2	0.3	0.2					
n-Nonadecane	1900	t	t	0.1					
15-*nor*-Labdan-8-ol *	1946	0.1	0.2	0.3			t		0.3
Tetradecyl angelate	2065			0.3					0.1
% Identification		96.1	86.9	73.2	66.7	59.1	54.0	85.1	96.1

Components	RI	*Cistus ladanifer*							
		EO			H			EOC	
		SD		HD	SD		HD		
		17 March	17 August	17 August	17 March	17 August	17 August	Min	Max
Grouped components									
Monoterpene hydrocarbons		79.6	47.7	28.8	0.3	0.1	0.2	46.2	79.7
Oxygen-containing monoterpenes		7.4	15.0	25.7	42.6	37.7	38.5	8.3	24.5
Sesquiterpene hydrocarbons		2.3	9.2	2.7			0.1	1.0	8.6
Oxygen-containing sesquiterpenes		3.0	10.1	8.9	1.3	1.2	1.7	1.2	2.6
Oxygen-containing diterpenes		0.1	0.2	0.3			t	t	0.3
Others		3.7	4.7	6.8	22.5	20.1	13.5	0.7	6.5
Yield (%, v/f.w.)		0.01	0.04	0.15				0.1	0.1

RI: In-lab calculated retention index relative to C_8-C_{21} *n*-alkanes on the DB-1column; EO: Essential oil; H: Hydrolate; EOC: Portuguese commercial essential oil samples; SD: Steam-distillation; HD: Hidrodistillation; Min: Minimum; Max: Maximum; t: traces (< 0.05 %); * Identification based on mass spectra only; Values ≥ 5 % in bold; v/f.w.: Volume/ fresh weight.

Table 2.7. Data on previous studies on *Cistus ladanifer* essential oils obtained by hydrodistillation or steam-distillation.

Country of Origin	CM	Plant Part	Plant condition	EP	Essential oil yield (%, v/w)	Main components (≥ 5%)*	Reference
Corsica	August 1990	Leaves and stems	Dnp	HD**	0.07	α-Pinene (39 %), viridiflorol (12 %)	Mariotti *et al.*, 1997
				HD***	0.16-0.41	α-Pinene (11-47 %), *trans*-pinocarveol (4-11 %), viridiflorol (5-11 %)	
Corsica	dnp	Aerial parts	Dry	HD	dnp	α-Pinene (47 %), camphene (5 %), globulol (6 %)	Rossi *et al.*, 2007
France	dnp	Leaves	Fresh	SD	0.081-0.119	§ α-Pinene (216), 2,2,6-trimethyl cyclohexanone (54), viridiflorol (57)	Robles *et al.*, 2003
Germany	Autumn	Leaves and steams	Fresh	SD	0.0-0.1	α-Pinene (43 %), camphene (12 %), δ-cadinene (7 %)	Gülz, 1984
Morocco	August 2004	Leaves and small branches	Fresh	HD	0.3-0.4	α-Pinene (5 %), camphene (12 %), bornyl acetate (17 %), ledol (8%), viridiflorol (19 %)	Greche *et al.*, 2009
Morocco	VP	Leaves		HD	1.4	1,8-Cineole (19 %), γ-terpinene (6 %), viridiflorol (16 %)	Viuda-Martos *et al.*, 2011
Morocco	May 2012	Leaves	Dry	HD	0.14	Camphene (16 %), 2,2,6-trimethyl cyclohexanone (7 %), borneol (11 %), terpinen-4-ol (6 %), δ-cadinene (6 %)	Zidane *et al.*, 2013
Portugal (South)	Summer	dnp	Dnp	HD	0.1	α-Pinene (20 %), *trans*- pinocarveol (7 %),	Miguel *et al.*, 2004

Country of Origin	CM	Plant Part	Plant condition	EP	Essential oil yield (%, v/w)	Main components (≥ 5%)*	Reference
						viridiflorol (6 %)	
Portugal (Center)	July-August 2001	Leaves and small branches	Fresh	HD	dnp	Viridiflorol (15 %)	Gomes *et al*., 2005
			Dry	HD	0.2-0.3	Globulol (5 %), viridiflorol (17 %)	
Portugal (North)	July-August 2001	Leaves and small branches	Fresh	HD	dnp	Viridiflorol (15 %)	
			Dry	HD	0.2-0.3	Viridiflorol (14 %), 15- *nor*- labdan-8-ol (5 %)	
Portugal (Center)	March 2015	Leaves	Young	HD	0.2-0.3	α-Pinene (40-44 %), 2,2,6- trimethyl cyclohexanone (6-8 %)	Torcato, 2015
			Mature	HD	traces-0.1	α-Pinene (9-22 %), 2,2,6-trimethyl cyclohexanone (4-7 %), *trans*-pinocarveol (2-6 %), viridiflorol (5-22 %), ledol (3-10 %)	
Spain	dnp	Leaves and stalks	dnp	ndp	dnp	# α-Pinene (24), camphene (13), α-copaene (8), *cis*-pinocamphone (6), bornyl acetate (6)	Costa *et al*., 2009
Spain	October 2006	Leaves	Fresh	HD	0.34	*trans*-Pinocarveol (20 %), terpinen-4-ol (6 %), bornyl acetate (7 %), viridiflorol (14 %)	Verdeguer *et al.*, 2012

CM: Collection moment if provided; EP: Extraction procedure; v/w: Volume/ weight; * Unless otherwise stated the data is provided in percentage; dnp: data not provided; HD: Hidrodistillation; SD: Steam-distillation; ** Hydrodistillation using an industrial apparatus; *** Hydrodistillation using a Clevenger-type apparatus; VP: Vegetative period; § Data given in concentration (μg/μL); # Data given in absolute amounts (g/100g).

4.2.2. Hydrolate volatiles

The oxygen-containing monoterpenes were the major constituents (SD, 38-43 %, HD, 39 %) of the hydrolate volatiles. The chemical composition was qualitatively similar with some quantitative differences for samples of different moments and different distillations procedure. 2,6,6-Trimethyl cyclohexanone (17 March, 12 %, 17 August, 9 %), *trans*-pinocarveol (17 March, 5 %, 17 August, 13 %), borneol (17 March, 9 %, 17 August, 7 %), terpinen-4-ol (17 March, 6 %, 17 August, 2 %) and 1-methyl cycloheptanol (17 March, 3 %, 17 August, 5 %) was the major compounds obtained by SD and *trans*-pinocarvol (8 %) and verbenone (9 %) by HD (Table 2.6).

4.2.3. Biological activities

4.2.3.1. Antimicrobial activity

In virtue of the significant number of infections leaded by both Gram-negative and Gram-positive bacteria and by the yeast *C. albicans*, it is important to find alternative approaches to combat these pathogens. In view of this, also *C. ladanifer* EOs were assessed against these microorganisms.

The EOs isolated from *C. ladanifer* showed weak antimicrobial activity against *E. coli*, *S. aureus* and *C. albicans* (Table 2.4). Chloramphenicol and amphotericin B demonstrated a higher activity (30-40 % and 13-15 %, respectively) than the EO (Table 2.4).

The obtained results are in line with those reported by Vieira *et al*. (2017) for *C. ladanifer* EO obtained by HD, against *E. coli* (10 mm) and *S. aureus* (11 mm) using the agar disc diffusion technique.

Cistus ladanifer hydrolates showed no antimicrobial activity against the microorganisms under study.

4.2.3.2. Antioxidant activity

4.2.3.2.1. Essential oil

By ABTS method the EOs showed a moderate inhibition capacity > 50 % (68-70 %) and by xanthine method showed a strong inhibition > 90 % (97-99 %), using 30 μL (9.9 μL/mL) and 50 μL (108 μL/mL) of EO, respectively (Table 2.5). There was not much difference in the antioxidant activity between the samples harvested in different periods of the year (Table 2.5).

The antioxidant activity of *C. ladanifer* EO was already reported by Zidane *et al*. (2013) using DPPH free radical scavenging assay, with comparable results.

4.2.3.2.2. Hydrolate

The SD hydrolate showed an activity less < 50 %, lower than the respective essential oils (Table 2.5). Again, there was no difference in the antioxidant activity observed in the samples harvested in different periods (Table 2.5). Inhibition of xanthine oxidase (25-26 %) and chelating metal ions (24-25 %) were the methods where higher antioxidant activity was observed.

4.2.3.3. Anti-inflammatory activity of hydrolates

The hydrolate of *C. ladanifer* (1 mL) showed a potent anti-inflammatory activity (94 %), but no differences between the two collection moments (17 March and 17 August). Acetylsalicylic

acid showed 95 % inhibition at 0.1 mg/mL.

Considering that Miguel (2010) reported the anti-inflammatory properties of 1,8-cineole and terpinen-4-ol, which are also present in C. *lusitanica* hydrolate, it would be interesting to verify whether the activity observed in the present study is due to these compounds.

Like for the genus *Cupressus*, several *Cistus* species have also been extensively used in traditional medicine for the treatment of inflammation associated diseases (Barros *et al*., 2013), which might be partly explained by the results here described.

5. Conclusions

The results from this study showed that *Cupressus lusitanica* and *Cistus ladanifer* forest wastes may have an added value, by turning these residues into useful products for the consumer. Before burning for energy purposes, or just being discarded, *C. lusitanica* and *C. ladanifer* forest wastes may be used to obtain bioproducts such as essential oils and hydrolates, which, in addition of being consumed de *per se*, can be used in co-formulations in the perfumery, beverages, food and health industries. Although these EOs were obtained from biomass wastes, they showed the same chemical characteristics as those obtained from plant material harvested for that purpose. In addition, these EOs have shown some biological properties, that may support their use for diverse purposes, namely, both *C. lusitanica* and *C. ladanifer* EOs showed a strong xanthine oxidase inhibiting activity.

This study reports for the first time the chemical composition of the hydrolate volatiles of these two species and their strong anti-inflammatory properties. These are richer in oxygen-containing monoterpenes when compared to the corresponding essential oils, which might be partly the reason for their anti-inflammatory characteristics. There is nowadays a growing interest in hydrolates, both by producers and consumers, not only because they are an essential oil production by-product, which would be important to value, but also because of its potential use in the food, drink, cosmetic, perfumer, aromatherapy, veterinary and agro-industries. For these reasons, it is important to gather information on the composition and biological activity of hydrolates.

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Chapter III

Bioproducts from forest biomass II. Bioactive compounds from the steam-distillation by-products of *Cupressus lusitanica* Mill. and *Cistus ladanifer* L. wastes

This chapter corresponds to the following publication:

Cláudia S. Tavares, Alice Martins, M. Graça Miguel, Florbela Carvalheiro, Luís C. Duarte, José A. Gameiro, A. Cristina Figueiredo, Luísa B. Roseiro, 2020. Bioproducts from forest biomass II. Bioactive compounds from the steam-distillation by-products of *Cupressus lusitanica* Mill. and *Cistus ladanifer* L. wastes. *Industrial Crops and Products*, 158: 112991. DOI doi.org/10.1016/j.indcrop.2020.112991.

1. Abstract

Obtaining essential oils and hydrolates from underutilized biomass is an economic and sustainable way for production of these high added-value bioproducts. However, this process still generates large amounts of residues as the by-products obtained during distillation, which can be a concern for the environment, but also adequate substrates for other applications. Considering this fact, the waste distilled by-products remaining after steam-distillation of underutilized biomass from *Cupressus lusitanica* and *Cistus ladanifer*, were evaluated as a natural source of other high value products with biological activities, namely, phenolic compounds. Thus, the remaining extracted solid residues (ESRs) were characterized and subject to further treatments by ultrasound-assisted extraction (UAE) with ethanol and 70 % acetone, in order to prepare phenolic-rich extracts thereof: ESRs(EtOH) and ESRs(70 % Ace). Together with the distiller condensation waters (DCWs), these extracts were characterized for their phenolic content (total phenols, tannins and flavonoids). Their antioxidant activity was also evaluated by different methodologies. The phenolic profile of DCWs, ESRs(EtOH) and ESRs(70 % Ace) from both waste species was obtained by capillary zone electrophoresis (CZE) and phenolic compounds were tentatively identified. Results obtained for *C. lusitanica* biomass are here disclosed for the first time. Generally, all samples revealed to be rich in phenolic compounds, being *C. ladanifer* biomass the one with higher phenolic content. DCWs presented values of 140 mgGAE/g for *C. lusitanica* and 210 mg GAE/g for *C. ladanifer*, from which ca. 60 % were tannins. Extracts obtained with 70 % acetone were the ones with the highest results, except for the antioxidant activity by xanthine oxidase and superoxide inhibition, which was higher in DCWs. Catechins were the major compounds found for both species, but gallocatechins and gallic acid were only identified in *C. ladanifer*. Hydroxycinnamic acid derivatives and salicylic acid were also identified in *C. ladanifer*, partly justifying the anti-inflammatory effect referred for this species.

2. Introduction

Following the Renewable Energy Directive (Directive 2009/28/EC), the Roadmap 2050 (2015) and the Paris Agreement to reduce greenhouse gas emissions by at least 40 % by 2030 (2015), the use of biomass as a renewable source of energy has been significantly encouraged, and forest residues such as bushes and aerial parts of trees, which play an important role in forest management, have been used mainly for fuel (Puy *et al*., 2011). However, the use of these bioresources for producing high added value products is becoming more and more important in the context of sustainability and bioeconomy, as biomass of natural origin can be recovered in biorefineries with environmental, economic and social benefits (Budzianowski, 2017; Ali *et al*., 2015). In the scope of a policy of valorisation of renewable energy sources, and in the context

of the valorisation of biomass according to the existing national potential, the Portuguese National Plan for the Promotion of Biorefineries (PCM, 2017) was launched, which reinforces the valorisation of the renewable energy sources through the sustainable use of biomass not only for energy, but also for various economic sectors. Generally, biorefineries are primarily energy-based, i.e. the plant is optimised primarily to generate bioenergy products from biomass, (namely biofuels, electricity and heat), while generating co-products that may be precursors of products of higher added value for non-energy applications. However, there are biorefineries that are optimised to generate (in mass percentage) mainly bioproducts, namely, biomolecules, intermediate chemicals, proteins, bioactive substances, etc (Cho *et al*., 2020; Mahmood *et al*., 2019; Chirat, 2017).

In our previous study, (Tavares *et al*., 2020), the potential for obtaining essential oils and hydrolates from the underutilised biomass of *Cupressus lusitanica* Mill. and *Cistus ladanifer* L., their chemical characterisation and several *in vitro* bioactivities were evaluated. Numerous biological activities have been attributed to the essential oils and these are value-added products from biomass that can be readily used in the perfume/cosmetic industry or as bioblocks for different other industries (Tavares *et al*., 2020). Nevertheless, the yield of distillation is considerably small and the process for obtaining essential oils and hydrolates from the biomass still generates large amounts of residue as the by-product obtained during distillation, which is of growing concern for the environment if not properly managed. In the present study, the by-products remaining after removing the essential oil through steam-distillation, namely, distiller condensation waters and the extracted solid residues were evaluated as natural sources of other high value products with biological activities, namely, phenolic compounds, which are valuable extractives from biomass (Volf and Popa, 2018), and can be mainly used as antioxidants for different industries, before an ultimate energy application of the solid residues. Identification of new sources of natural antioxidants is a priority for example, for the food and feed industries, as the safety of the widely used authorized preservatives, such as butylated hydroxytoluene and butylated hydroxyanisole is very controversial (Wollinger *et al*., 2016). Thus, the aim of this work was to test the potential of the remaining residues from steam-distillation as source of antioxidant compounds and broaden the utilization of these biomasses. Figure 3.1 illustrates the complete valorisation potential of these biomasses, highlighting the procedure described in the present study.

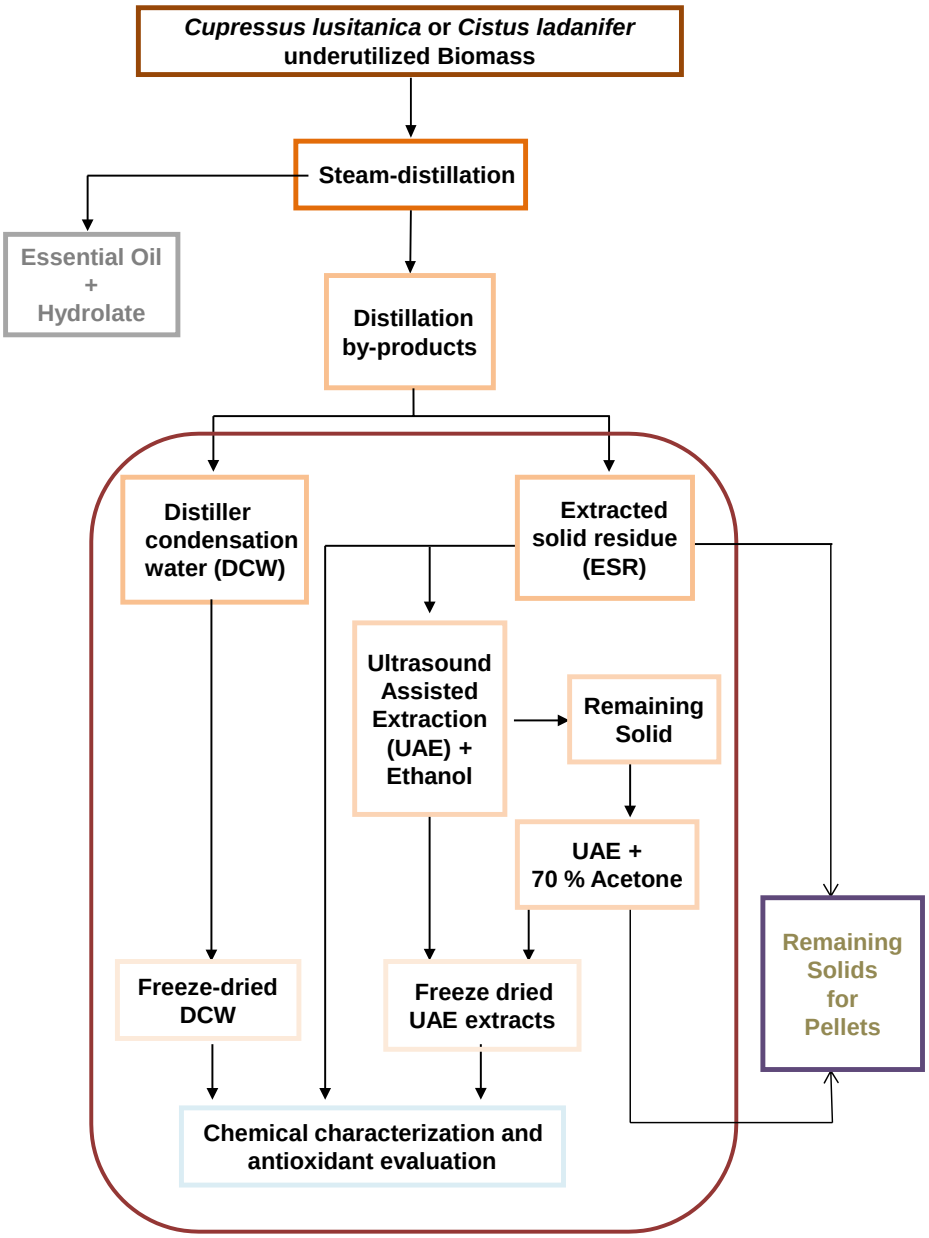

Figure 3.1. Illustrative scheme for the valorisation of *Cupressus lusitanica* or *Cistus ladanifer* underutilized biomass, highlighting the present study (round corner rectangle).

3. Materials and methods

3.1. Plant material and steam-distillation by-products

Cupressus lusitanica Mill. and *Cistus ladanifer* L. underutilized biomasses (aerial parts) were collected separately for obtaining essential oils and hydrolates by steam-distillation, which was performed at a semi-industrial scale using a stainless-steel distiller (1100 L, Vieirinox®, Aveiro, Portugal) at Silvapor premises, as described in Tavares *et al*. (2020). Circa 100 Kg of aerial parts were subject to steam-distillation to get an average essential oil yield <0.3 % for *Cupressus* and <0.04 % for *Cistus*, and 20 litres of hydrolate from each biomass (Tavares *et al*.

2020). After steam-distillation of each of these biomasses, samples of the remaining extracted solid residues (ESRs) were collected and taken to the lab for further sequential ultrasound assisted extraction (UAE), which is a simple and efficient extraction method that prevents possible chemical degradation of the targeted compounds (Ghafoor *et al*., 2009), using ethanol (EtOH) and 70 % acetone (70 % Ace), and characterization of the corresponding extracts. Also, 1.5 litres of the distiller condensation waters (DCWs) were obtained from each biomass and distillation assay, collected and freeze-dried for further analysis.

3.2. Characterization of the extracted solid residues (ESRs)

Representative samples of *Cupressus lusitanica* Mill. and *Cistus ladanifer* L. ESRs obtained after steam-distillation were air-dried for one week at open air conditions, then chemically characterized for their moisture, ash, carbohydrate, Klason lignin, soluble lignin and protein content. After milling to a particle size smaller than 0.5 mm, the moisture content was determined by oven-drying at 105 °C to constant weight. Ash content was determined at 550 °C using NREL/TP-510-42622 protocol (Sluiter *et al*., 2008). The quantification of macromolecular compounds was determined by sequential quantitative acid hydrolysis with 72 % (w/w) H_2SO_4 and 4 % (w/w) H_2SO_4 following a method based on NREL/TP-510-42618 protocol (Sluiter *et al.*, 2012). The amounts of glucan, xylan, arabinan, galactan and mannan were calculated based on the concentrations of sugars in hydrolysates obtained after quantitative acid hydrolysis. An HPLC (Agilent, Germany) was used, equipped with RI detector and an Aminex HPX-87P column (Bio-Rad, Hercules, CA, USA) operating at 80 ºC, in combination with a microguard CarboP column (Bio-Rad), and using water as the mobile phase at the flow rate 0.5 mL/min. The acid-insoluble residue was considered as Klason lignin, after correction for ash. The acid-soluble lignin was determined in the filtrate of sugars in hydrolysates by UV spectroscopy at 206 nm using 110 L / (g cm) as absorptivity (extinction coefficient) (TAPPI, 1991). The determination of protein was carried out according to the Kjeldahl method (AOAC, 1975) using the N×6.25 conversion factor.

3.3. Preparation of phenolic-rich extracts from extracted solid residues

The ESRs obtained after steam-distillation from either *C. lusitanica* and *C. ladanifer* were mixed with ethanol at a solid:liquid ratio of 1:20 and subjected to ultrasound-assisted extraction (UAE) at 30 °C for 30 min, using a Transsonic T700 sonifier (320 W, 35 kHz) (Elma GmbH & Co, Germany), according to a previously used UAE method for phenolics from biomass (Roseiro *et al*., 2013a). Extracts were then filtered through filter paper (Whatman nº. 1), concentrated under vacuum at 45 ºC in a Rotavapor R-210 BUCHI (with vacuum controller V-850 and heating Bath B-491, also from BUCHI) to obtain the ethanolic extract. This procedure was repeated 3 times. The remaining UAE solids were further extracted with 70 % acetone at a

solid:liquid ratio of 1:20 using the same procedure, and the acetone extracts were pooled, concentrated under vacuum and freeze-dried at -56 ºC in a Heto Power Dry LL3000, Thermo Scientific. Figure 3.2 shows a schematic drawing of the procedure. Remaining solids from both UAEs solvent systems of each biomass were oven dried at 45 ºC for one week and stored for future trials to produce pellets.

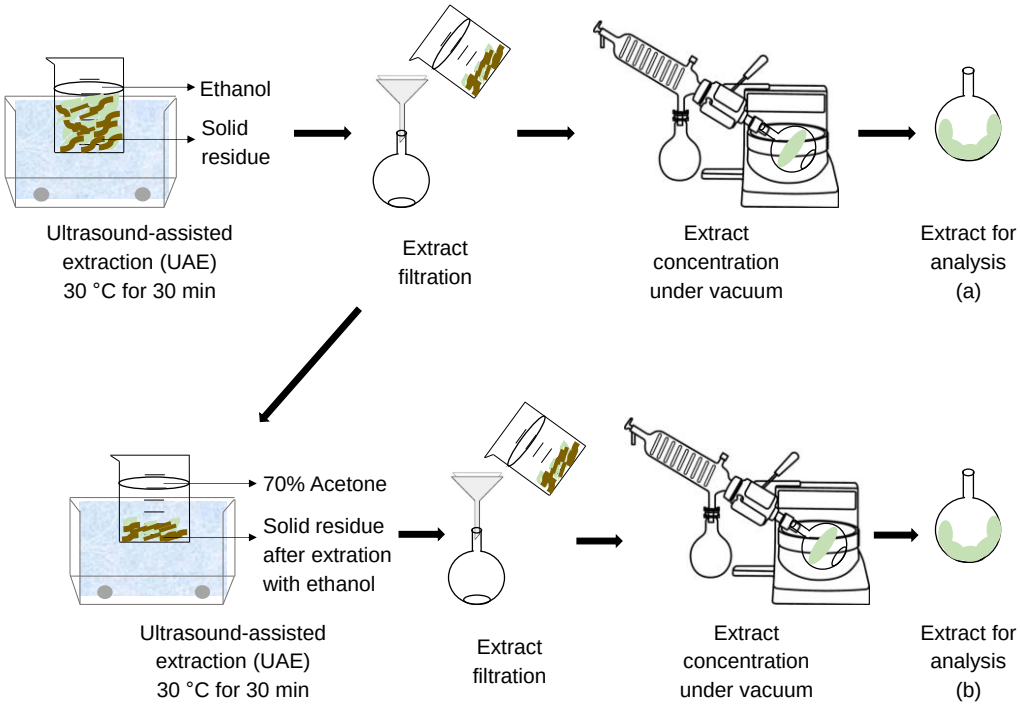

Figure 3.2. Schematic representation of the sequential ultrasound-assisted extraction (UAE) from the extracted solid residues (ESRs), using ethanol [extract for analysis (a)] followed by 70 % acetone [extract for analysis (b)].

3.4. Phenolic composition from distiller condensation waters (DCWs) and ultrasound-assisted extracts (UAEs) of extracted solid residues (ESRs)

3.4.1. Total phenolics, tannins and non-tannins content

Total phenolics were determined in the DCWs and all the UAEs extracts for both *C. lusitanica* and *C. ladanifer* biomass residues, by the Folin–Ciocalteu colorimetric method according to the procedure described in Roseiro *et al*. (2013b), adapted to a microplate format using spectrophotometric detection and microtiter 96-well plates. Briefly, reconstituted samples (1 mg/mL) of distiller condensation waters and UAE extracts (0.1 mL; or water for blank) were mixed with 0.4 mL distilled water, 1/1 (v/v) diluted Folin–Ciocalteu reagent (0.25 mL) and 20 % m/v Na_2CO_3.10 H_2O (1.25 mL). Aliquots of 200 µL were placed in each microplate well. Absorbance was measured at 725 nm on a microplate reader (Multiscan GO, ThermoFischer Sc.). A calibration curve of gallic acid was prepared. Tannins content was determined based on

the same methodology as above (Roseiro *et al*., 2013b), after removal of tannins by their adsorption on an insoluble matrix (polyvinylpolypyrrolidone, PVPP). The non-adsorbed phenolics (non-tannins) in the supernatant were transferred into the microplate wells and determined as previously described. Calculated values were subtracted from total phenolics content to obtain the total tannins content. Results were expressed as mg gallic acid equivalent (GAE) / g of extract.

3.4.2. Flavonoids content

Flavonoids content were determined in the DCWs and all the UAEs extracts for both species according to Miguel *et al*. (2014) with some modifications. Briefly, 0.25 mL of 2 % aluminium chloride-ethanol solution was added to 0.25 mL of reconstituted sample or standard in a test tube. After 1 h at room temperature, absorbance was measured at 420 nm using a UV-Vis Shimadzu UV-160A spectrophotometer. Quercetin was used as a standard for the calibration curve. Results were expressed as mg quercetin equivalent (QE) / g of extract.

3.4.3. Phenolic profile by Capillary Zone Electrophoresis (CZE)

Phenolic profile of DCWs and all the UAEs extracts was obtained by capillary zone electrophoresis (CZE) using an Agilent Technologies CE system (Waldbronn, Germany) equipped with a diode array detector (DAD), as described in Roseiro *et al.* 2013b. Electropherograms (e-grams) were recorded at 200 and 280 nm, and phenolic compounds were identified by electrophoretic comparisons (migration times and UV spectra) with data from authentic standards run under the same conditions and stored in library.

3.5. Antioxidant Activity

The antioxidant activity of DCWs and all the UAE extracts for both *C. lusitanica* and *C. ladanifer* biomass by-products obtained by steam-distillation was determined using different methodologies as referred previously in Tavares *et al*. (2020) and summarized below. These methodologies were chosen according to some of the standardized antioxidant method criteria, namely, for being simple, rapid and reproducible with chemicals and instrumentation readily available using methods with a defined endpoint and chemical mechanism, for both hydrophilic and lipophilic antioxidants, and being representative of biomolecules.

3.5.1. ABTS cation radical decolourisation assay

The ABTS radical scavenging was carried out as reported by Re *et al*. (1999) adapted to a microplate format using spectrophotometric detection (Multiscan GO, ThermoFischer Sc.) and microtiter 96-well plates. Aliquots of the reconstituted DCWs and UAEs extracts (30 µL) were

added to the radical solution (3 mL) and 200 µL of each and placed in each microplate well. Trolox (*(*±*)*-6-Hydroxyl-2,5,7,8-tetramethylchromane-2-carboxylic-acid) was used as standard and results were expressed as Trolox equivalent antioxidant capacity (TEAC).

3.5.2. Inhibition of superoxide anion radical formation

Scavenging ability of superoxide anion radical was evaluated according to Soares (1996) with some modifications. In brief, reconstituted samples (60 µL) were used and the reaction mixture was incubated at room temperature for 10 min and the absorbance reading was performed at 560 nm in a UV/VIS spectrophotometer. Ascorbic acid was used as standard and results were expressed as ascorbic acid equivalent (AAE).

3.5.3. Inhibition of xanthine oxidase

Xanthine oxidase inhibiting activity followed the Umamaheswari *et al*. (2013) method using allopurinol as standard and 50 µL of the reconstituted samples. The assay mixture was incubated for 30 min, after which the reaction was stopped and the absorbance was measured at 290 nm in an UV/VIS spectrophotometer. Results were expressed as allopurinol equivalent (AE).

3.5.4. Chelating metal ions

Chelating of ferrous ions by the reconstituted DCWs and UAEs samples (200 µL) was evaluated according to Wang *et al*. (2004) method. EDTA (ethylenediamine tetra-acetic acid) was used as standard and results were expressed as EDTA equivalent (EDTAE).

3.6. Statistical analysis

All analytical determinations were carried out in quadruplicate and results are presented as mean values with their corresponding standard deviations.

4. Results and discussion

4.1. Chemical composition of the extracted solid residues

Literature data for *Cupressus lusitanica* other than essential oil is very scarce, particularly when compared with literature for *Cistus ladanifer*. To the best of our knowledge, there are no published results concerning the chemical composition of *C. lusitanica* aerial parts neither for other *Cupressus* trees. Table 3.1 compares the results here obtained with results in literature for *Juniperus* spp, which are in the same family (Cupressaceae) and for *Pinus radiata* and *Picea*

abies wood, both the same order as *Cupressus* (Pinales) and also softwood trees. Table 3.2 shows the composition of *C. ladanifer* aerial parts used in the present study and compares it with literature results. It can be observed from Table 3.1 that the composition of the extracted solid residue for *C. lusitanica* differs largely, which would be expected, considering that, not only this has suffered an extraction by steam-distillation, but also its native biomass consisted on leaves, small branches and globular seed cones from the tree top, and not the wood from the trunk of the tree. Only the cellulose value was similar to the one for *Juniperus sibirica* needles, but conversely, Klason lignin was within the range of values found for the other species wood.

Table 3.1. Chemical composition of the extracted solid residue (ESR) from distilled aerial parts of *Cupressus lusitanica* and comparison with other species of the same family (Cupressaceae) and order (Pinales).

	Chemical composition (% w/w)							
	Cupressus lusitanica	*Juniperus sibirica*	*Juniperus communis*		*Picea abies*	*Pinus radiata*		
Plant part	**ESR**	Needles	Wood	Wood	Wood	Wood	Wood	Wood
Cellulose								
Glucan	**14.6**	18.1-20.9	38.0-41.0	61.9	44.0	45.3	31.1-42.5	44.9-54.1
Hemicellulose	**17.7**			37.9	23.3	22.2	21.4-26.0	6.1-11.1
Xylan	**7.5**			11.9	6.0	6.4		
Arabinan	**2.5**			1.4	2.0	1.5		
Galactan	**3.3**			7.5	2.3	2.1		
Manann	**4.4**			17.1	13.0	12.2		
Klason lignin	**39.0**	15.2-19.9	30.0-32.0	30.1	27.5	26.8	29.4-39.0	26.3-30.0
Soluble lignin	**2.5**							
Protein	**6.6**							
Ash	**5.7**				1.6	0.2		
Others (by difference)	**13.9**							
Reference	**Present study**	Artemkina *et al*., 2016	Bogolitsyn *et al*., 2015	Hänninen *et al*., 2012	Sassner *et al*., 2008	Araque *et al*., 2008	Berrocal *et al*., 2004	Uprichard, 1971

Table 3.2 shows results from the present study for *C. ladanifer* and from literature, including the ones disclosed by Alves-Ferreira *et al*. (2019a,b and 2017), which are within the same broad research study. Results show that Klason lignin (37 % w/w) was much higher in the present study than in the others, particularly when compared to results from Alves-Ferreira *et al*. (2019b and 2017), and also higher than the carbohydrate content (26.2 % w/w). The later agree with the ones previously determined by Alves-Ferreira *et al*. (2019b and 2017). Also, for other studies, the composition of the starting material used was different from the one here described. Alves-Ferreira *et al*. (2019a) and Fernandes *et al*. (2018) used the extracted solid residue from steam-distillation and the raw material, respectively, after being subject to Soxhlet extraction with several solvents, and obtained lower results for Klason lignin (29 % and 32 % w/w) but

higher results for carbohydrate content (47 % and 41 % w/w), respectively, when compared to our study, nevertheless, both data were within the same range. However, Carrión-Prieto *et al*. (2017) and Ferro *et al*. (2015), which used the original raw material, obtained values of 25 % and 16 % w/w for lignin and 65 % and 42 % w/w for carbohydrate, respectively, showing their heterogeneity. Despite all studies refer to the aerial parts, the fact that one has more leaves in its composition and the other more branches, may justify the difference observed in lignin content. Also, in addition to the differences in the starting material (extracted by different procedures versus raw), the place and time of harvest and in particular the age of the plant are also factors that can influence the lignocellulosic composition of this species, thus justifying the observed differences. Nevertheless, the high content in lignin of the ESRs for both *C. ladanifer* and *C. lusitanica* species here revealed suggests an additional potential for its valorisation.

Table 3.2. Chemical composition of the extracted solid residue (ESR) from distilled aerial parts of *Cistus ladanifer* obtained in the present work and comparison with previous studies in literature.

Chemical composition (% w/w)							
	Aerial parts						
Plant part	**ESR**	ESR	ESR	ESR(Sx)	RM(Sx)	RM	RM
Cellulose							
Glucan	**10.6**	17.8	16.1	27.8	26.6	55.0	34.9
Hemicellulose		12.3				10.2	6.6
Xylan	**9.3**		8.0	15.7	12.0		
Arabinan	**1.7**		2.5	3.6	2.5		
Galactan	**2.4**						
Manann	**2.2**						
Klason lignin	**37.0**	19.3	17.0	29.4	32.1	25.3	15.6
Soluble lignin	**8.0**	1.7	1.8	2.9			
Protein	**4.6**		5.7	7.3			9.2
Ash	**5.7**	4.8	4.3	4.2	2.9		3.1
Others (by difference)	**18.5**			7.4		9.5	
Reference	**Present study**	Alves-Ferreira *et al*., 2019b	Alves-Ferreira *et al*., 2017	Alves-Ferreira *et al*., 2019a	Fernandes *et al*., 2018	Carrión-Prieto *et al*., 2017	Ferro *et al*,. 2015

ESR(Sx): Soxhlet extract of ESR with different solvents; RM(Sx): Soxhlet extract of RM with different solvents; RM: Raw material without any previous treatment.

4.2. Total phenolics, flavonoids and tannins content

The yield of the UAE phenolic-rich extracts obtained from the ESRs of *C. lusitanica* and *C. ladanifer*, and also the polyphenolic content for both UAE extracts and DCWs for both biomass species, are detailed in Table 3.3. The results show that the yield of phenolic-rich extract for *C. ladanifer* was ca. 3 times higher than the yield obtained for *C. lusitanica*, independently of the extraction solvent, which indicates that this biomass is potentially richer

in these compounds. The total phenolics, tannins and flavonoids content obtained show again that *C. ladanifer* extracts are richer in these compounds than *C. lusitanica* extracts, which are in agreement with the yields obtained and the phenolic profiles shown below (Figure 3.3).

The 70 % acetone extracts for both species presented higher total phenolics, flavonoids and tannins content than the ethanol extracts. The DCW of *C. lusitanica* and *C. ladanifer* also showed the presence of phenolic compounds, mostly tannins. Regarding *C. lusitanica*, the total phenolic content for DCW was lower than UAE extracts, while for *C. ladanifer*, the total phenolic content of DCW was superior than UAE extracts with ethanol, but lower than UAE extracts with 70 % acetone. Overall, it was found that extraction with 70 % acetone was more efficient in removing phenolic compounds than the other solvents used for both species.

Although it is known that *Cupressus* trees are rich in phenolics, flavonoids and tannins (Harraz *et al*., 2018), there are very few studies about their composition in *C. lusitanica*. Guimarães *et al*. (2010) were the first to disclose the phenolic estimation and antioxidant activity of a 50 % methanolic extract from *C. lusitanica* fresh leaves harvested in North-eastern Portugal, revealing values of 30 mg GAE / g extract. The reported value was much lower than that obtained in the present study, probably due to the different material used (fresh leaves against dried aerial parts) and also different extraction solvents and conditions. No references were found in literature for the flavonoids and tannins content of *C. lusitanica*. The only results found for *Cupressus* genus was the study of Selim *et al*. (2014), which determined the total flavonoid content of a methanol extract from *Cupressus sempervirens* aerial parts (leaves) as being 53 mg QE / g of sample, which is approximately ten times higher than the values here reported for *C. lusitanica*. However, some data could be found for other Cupressaceae species, particularly for *Juniperus* species. A study of water and 80 % ethanol extracts of chopped dried leaves from five *Juniperus* species disclosed a total phenol content ranging from 4.0 – 139 mg GAE / g for the water extract and 111 – 206 mg GAE / g for the 80 % ethanol extract (Orhan *et al*., 2011). The same study reported the flavonoid content in quercetin equivalents (mg QE / g extract). Even though results are within a wide range according with the different *Juniperus* species, results from the present study are within the same order of magnitude with the ones reported by Orhan *et al*. (2011) for total phenols of *C. lusitanica* extracts obtained with the same solvent system. However, flavonoid content for the 80 % ethanolic extracts were generally more than ten times higher than the ones of water extracts for *Juniperus* species, while our results present flavonoid content for *C. lusitanica* of 5 to 6 times higher for ethanol and 70 % acetone extract than for water extract, respectively.

Regarding *C. ladanifer*, the previous study by Alves-Ferreira *et al*. (2019b), obtained values between 271-286 mg GAE/g extract for ESR(50 % EtOH) by UAE at 50 ºC for 60 minutes. These values are higher than the ones here obtained with pure ethanol, but within the range of the ones obtained with 70 % acetone extracts. In the same study, flavonoids (33-39 mg CE/g extract) and tannins (22-26 mg CE/g extract) were determined. The flavonoids content were

higher than the ones in the present study, but tannin values were lower. These latter results complement the ones here disclosed and show the effect of different conditions of extraction (higher temperature and time) and also of solvent used (50 % ethanol allows the extraction of water soluble glycosides and sugars, which are also accounted in the Folin method).

Flavonoids and tannins were determined using a different procedure than in the present study, also justifying these differences. Dudonné *et al*. (2009) found values of 103 mg GAE/g extract of total phenolics for aqueous extracts of initial plant material harvest in Spain, which were lower than the results shown in Table 3.3, independently of the extraction solvent. The differences observed may also be due to the origin and conditions of the material used. Also, total phenolics and flavonoids content of ethanolic (95 %) and acetone/water (60:40) extracts obtained by reflux of wood/stalks, bark and leaves of *C. ladanifer* was determined by Andrade *et al*. (2009), disclosing values of 255 and 335 mg GAE/g extract, respectively, and 21 and 23 mg QE/g extract, respectively. These authors also concluded that acetone extracts give rise to more compounds than ethanol. Values were higher comparing with the present study for ethanolic and acetone/water extracts, which is expected considering that they also include results for bark. Again, these variations are also dependent of several factors, including the state of the material, harvesting time and area, and the extraction conditions. Nevertheless, *C. ladanifer* extracts here studied showed to be rich in phenolics, supporting the additional potential for valorisation of these residues as a source of bioactive compounds.

Table 3.3. Extraction yield, total phenolics, flavonoids and tannins of distiller condensation waters and the different extracts obtained from *Cupressus lusitanica* and *Cistus ladanifer* distillation by-products.

	Cupressus lusitanica			***Cistus ladanifer***		
	DCW	**ESR (EtOH)**	**ESR (70%Ace)**	**DCW**	**ESR (EtOH)**	**ESR (70%Ace)**
Extraction yield (%)		4.02	3.85		13.3	11.0
Total Phenolics (mg GAE/g extract)	139.7 ± 0.2	178.9 ± 0.4	251.3 ± 0.6	209.6 ± 0.2	177.5 ± 0.2	275.6 ± 0.0
Flavonoids (mg QE/g extract)	1.3 ± 0.1	4.5 ± 0.1	6.3 ± 0.0	11.5 ± 0.8	12.3 ± 0.2	15.2 ± 0.1
Tannins (mg GAE/g extract)	86.8 ± 0.3	23.0 ± 0.2	82.2 ± 0.4	133.3 ± 1.2	110.5 ± 0.6	116.6 ± 0.8

DCW: Distiller condensation water; ESR: Extracted solid residue; EtOH: Ethanol; Ace: Acetone; GAE: Gallic acid equivalents; QE: Quercetin equivalents.

4.2.1. Phenolic profile by Capillary Zone Electrophoresis

The phenolic profile from the DCWs and the different UAEs extracts obtained from *C. lusitanica* and *C. ladanifer* distillation by-products are shown in Figure 3.3. Both species

extracts present very rich and complex, nevertheless different phenolic profiles. Each species shows a characteristic profile, similar for all the extracts within each species, except for the extracted solid residue ethanolic extract of *C. lusitanica*. It is noteworthy from the e-grams in Figure 3.3 that *Cistus* extracts present a higher and more complex phenolic content than *Cupressus* extracts. Phenolic compounds identification and the % matching with the available authentic standards are also shown in the e-grams. Catechins were the major compounds found for both species extracts and also 3-hydroxybenzoic acid was found as a common compound. Gallocatechins, hydroxycinnamic acid derivatives and gallic acid were only identified in *Cistus* extracts.

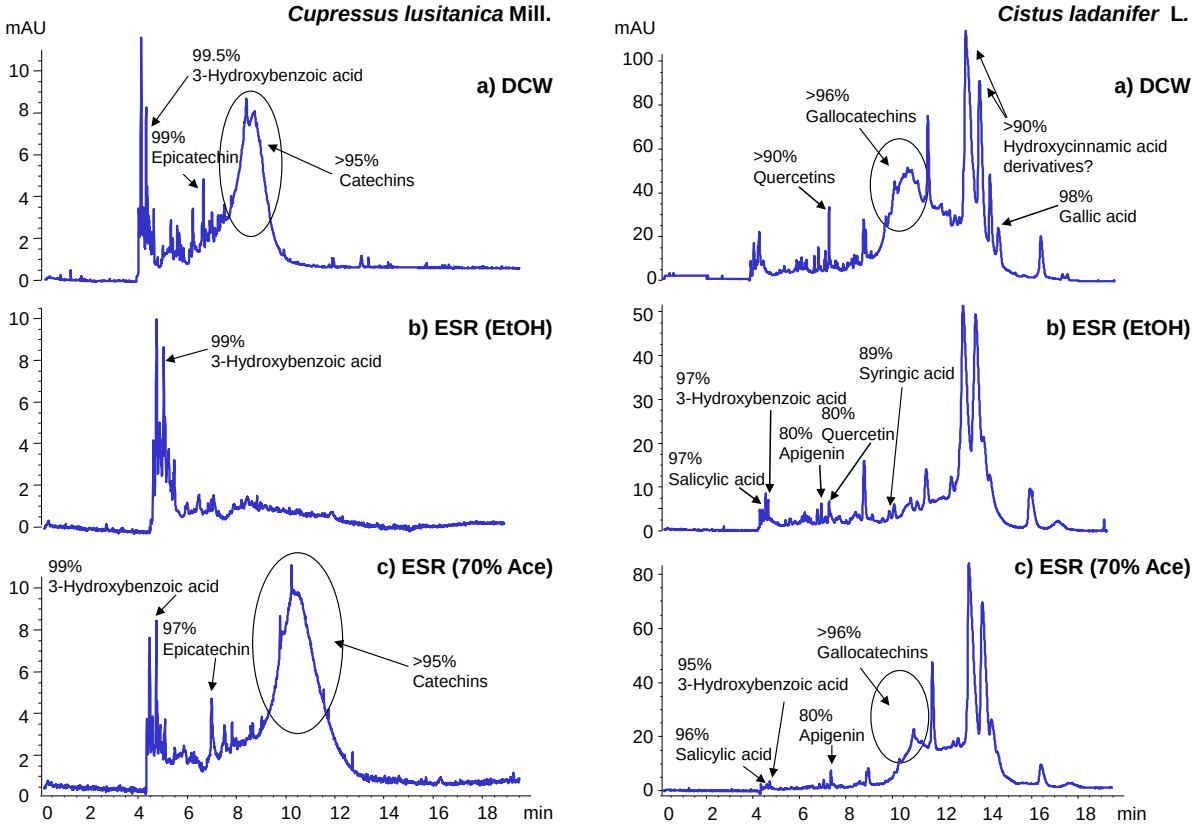

Figure 3.3. Electropherograms at 280 nm showing the phenolic profiles obtained for obtained for *C. lusitanica* and *C. ladanifer* wastes distilled by-products: a) distiller condensation waters (DCWs), and ultrasound-assisted extracts from the extracted solid residues obtained with b) EtOH (ESR(EtOH) and c) 70 % acetone (70 % Ace). Matching % was obtained by comparison with authentic standards. See text for CZE conditions.

As previously mentioned, results for the phenolic composition of *Cupressus* sp. are scarce, particularly for *C. lusitanica*. Cowan *et al.* (2001) reported two lignans (arctigenin and matairesinol) in a dichloromethane extract of stems from *C. lusitanica* and referred also the presence of tropolones in this species. Romani *et al.* (2002) reported a method of high performance liquid chromatography with diode-array detector combined with mass

spectrometry (HPLC-DAD-MS), for the identification and quantification of flavonoids and bioflavonoids present in 70 % hydroalcoholic extracts of Cupressaceae green leaves, including *C. lusitanica*. In their study, Romani *et al*. (2002) identified quercetin-glucoside and quercetin-rhamnoside, cupressusflavone, amentoflavone, robustaflavone and methylamentoflavone in *C. lusitanica* fresh leaves.

The current study refers to by-products and extracts from a steam-distillation procedure of 1 h:30 min (Tavares *et al*., 2020), which have suffered hydrolysis and thermal degradation in the different preparation steps. Therefore, it was only expected to find non-conjugated forms of the flavonoids and phenolics. Nevertheless, only 3-hydroxybenzoic acid and catechins, could be identified with matching confidence (≥ 95 %), namely epicatechin. Catechin and epicatechin are the building blocks of proanthocyanidins, a type of condensed tannins, which justifies the values found for the tannins content in *C. lusitanica* DCW and ESR (70 % Ace) extracts.

Opposite to *C. lusitanica*, *C. ladanifer* phenolic composition is well known and studied, as this is an important aromatic plant widely used in the perfumery industry. Phenolic compounds identified in *C. ladanifer* steam-distillation by-products, with the available standards, (Figure 3.3), are in agreement with literature data, although these compounds content in *Cistus* species are highly variable (Papaefthimiou *et al*., 2014). Our results revealed high concentrations of tannins, which have also been determined in various *Cistus* species, including *C. ladanifer*, such as gallic acid, as reported by Barrajón-Catalán *et al*. (2011). Several flavonoids belonging to the groups of flavones, flavonols and flavon-3-ols, such as apigenin, quercetin, gallic acid and gallocatechins here identified, were previously detected in *C. ladanifer* (Chaves *et al*.,1997; Fernández-Arroyo *et al*., 2010; Barrajón-Catalán *et al*., 2011) and also in the Soxhlet ethanolic extract of *C. ladanifer* analysed by Alves-Ferreira *et al*. (2019b). Although hydroxycinnamic acid derivatives in *C. ladanifer* were reported before (Chaves *et al*., 2001; Herranz *et al*., 2006), they were not present in the Soxhlet ethanolic extract analyzed by Alves-Ferreira *et al*. (2019b), but were here identified with a matching > 90 %.

4.3. Antioxidant activity

The antioxidant potential of UAE extracts and DCW of *C. lusitanica* and *C. ladanifer* distillation by-products are summarized in Table 3.4. In general, extracts of *C. ladanifer* showed higher antioxidant activity than those of *C. lusitanica*. Also, UAE with 70 % acetone were the ones with highest results determined by all methods for both species, except for superoxide, which was higher for DCWs. The results here obtained show a valid assessment for the potential use of DCWs and ESRs extracts as natural antioxidants in different industries, such as food, cosmetic and pharmaceutical. The potential antioxidant activity of these extracts was previously evaluated, and their results expressed in terms of % inhibition (Tavares *et al*., 2019) and the same tendency was observed. Antioxidant activity is usually associated with the presence of phenolic compounds (Ballesteros *et al*., 2017). The activity observed in the present study may

be due to the type of phenolic compounds, namely catechins and gallocatechins, as well as other flavonols and flavanols. Only one study in literature refers the antioxidant activity of *C. lusitanica* extracts (Guimarães *et al*., 2010) suggesting a maximum of 80 % DPPH inhibition for this species methanolic extracts.

Cistus species rich in phenolic compounds, especially flavonoids, are known to demonstrate significant antioxidant activity (Papaefthimiou *et al*., 2014), namely, aqueous extracts were able to generate strong antioxidant activities in a dose-dependent manner, using several free radical scavenging methods. Water and ethanol extracts of this species aerial parts showed high scavenging ability of DPPH radical, the water extracts showing 70-95 % inhibition and 95 % in case of the ethanolic extracts (Zidane *et al*., 2013). Guimarães *et al*. (2010) also obtained a maximum inhibition of 90 % with the leaf methanolic extracts, using the DPPH method.

Table 3.4. Antioxidant activity of distiller condensation waters and the different extracts obtained from *Cupressus lusitanica* and *Cistus ladanifer* distillation by-products.

	Cupressus lusitanica			***Cistus ladanifer***		
Antioxidant activities	**DCW**	**ESR (EtOH)**	**ESR (70%Ace)**	**DCW**	**ESR (EtOH)**	**ESR (70%Ace)**
ABTS TEAC (mM/g extract)	1.6 ± 0.0	0.8 ± 0.2	2.0 ± 0.1	2.6 ± 0.0	1.8 ± 0.1	4.1 ± 0.0
Superoxide AAE (mg/g extract)	768.4 ± 2.7	724.5 ± 2.4	753.2 ± 2.2	813.0 ± 6.2	703.8 ±1.3	795.0 ± 2.8
Xanthine AE (mg/g extract)	85.5 ± 0.2	70.6 ± 0.2	73.5 ± 0.2	87.6 ± 0.7	71.4 ± 0.1	76.4 ± 0.3
Chelating EDTAE (mg/g extract)	16.7 ± 0.1	14.7 ± 0.7	16.3 ± 0.1	15.1 ± 0.2	13.8 ± 0.0	15.8 ± 0.1

DCW: Distiller condensation water; ESR: Extracted solid residue; EtOH: Ethanol; Ace: Acetone; ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; TEAC: Trolox equivalent antioxidant capacity; AAE: Ascorbic acid equivalent; AE: Allopurinol equivalent; EDTAE: EDTA equivalent.

The results here obtained for the antioxidant activity support the fact that aerial parts of cypress trees (Harraz *et al*., 2018; Ibrahim *et al*., 2009; Kuiate *et al*., 2006) including *C. lusitanica* (Romani *et al*., 2002; Selim *et al*., 2014) and also species of Cistaceae, including *C. ladanifer* (Barros *et al*., 2013; Attaguile *et al*., 2000) have been traditionally used as remedies in folk medicine to treat several diseases, including anxiety, headaches, asthma, infection, inflammation, diabetes and various types of cancer, among others. These effects are mainly attributed to the bioactivities of essential oils and secondary metabolites such as phenolics present in these species, in particular to their antioxidant and anti-inflammatory activity (Alam *et al*., 2016). The same bioactive compounds are also present in these species biomasses. It is important to refer that hydroxycinnamic acid derivatives were identified in DCW and ESR

extracts of *C. ladanifer*, and salicylic acid was found in ESR (70 % Ace), partly justifying the anti-inflammatory effect referred for this species (Barros et. al., 2013). In fact, recently, the anti-inflammatory activity of steam-distillation hydrolates and by-products from both *C. lusitanica* and *C. ladanifer* biomasses were evaluated for the first time, with *C. ladanifer* evidencing higher activity for all the samples tested (Tavares *et al*., 2019 and 2020).

DCWs and ESRs extracts are thus an abundant source of natural phenolic compounds, tannins in particular, that can be exploited as building blocks in different industries (Rahim *et al*., 2018), not only as natural antioxidants, dyes and food additives, but also as raw materials for producing medical drugs or other chemicals such as polymers (Li *et al*., 2019; Cavaca and Afonso, 2018; Duval and Avérous, 2016; Garcia *et al*., 2016; Ramires *et al*., 2010).

5. Conclusions

Distiller condensation waters and extracted solid residues resulting from steam-distillation of essential oil from wastes of *Cupressus lusitanica* and *Cistus ladanifer*, were here characterized and their phenolic composition as bioactive compounds was evaluated. The ethanol and 70 % acetone extracts obtained from these residues and the distiller condensation waters were here studied for the first time and have remarkable phenolic content and antioxidant activity, the extent of which depending on the solvent used and the original species. Results indicate some correlation of the antioxidant activity with the type of phenolic compounds identified, namely catechins and hydroxycinnamic acid derivatives, and other flavonoids such as quercetins. Thus, the present work together with previous researches shows that forest biomass has a great potential as a renewable resource for recovery of bioproducts, in particular phenolic compounds, to be used as building blocks in different industries. Within a biorefinery context and towards zero waste, the use of the remaining extracted solids for pellets production, alone or together with other biomasses, is currently an ongoing study.

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Chapter IV

Valorisation of forest biomass and Economic viability analysis

1. Introduction

Valorisation of forest biomass is of upmost importance, not only at environmental level, but also for social economy. Hence, this chapter intends to show the possible value that may be given to the different fractions that result from the steam-distillation process at Silvapor - Ambiente e Inovação, Lda., and for biomasses of *Cupressus lusitanica* Mill, and *Cistus ladanifer* L. Essential oils (EOs), hydrolates (Hs), distiller condensation waters (DCWs) and extracted solid residues (ESRs) are the different fractions that can be directly or indirectly valued from the initial forest biomass waste.

The EOs market is nowadays well established, while further growth is predicted. In 2018, its global market value comprised 7.03 billion dollars, being expected to increase to 15 billion dollars in 2026 (FBI, 2019). The EOs odoriferous and biological properties, allow their use in numerous applications by incorporation in: i) the manufacture of food and beverages (Sarkic and Stappen, 2018), ii) products for personal use, such as perfumes, creams, deodorants, shampoos, bath lotions, soaps and mouth lotions, iii) household products, such as flavourings, detergents and cleaning products (Mason *et al*., 2015), iv) pharmaceutical products (Lawal and Ogunwande, 2013) and v) alternative therapies such as aromatherapy (Proença da Cunha *et al*., 2012). Besides all benefit properties of EOs, their toxicity should also be taken into consideration. In cosmetics and perfumes, the amount of added EOs should not exceed 5 %., whereas in food, should be < 0.1 % (Proença da Cunha *et al*., 2012). In addition to the amount of EO added, also the quality of the EO is important. The International Organization for Standardization (ISO), of which Portugal is a member, through the national standards organization Instituto Português da Qualidade (IPQ), has contributed to develop and revise EOs technical standards to ensure their quality, safety and efficacy and facilitate world trade (Figueiredo *et al*., 2014).

Although commercialised by some national producers, such as Ervitas Catitas (Ervitas Catitas, 2020), there is no international or national standard for *C. lusitanica* EO, nor documented reference to its use in food and drinks, in perfumes and pharmaceuticals. The EO extracted from *C. ladanifer* is very appreciated in the perfume and cosmetic industries (Barrajón-Catalán, 2016; Alves-Ferreira *et al*., 2017). It is also used as essence in detergents (Proença da Cunha *et al*., 2012). Due to its international relevance, an ISO standard is being prepared on this EO.

Likewise, the Hs may be used directly or incorporated into the formulation of other products. Currently, Hs have still a small market share when compared to EOs. However, it is predicted a growth of 5.2 % between 2019 and 2024, reaching a global market value of 437 million dollars by the end of this period (MRF, 2019). Despite the reduced use of Hs when compared to EOs, they also have a vast number of applications, namely as aromatizing in detergents and air fresheners. In food industry, Hs have been used as aromatics in food and drinks. In perfumery and cosmetics, their use is even more frequent, from perfumes to creams and shampoos. In

aromatherapy, they are fairly used in massage, baths, inhalations, flavouring, sprays and compresses. Hs find application in all these fields due to their aromatic, aphrodisiac, calming, antibacterial, antifungal, antiseptic, astringent, analgesic, antioxidant, anti-inflammatory and cicatrizing properties (Rao, 2013). Concerning the Hs from *C. lusitanica* and *C. ladanifer*, beyond its traditional uses, there are no references to their use in food, cosmetic and pharmaceutical industries. However, Hs from both species are commercialised in Portugal by producers such as Ervitas Catitas (Ervitas Catitas, 2020). In addition, *C. ladanifer* H is also commercialised by Aromas do Valado and Plena Natura (Aromas do Valado, 2020; Plena Natura, 2020).

Of all the four steam-distillation fractions, the DCWs are so far, the least exploited. As referred in Chapter III, these residual waters resulting from the steam-distillation process showed interesting bioactive compounds, such as phenolic compounds, that can be used for different industries, as natural antioxidants. Phytochemical antioxidants can be used for many products, such as pharmaceuticals, functional foods, food supplements, cosmetics, among others. Although these DCWs still need further processing, they can be sold as starter material for production of other products, provided they are adequately preserved, *e.g.* freeze-dried.

Another fraction resulting from the distillation process is the ESRs. In general, these residues are discarded or used for burning, being the last option an application of low economic value (Alves-Ferreira *et al*., 2017). A more economical valorisation for these ESRs could be transformation into solid biofuels such as pellets. Pellets consist of small cylindrical-shaped biomass produced from waste resulting from the wood processing industry, in particular sawdust and chips (Martos, 2020). They are a solid renewable biofuel used for production of thermal energy in various sectors, including the domestic, public (e.g. hospitals, nursing homes, schools, swimming pools and hotels), agricultural (e.g. greenhouses) and agri-food (e.g. bakeries) sectors (EPC, 2015). There are several advantages associated with the use of pellets, such as (i) valorisation of underused endogenous forest resources, (ii) CO_2 neutral emission, (iii) sustainable and renewable biofuel, (iv) easy to transport and store, (v) clean, safe and easy to use and (vi) more economical than fossil fuels (Martos, 2020). In addition, and in context of this study, one of the great advantages of pellets production for Silvapor additional to direct commercialization is the possibillity of using them also to feed the boiler to steam-distillation process, thus closing the valorisation cycle.

Considering that the present study was carried out in collaboration with a company in order to provide them different valorisation options for the large amounts of waste resulting from their activities, it was also considered important to evaluate the economic viability of this project. In this way, a simplified economic viability analysis was carried out. Economic viability analysis is an important tool for any company that wants to know if it is worth to invest in a particular project. The evaluation of an investment project can be carried out through flow indicators, being cash flow one of the most used. Cash flow is associated to the costs or benefits

expected to result from an investment project over a specified period. Three types of cash flows are considered: i) operating cash flow, ii) investing cash flow and iii) financing cash flow (Saias *et al*., 2006). There are several models based on cash flows that can be used to evaluate the viability of a project. The most used ones are Net Present Value (NPV), Internal Rate of Return (IRR), Pay-Back Period (PBP) and Project Profitability Index (PPI). Among them, the most used and least contested is the NPV (Soares *et al*., 1999).

In the present work, a preliminary approach was conducted by using both *C. lusitanica* and *C. ladanifer* biomasses in the small-scale valorisation of the steam-distillation fractions obtained thereof, namely, using EOs for the production of two types of handmade solid soaps: glycerine-based and olive oil-based, and using the ESRs for pellets production. Additionally, a simplified economic viability analysis was carried out with the aim of assessing the profitability of the forest biomass valorisation in a small-scale process for Silvapor Company towards a small-scale biorefinery.

2. Materials and methods

2.1. Artisanal small-scale production of soaps

2.1.1. Glycerin-based solid soap

Three types of glycerine-based soaps were produced, with, and without addition of a colorant, EO and dried plant parts. Blue, red and yellow food colorants (Vahiné) were used. In preliminary experiments the colorants were tested separately and mixed together to obtain the desired colors, namely brown (6:7:2 drops of red, yellow and blue colorants - one drop approximately 0.1 ml) and green (4:3 drops of yellow and blue colors).

To prepare the soaps, “crystal glycerine” (commercial solid form of glycerol for soaps production, 1 kg, Lacrilar) was sliced in small portions in a glass beaker and melted in a water-bath without shaking. After cooling to 50°C, the glycerin was divided into three parts: 1) without any other ingredient (control sample), 2) with *C. ladanifer* EO [2 % v/v, according Mourato and Falcão (2014)] plus brown color and 3) with *C. lusitanica* EO (2 % v/v), green color and dried *C. lusitanica* aerial parts. Each portion of glycerine without, or with the added, ingredients was poured into different silicone moulds and left for 24 h, at room temperature, to solidify. Whenever needed the soap surface was sprayed with alcohol to remove the bubbles formed. After 24 h, the soaps were demoulded and stored in a dry and cold place or kept at room temperature without individual wrapping. Three independent batches of soaps were produced. Organoleptic (colour, appearance and odour) and physico-chemical (weight and pH) characteristics were monitored monthly for 4 months. Soaps with *C. lusitanica* EO, without colorant or biomass were also prepared, however the organoleptic evaluations were only performed on soaps 3). The measurement of pH was carried out according the European Standard EN 1262:2003, using a pH meter (Chemtrix Type 45AR pH controller) and an

aqueous soap solution (1 %).

2.1.2. Olive oil-based solid soap

Two types of olive oil-based soaps were produced, with, and without *C. lusitanica* or *C. ladanifer* EO according to Mourato and Falcão (2014). Olive oil (400 g) and coconut oil (100 g) were mixed in a glass beaker and heated to 120-130 °C in a waterbath. Separately, the lye water was prepared, in a fume hood under safety conditions (wearing lab coat, gloves and goggles). Sodium hydroxide (lye or caustic soda) (66 g) was slowly and carefully added to the distilled water (155 g) and gently stirred until the lye has fully dissolved. Once both the lye water and the oils mixture cooled to 45˚C, the lye water was added to the oils mixture, first gently stirred by hand for a few seconds, and then with a stick blender until a light trace was reached. The mixture was divided into two portions: 1) without EO (control sample) and 2) with either *C. lusitanica* or *C. ladanifer* EO (2 % v/v), added under constant stirring with a glass rod until a homogeneous mixture was obtained. Each portion of soap mixture was poured into different silicone moulds, which were covered with cling film, and left, at room temperature, for 24 h to 48 h. After this period, the cling film was removed, and the soaps were demoulded and stored in a dry and cold place and were kept at room temperature without individual wrapping. Three independent batches of soaps were produced. Colour, appearance, odour, weight, and pH parameters were monitored monthly for 4 months. The measurement of pH was carried out as previously described according the European Standard EN 1262:2003.

2.2. Pellets production

Cylindrical pellets of *C. lusitanica* (wood) and *C. ladanifer* (non-wood) extracted solid residues resulting from the steam-distillation process were produced. These ESRs were air-dried for one week under open air conditions at room temperature. The biomass was then crushed in a Viking® GE 355 crusher and sieved (ASTME 11 Ser.No.246762) until < 4 mm in diameter particles was obtained. When required, the particles whose dimensions were larger than those definded by the pelletizer were milled in a Waring® Commercial Blendor. This was then inserted in a domestic pelletizer (PL T-100 Smartwood), with a production capacity of around 70 kg pellets/hour, with 20 mm diameter and 6 mm length. The production process used is schematized in Figure 4.1. Prior to characterization, the pellet samples were ground (0.25 mm) using a microfine grinder drive (MF 10 basic IKA WERKE) and then characterized in terms of i) length and ii) diameter using an electronic digital calliper (POWERFIX) and iii) net calorific value, iv) moisture, v) ash, vi) volatile matter, vii) carbon, viii) hydrogen, ix) chlorine, x) nitrogen and xi) sulphur content. The tests were carried out in the Accredited Laboratory (IPAC L0041 ISSO/IEC 17025) of Biofuels and Biomass - Bioenergy and Biorefinery Unit of LNEG (Laboratório Nacional de Energia e Geologia, Campus do Lumiar)

according to the standards referred in Table 4.2.

Figure 4.1. Flowchart for pellets production from *C. lusitanica* and *C. ladanifer*.

2.3. Evaluation of the investment project

The profitability of project investment was therefore evaluated by the Net Present Value (NPV) model through the determination of cash-flows, on a 6-year time frame and considering investment, costs and sales over each year. The NPV was calculated according to Soares *et al*. (1999) following the formula (1):

$$NPV = \sum \frac{CFG_t}{(1+r)^t} = \frac{(OCF+ICF)_t}{(1+r)^t} \quad (1)$$

Where:

CFG_t : Represents the global cash flow obtained in period *t*, being calculated based on net cash flow which corresponds to the sum between operating cash flow, OCF (which consists in the money received or spent as a result of the main activities of the company) and investing cash flow, ICF (which corresponds to money received or spent in investments or acquisitions).

t : Represents the period of time (from 1 to n),

n : Represents the number of predicted time periods,

r : Represents the discount rate, in other words the rate of return that the investors expected.

The OCF and ICF was calculating following the formulas (2) and (7), respectively. The OCF took into account the Result after tax (RAT), Financial charges (FC), Depreciation and amortization (D.A), Result before tax (RBT), Tax (T), Operational result (OR), Gross result (GR), Personnel costs (PC), External supplies and services (ESS), Total expected profits (TEP) and Costs of good acquired and materials consumed (CGAMC). The ICF corresponds to the total investment (TI).

OCF = RAT + FC + D.A *(2)*

RAT = RBT – T *(3)*

RBT = OR – FC *(4)*

OR = GR – (D.A + PC + ESS) *(5)*

GR = TEP – CGAMC *(6)*

ICF = TI *(7)*

The calculations needed for the determination of NPV were made considering the expected investment, costs and profits. For the calculation of the investment, all equipment, materials, and furniture needed for the project were considered (Table 4.5). The interest and amortization as well as financial charges were also considered (Table 4.6). The expected costs can be divided into: i) costs of goods acquire and materials consumed (CGAMC), ii) external supplies and services (ESS) and iii) personnel costs (Table 4.7). Essential oils, hydrolates, soaps and pellets were the products considered for sales (Table 4.8). All parameters described above have been considered for the calculation of the operating cash flow and investing cash flow used for determination of NPV to a discount rate of 10.7 % (*r*) (Table 4.9).

3. Results and discussion

3.1. Artisanal small-scale production of soaps

3.1.1. Glycerin-based solid soap

Three types of glycerin-based soaps were produced, 1) the control sample without any other ingredient, 2) with *C. ladanifer* EO and colorant and 3) with *C. lusitanica* EO, colorant and *C. lusitanica* dried aerial parts (Figure 4.2). The colour and appearance did not change over the 4 months of evaluation. Nevertheless, the odour intensity vanished over time, particularly from those kept at room temperature without wrapping, for one month.

Four months after production, the average weight decreased around 4 % for all samples (Table 4.1). The observed weight losses can be related with the natural evaporation process. The measurement of the soaps pH 4 months after the first evaluation and 24 h after production, did not show major changes, neither over time, nor between the control sample and those to which essential oil was added (Table 4.1). The average pH of the produced soaps (pH 9) was within the limits of the pH range of the commercial soaps (pH 7 to 9). Although most of the soaps in market present an alkaline pH, from the dermatological point of view, the ideal pH should be acid, about 5.5, since this corresponds to the skin pH (Tarun *et al*., 2014). Alkaline soaps increase the skin surface pH, diminishing the skin antibacterial capacity and barrier function (Duncan *et al*., 2013).

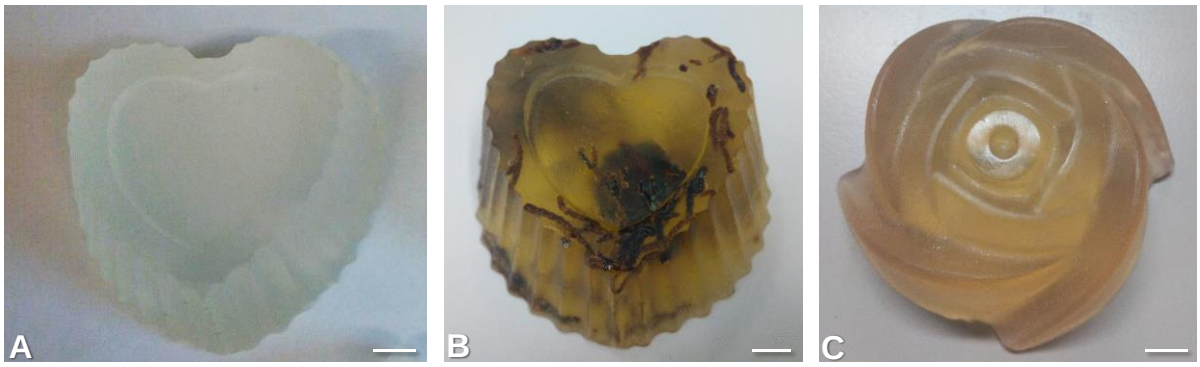

Figure 4.2. Examples of glycerin-based solid soaps: control sample (A), with *C. lusitanica* EO, colorant and dried aerial parts (B) and with *C. ladanifer* EO and colorant (C) (bar = 1 cm).

3.1.2. Olive oil-based solid soap

Two types of olive oil-based soaps were produced, 1) without EO (control sample) and 2) with either *C. lusitanica* or *C. ladanifer* EO (2 % v/v). The odor did not change over the 4 months of evaluation. The colour and appearance changed over the first 24 h after production, with a whitish film appearing at the surface (Figure 4.3 A and B). This effect is known as “ash”, which results from the reaction of sodium hydroxide (lye or caustic soda) with carbon dioxide

in the presence of air. Normally, it emerges in the region of the soap that has direct contact with air over the drying step. A way to avoid this is by covering the soap with a film so that contact with air can be avoided (Mourato and Falcão, 2014). Despite having been covered with a protective film during the first 24 h, some soaps showed the "ash" effect (Figure 4.3 A and B). This may indicate that the soaps were not completely dried prior to wrapping. The procedure was repeated, but, this time, the soaps were stored for 48 h before removing the film and from the silicone moulds. In this case the ash effect did not develop (Figure 4.3 C).

Table 4.1. pH and weight values of glycerine and olive oil-based solid soaps after 24 h following production and after 48 h and 4 months storage.

Glycerine-based			Olive oil-based		
Sample	**pH**	**Weight (g)**	**Sample**	**pH**	**Weight (g)**
CS_24 h	9.2 ± 0.0	49.2 ± 0.0	CS_48 h	11.1 ± 0.0	78.5 ± 0.0
CS_4 M	9.3 ± 0.0	47.5 ± 0.0	CS_4 M	9.6 ± 0.0	57.7 ± 0.0
ClaS_24 h	9.4 ± 0.0	50.4 ± 0.0	ClaS_48 h	11.2 ± 0.0	79.5 ± 0.0
ClaS_4 M	9.4 ± 0.0	48.3 ± 0.0	ClaS_4 M	9.3 ± 0.0	58.6 ± 0.0
CluS_24 h	9.4 ± 0.0	71.2 ± 0.0	CluS_48 h	11.3 ± 0.0	79.2 ± 0.0
CluS_4 M	9.3 ± 0.0	68.4 ± 0.0	CluS_4 M	9.3 ± 0.0	58.6 ± 0.0

CS: Control soap; M: month; ClaS: *C. ladanifer* soap; CluS: *C. lusitanica* soap.

Figure 4.3. Examples of olive oil-based solid soaps: control sample 24 h following production (A), and with the addition of *C. lusitanica* EO, 24 h (B) and 48 h (C) following production (bar = 1 cm).

The olive oil-based solid soaps showed a higher reduction of weight over time than the glycerine-based soaps. Four months after production the average weight decrease was around 36 % for all samples (Table 4.1). The measurement of the soaps pH 48 h and 4 months showed differences. Initially, olive oil-based soaps presented an alkali pH around 11, that decreased to pH 9 after 4 months. The initial pH is related with the high lye concentration which decreases with the curing time. This 4-month period is thus of high relevance since a soap with a pH > 10

may be too aggressive to the skin, and its use should be avoided. As previously mentioned, ideally the pH of the soap should be close to the skin pH. However, a soap manufactured with this cold process is naturally alkaline, having a pH ranging from 8 to 10, so that the skin can be smoothly cleaned without causing allergies and being thus compatible with body washing (Mourato and Falcão, 2015). Therefore, the produced soaps presented a pH within the expected range.

Comparing both types of solid soaps, the olive oil-based soaps surface was more prone to changes than the surface of the glycerin-based ones. On the other hand, the odour of the olive oil-based soaps remained over larger periods than that of the glycerin-based ones. This may be due to the soap's own composition, preserving volatiles for longer time.

There are no references to the commercialization of soaps with *C. lusitanica* EO in Portugal. On the other hand, Herdade Vale Covo (Herdade Vale Covo, 2020) is a Portuguese company that commercializes soaps with *C. ladanifer* EO.

Considering the aromatic factor and the results obtained in the present study for antioxidant activity demonstrated by *C. lusitanica* and *C. ladanifer* EOs, it would be interesting to study the possibility to use these soaps against the natural oxidation of the skin. In addition to the benefits of the use these soaps might have, it is equally important to evaluate the prossible side effects. Therefore, before these artisanal products can be marketed with due safety, they should be subjects to toxicity and allergology analyses.

3.2. Pellets production

C. lusitanica and *C. ladanifer* pellets were evaluated for length and diameter according to ISO 17829:2015, for net calorific value according to ISO 18125:2017, for moisture according to ISO 18134-3:2015, for ash and volatile matter according ISO 18122:2015 and ISO 18123:2015, respectively, for carbon and hydrogen according EN 15104:2011, for chlorine and sulfur according EN 15289:2011 and for nitrogen according EN 15104:2011 (Table 4.2).

Table 4.2. Results of physical and chemical analysis of *Cupressus lusitanica* and *Cistus ladanifer* pellets.

Parameters (Units)	***C. lusitanica***	***C. ladanifer***	**ISO/EN Test Method**
Length (mm)	18.4 ± 5.3	18.1 ± 4.3	ISO 17829:2015
Diameter (mm)	6.1 ± 0.0	6.1 ± 0.0	ISO 17829:2015
Net calorific value (MJ/Kg)	20.9	20.3	ISO 18125:2017
Moisture (w-%, as received)	7.8 ± 0.2	8.5 ± 0.2	ISO 18134-3:2015
Ash (w-% dry 105˚C)	6.1 ± 0.4	4.8 ± 0.3	ISO 18122:2015
Volatile matter (w-%, as received)	74.9 ± 0.5	74.6 ± 0.5	ISO 18123:2015
Carbon (w-% dry 105˚C)	51.5	50.9	EN 15104:2011
Hydrogen (w-%, as received)	5.7	5.4	EN 15104:2011
Chlorine (w-% dry 105˚C)	0.25	0.22	EN 15289:2011
Nitrogen (w-% dry 105˚C)	1.06	1.03	EN 15104:2011
Sulfur (w-% dry 105˚C)	0.05	0.06	EN 15289:2011

Results for the *C. lusitanica* pellets revealed that, with exception for ashes, chlorine and nitrogen contents, all the other parameters were according to ISO 17225-2:2014 reference values for wood pellets (Table 4.3).

Table 4.3. Specifications of pellets produced from woody and non-woody biomass according to ISO 17225-2:2014 and ISO 17225-6:2014 standards.

	Wood pellets ISO 17225-2:2014			**Non-woody pellets ISO 17225-6:2014**		
Parameters (Units)	**ENplus® A1**	**ENplus® A2**	**ENplus® B**	**A**	**B**	**ISO/EN Test Method**
Length, L (mm)		3.15 < L < 40.0		3.15 < L < 40.0		ISO 17829:2015
Diameter (mm)		6.0-8.0 ± 1.0		6.0-8.0 ± 1.0		ISO 17829:2015
Moisture (w-%, as received)	≤ 10.0	≤ 10.0	≤ 10.0	≤ 12.0	≤ 15.0	ISO 18134:2015
Ash (w-% dry)	≤ 0.7	≤ 1.2	≤ 2.0	≤ 6.0	≤ 10.0	ISO 18122:2015
Net calorific value (MJ/Kg)	≥ 16.5	≥ 16.5	≥ 16.5	≥ 14.5	≥ 14.5	ISO 18125:2017
Nitrogen (w-% dry)	≤ 0.3	≤ 0.5	≤ 1.0	≤ 1.5	≤ 2.0	ISO 16948:2015
Chlorine (w-% dry)	≤ 0.02	≤ 0.03	≤ 0.03	≤ 0.10	≤ 0.30	ISO 16994:2016
Sulfur (w-% dry)	≤ 0.04	≤ 0.05	≤ 0.05	≤ 0.20	≤ 0.30	ISO 16994:2016

ENplus: World-leading wood pellets certification; A: Pellets produced from herbaceous biomass; B: Pellets from fruit biomass or aquatic biomass.

Further than the ash values obtained being above the ISO 17225-2:2014 specifications, they were also not comparable to those obtained by other authors for softwoods like *Pinus* spp. (Table 4.4). It should be mentioned that, in the present study, pellet samples were essentially made of leaves and small stems. This may be the reason for the higher amount of ashes when compared to other samples mostly made of wood. It is known that the amount of ashes is essential for the pellets energetic excellence, since high levels contribute to the reduction of the calorific value. This is because not every element that constitutes the ashes contributes to the combustion process (Garcia *et al*., 2018). On the other hand, more and more customers look for biofuels that produce fewer ashes for hygienic reasons. Given this, an alternative would be the addition of other biomass materials (e.g. wood chips) in the production of pellets from *C. lusitanica*.

Chlorine and nitrogen contents were also above the reference. In general, the chlorine content in solid woody biofuels is relatively low, < 1 % (Jenkins *et al*., 1998). Although most of the chlorine is left in the ashes, part is also released in the form of hydrogen chloride and thus causes corrosion of the equipment (Barros, 2013). Even though the chlorine content obtained in the *C. lusitanica* pellets was below < 1 %, it was above the ISO 17225-2:2014 reference values (Table 4.3). This was also higher than the chlorine content from other softwoods (Table 4.4). The nitrogen contents should not be above those in the standard since it participates in the formation of nitrogen oxides during combustion while being harmful to the environment when exhausted. Usually, both biomasses from agriculture activities and leaves

from trees show nitrogen contents above woody biomass (Barros, 2013). Given that the sample is mostly made of leaves, this may justify the higher nitrogen contents that was obtained, and when compared to the corresponding standard and other softwoods (Table 4.4). Again, a possible solution may be the addition of wood parts of *C. lusitanica* or other biomasses, like chips.

Table 4.4. Data extracted from previous studies on some woody and non-woody pellets and biomass.

Parameters	**Wood pellets**			**Non-woody biomass**			
(Units)	***Pinus* spp.***	***Pinus* spp.***	***Pinus* spp.***	**Miscanthus**	**Peanut shell**	**Straw**	**Sugarcane bagasse**
Net calorific value (MJ/Kg)	19.2	17.0	16.7-17.9	16.9	17.0	15.7	16.5
Ash (w-% dry 105˚C)	0.6	0.9	0.2-0.3	1.8	3.6	6.2	1.9
Volatile matter (w-%, as received)	dnp	dnp	dnp	82.9	74.1	76.3	dnp
Carbon (w-% dry 105˚C)	51.8	dnp	49.1-49.8	48.9	47.0	43.8	50.5
Hydrogen (w-% dry 105˚C)	6.1	dnp	6.0-6.1	6.7	6.8	6.8	6.1
Chlorine (w-% dry 105˚C)	0.01	0.01	0.06-0.07	dnp	dnp	dnp	0.03
Nitrogen (w-% dry 105˚C)	0.2	0.2	0.05-0.1	0.4	1.3	0.6	0.2
Reference	Garcia *et al*., 2018	Duca *et al*., 2014	Alakangas, 2005	Sher *et al*., 2017	Sher *et al*., 2017	Sher *et al*., 2017	Garcia *et al*., 2018

*Mean values; dnp: data not provide.

There are no standards reporting on carbon and hydrogen contents in pellets. However, these are elements that have a positive influence in the calorific power. Generally, a woody material presents carbon and hydrogen contents of around 50 % and 5-6 %, respectively (Jenkins *et al*., 1998). The carbon and hydrogen contents of the *C. lusitanica* pellets obtained in the present study are comparable to the ones indicated for the woody materials, while being also similar to the ones found in literature for softwoods (Table 4.4).

Besides the calorific power, the high volatiles content is another characteristic that make biomass a suitable fuel. In opposite to coal, the main source of energy in biomass comes from burning the volatiles that contributing to the high output performances (Werther *et al*., 2000). Like for the parameters mentioned before, there are also no standards for the volatiles content. In the present work, *C. lusitanica* pellets show volatile values of 69 %, which is below the ones obtained for other softwoods, e.g. 82 % (Oliveira *et al*., 2017) and 85 % (Amorim *et al*., 2015) for Pinus spp. The low values could be related to the fact that biomass undertook a previous steam-distillation step, where part of the volatiles was extracted.

Results for *C. ladanifer* pellets showed that all parameters were according to the ISO 17225-6:2014 standard for non-woody pellets (Table 4.3). Like in woody pellets, there are also no mandatory parameters for the carbon, hydrogen and volatile contents in non-woody ones. Although no information on *C. ladanifer* pellets was available, it is possible to find information on other non-woody species such as *Miscanthus* spp., peanut shell, sugarcane bagasse and straw (Table 4.4). It was found that the values obtained in the present work are similar to the ones found in literature. The exception is the chloride content that was found to be 20 times above the values of sugarcane bagasse, for example. Also, the volatile content is below the ones for non-woody species. Again, the fact that the biomass went a pre steam-distillation step may justify these results.

Therefore, with the present data it was verified that the production of pellets from solid residues obtained after steam-distillation from both *C. lusitanica* and *C. ladanifer*, could be a viable pathway towards valorisation of these residual fractions. It allows reusing a material that is already considered as waste throughout its transformation into an added-value product. In addition to the production of pellets from the ESR of each species individually, tests were carried out by joining the ESRs of the two biomasses, with good apparent results for the final pellets, although no further assays have been performed. Thus, joining these extracted solid fractions from steam-distillation with several other biomasses. to produce pellets, including the ones with no value for EO extraction, constitutes another option for integrated valorisation of these wastes for the company.

3.3. Evaluation of the investment project

The profitability of project investment was evaluated by the Net Present Value (NPV). For calculation of the NPV, the net cash flow (sum between operating and investing cash-flows) (*CFG*) were determined considering three main parameters, i) expected investment, ii) expected costs and iii) expected profits, which will be described below. The calculation was made a 6-year time (t) to a discount rate of 10.7 % (r).

3.3.1. Expected investment

For the calculation of the investment, all equipment, materials, and furniture needed for the project were considered. Except for the physical space that already existed, all the investment described below corresponds to what was acquired, or it is necessary to acquire for the execution of this project at Silvapor. The necessary equipment includes the distiller and a pelletizer, and the values correspond to the ones found at Vieirinox and Ecofricalia, respectively. The cold equipment corresponds to fridges and freezer cabinets, the cost having been calculated based on market values. The laboratory resources include glassware (flasks, graduated beakers, gobelets, funnels, separatory funnel, graduated pipettes, and Pasteur

pipettes), stainless steel based material (universal holder and rings), among other materials. The required values were calculated using the Labbox 2019 catalogue. Office supplies include a computer, scientific books, notebooks and writing materials, being the purchasing values estimated based on market prices. The estimated cost of the necessary furniture has also been calculated, which includes storage cupboards, countertop, desk, and chairs. In this way, it is expected that total investment (TI) value will be 20600.00 € including VAT (value-added tax) (Table 4.5).

Table 4.5. Predicted project investment costs.

Investments	**Company source**	**Price (€)***
Distiller	VieiraInox	15000.00
Pelletizer	Ecofricalia Sostenible	2300.00
Cold equipment	Radio Popular	900.00
Laboratory material	Labbox Export: Lab supplies	700.00
Office material	Staples	700.00
Furniture	Leroy Merlin	1000.00
Total investment (TI)		**20600.00**

*Price including VAT (value-added tax) 23 %

The value of each investment was multiplied by the amortization rate (0.125), obtaining the value for the annual amortization. The sum of the annual amortization of each investment corresponds to the value of the depreciations and amortizations. From banking point of view, the value of the loan was the value of the total investment required. The medium/long-term loan interest rate used it was based on the value tabulated by the national Caixa Geral de Depósitos bank at the date of the present study, 0.05 €/year. The interest was calculated by multiplying the outstanding capital (portion of the capital amount which has not been repaid by the borrower to the lender) by the interest rate, while the amortization was calculated by the difference between the instalment value (a partial payment on a financial obligation) and interest (Table 4.6). The outstanding capital in the following years was always calculated by the difference between the outstanding capital of the previous year and the respective amortization of that year (Table 4.6). The financial charges (total cost of borrowing, including interest charges and other charges paid by the borrower for availing the loan facility), was calculated by the sum of the interest with the instalment value. Depreciations, amortizations, and financial charges were used to calculate the OCF and the value of total investment was considered as ICF.

Table 4.6. Bank interest and amortization over a period of 6 years.

Interest and Amortization			
Bank lending (€)			20600.00
ML-term loan interest rate (€)/year			0.05
Instalment value (€)/year			3433.30
Years	**Outstanding capital (€)**	**Interest (€)**	**Amortization (€)**
1 (T0)	20600.00	1015.60	2417.80
2	18182.20	896.40	2536.90
3	15645.30	771.30	2662.00
4	12983.30	640.10	2793.30
5	10190.00	502.40	2931.00
6	7259.10	357.90	3075.50

ML-term: Medium and long-term.

3.3.2. Expected costs

The expected costs of this project can be divided into three large groups: i) reagents, which correspond to the costs of goods acquired and materials consumed (CGAMC), ii) fuel, energy, chemical analysis, shipping fees, design/prints, communication, vigilance and safety, which correspond to external supplies and services (ESS) and yet iii) personnel costs (PC). In all, total cost will be 15947.00 € (Table 4.7).

Table 4.7. Expected individual and overall costs for the project per year.

Costs	**Price (€)* / year**
CGAMC	
• Reagents	300.00
ESS	
• Fuel	210.00
• Energy	240.00
• Chemical analysis	380.00
• Shipping fees	400.00
• Design/Prints	200.00
• Communications	310.00
• Vigilance	270.00
• Safety	72.00
Personnel costs	
• Operators	13550.00
Expected total cost	**15947.00**

Price including VAT (value-added tax) 23 %, CGAMC: Costs of goods acquired, and materials consumed; ESS: External supplies and services.

For the calculations, the costs of water and cleaning of the workspace were not considered, because these are not additional costs to this project.

For the costs related to reagents, a value was stipulated considering the prices charged by LaborSpirit and Norauto companies. The reagents considered were distilled water (2.49 € / 5 L com VAT), ethanol (1.71 €/ 25 L + 23 % VAT) and acetone (4.0 €/ 2 L + 23 % VAT).

The cost of fuel contemplated the transport of biomass (approximately 84 km round-trip) and was calculated considering an average fuel consumption of the vehicle of 10 L / 100 Km and the price of diesel at Idanha-a-Nova region at the time of execution of this study (1.267 €, Intermarche).

The cost of energy included the consumption of i) cold equipment (173 kWh / year for a fridge with a gross capacity of 213 L and 193 kWh / year for a freezer cabinet with a gross capacity of 250 L, on a daily over the year), ii) pelletizer (consumption of 4.0 kW, considering that it works 60 days/year), iii) to the lighting of the facility itself (consumption of 10 W, considering that it is switched on during half of the year) and iv) other equipment, such as a desktop PC (consumption of 200 W, considering that it is connected all days of the year). For the overall calculations, the present energy cost at Castelo Branco city in Portugal, 0.153 €/kWh, was considered.

Costs with the chemical analysis of the essential and volatile oils from hydrolates, were calculated based on current pricing table at Centro de Biotecnologia Vegetal, CESAM Lisboa, FCUL, 67.50 € + IVA / sample and 87.50€ + IVA / sample, respectively.

The postage prices were calculated based on DHL carrier pricing list.

For design and printing, the value was established based on the ones practiced by several companies in the market.

It was also stipulated that the gross salary of a graduate employee would be 950.00 €/month. On this amount will also be taken into account the costs for the social security (*Segurança Social*) (11 %), accident at work insurance (250 €/year), Christmas and holiday subsidy and the meal allowance (4.77 €/working day).

CGAMC, ESS and PC costs were considered for the calculation of the OCF.

3.3.3. Project viability

As a result of the valorisation of forest biomass studied during this project, it is expected that different added value products will be obtained and can be commercialised directly by Silvapor. These products are the essential oils and hydrolates of *C. lusitanica* and *C. ladanifer*, soaps with EO extracted from both species and also pellets produced with extracted solid residues of *C. lusitanica* and *C. ladanifer*. The estimated profits with the commercialization of these products for one year will be 17540.00 € (Table 4.8). All sales estimations were stipulated separately for *C. lusitanica* and *C. ladanifer*. Based on prior knowledge of seasonality to obtain the best EO yield, the total expected profits was made considering the amount of EO and H

produced for one month in year (considering 16 days/month to be distilled) for each species.

Table 4.8. Expected yearly profits from sales. Sold products result from the valorisation of *Cupressus lusitanica* and *Cistus ladanifer* biomass.

	Expected sales and profits / year		
Products	**Base price (€) / amount**	**Expected quantity sold**	**Expected profit / year (€)**
C. lusitanica			
Essential oil	9.00 / 10 mL	6480.0 mL	5832.00
Hydrolate	3.00 / 125 mL	144000.0 mL	3456.00
Soap with essential oil	2.50 / unit	100.0 unit	250.00
Pellets	4.00 / 15 kg	240 unit	960.00
C. ladanifer			
Essential oil	22.00 / 10 mL	1080.0 mL	2376.00
Hydrolate	3.00 / 125 mL	144000.0 mL	3456.00
Soap with essential oil	2.50 / unit	100.0 unit	250.00
Pellets	4.00 / 15 kg	240 unit	960.00
Total expected profits (TEP)			**17540.00**

In the case of *C. lusitanica*, only one Portuguese company sells this EO, at 7.5 € / 10 mL. Considering cypress species, the EO of *C. sempervirens* is the most commercialized, with prices ranging from 2.5 and 10 € / 10 mL. For *C. ladanifer* EO, the national market prices range between 20.9 and 23.0 € / 10 mL. For both species EOs, a current average value was considered in the calculations.

Considering the trials carried out in the present study and the quantities of *C. lusitanica* and *C. ladanifer* EO obtained, it was estimated that the distillery has the capacity to perform three distillations/day, using 100 Kg of biomass/assay. For each distillation, the average amount of EO of *C. lusitanica* and *C. ladanifer* obtained should be about 190 mL and 30 mL, respectively, that is, an average yield of 0.19 % on a dry weight and 0.03 % on a fresh weight, respectively. In case of Hs, the average amount obtained per distillation should be about 20 L. Taking into account the market and demand, the present analysis was carried out considering that 75 % of EOs and 15 % of Hs produced for one month would be sold in the national and international markets every year.

It was also considered that sales of solid handmade soaps would be around 100 units/year. For pellets production, it was considered that 1200 kg of ESRs may be produced per week and the pelletizer average production would be about 80 bags of 15 kg pellets/week, or 320 bags/month. In the national market, it would be expected that 75 % of the production would be sold/year, equivalent to 240 bags of 15 kg for each species. Total expected profits were considered for the calculation of the OCF.

Considering all the parameters mentioned above, it was possible to determined the OCF and

ICF following the formulas (2) and (7), respectively, and consequently NPV following the formula (1), considering a 6-year time (*t*) and a discount rate of 10.7 % (*r*). A statement table was built with all this information (Table 4.9).

Table 4.9. Valorisation project estimations for the underutilized forest biomass. It accounts for a period of 6 years and was calculated based on the NPV.

Income statement (€)						
	2020	**2021**	**2022**	**2023**	**2024**	**2025**
Total expected profits (TEP)	17 540.00	19 065.98	20 724.72	22 527.77	24 487.69	26 618.12
CGAMC	300.00	326.10	354.47	385.31	418.83	455.27
Gross result (GR)	17 240.00	18 739.88	20 370.25	22 142.46	24 068.86	26 162.85
ESS	2 082.00	2 263.13	2 460.03	2 674.05	2 906.69	3 159.57
Personnel costs (PC)	13 550.00	13 550.00	13 550.00	13 550.00	13 550.00	13 550.00
Depreciation and amortization (D.A)	2 575.00	2 575.00	2 575.00	2 575.00	2 575.00	2 575.00
Operational result (OR)	-967.00	351.75	1 785.22	3 343.41	5 037.16	6 878.27
Financial charges (FC)	4 448.91	4 329.72	4 204.65	4 073.41	3 935.70	3 791.20
Result before tax (RBT)	-5 415.91	-3 977.97	-2 419.42	-730.00	1 101.46	3 087.07
Tax (T)	-1 137.34	-835.37	-508.08	-153.30	231.31	648.28
Result after tax (RAT)	-4 278.57	-3 142.60	-1 911.34	-576.70	870.16	2 438.78
Operating cash flow (OCF)	2 745.34	3 762.12	4 868.30	6 071.71	7 380.86	8 804.99
Residual value (RV)						5 150.00
Investing cash flow (ICF)	-20 600.00	0.00	0.00	0.00	0.00	0.00
Net cash flow (NCF)	-17 854.66	3 762.12	4 868.30	6 071.71	7 380.86	8 804.99
Updated net cash flow	-17 854.66	3 399.71	3 975.54	4 480.63	4 922.03	5 306.10
Accumulated cash flow	-17 854.66	-14 454.95	-10 479.41	-5 998.78	-1 076.75	4 229.35
Net present value (NPV)						**4 229.35 €**

CGAMC: Costs of good acquired and materials consumed; ESS: External supplies and services.

Some considerations were taken into account for the calculation of the parameters, namely i) yearly growth rate of 8.7 %, because is expected that the EOs market have an annual growth in this value order until 2026 (AMR, 2020), ii) tax (T) of 21 %, according to the value tabulated at the date of the present study (ATA, 2020) and iii) discount rate of 10.7 %. According to the income statement, it was estimated that the value of NPV would be 4229.35 €. The results of the simplified economic viability study demonstrated that this project is considered viable if all the conditions mentioned above are met. This study showed that, in addition to the costs with i) investment, ii) CGAMC, iii) ESS and iv) PC, if the company manages to sell in a year v) 75 % of the EOs and 15 % of Hs produced in one month/year for each species, vi) 100 units of soaps with EO of both species and vii) 75 % of the pellets produced from *C. lusitanica* e *C. ladanifer* ESRs, after 6 years the project will generate a profit in the order of 4229.35 € considering only two months of production/year. This means that, despite the anual profits (NCF), it is expected that it is only after the end of the 6 years that all investment is settle, being effective gains in the order of 4229.35 €.

4. Conclusions

From the four different fractions obtained out of the steam-distillation process, only the essential oils and the extracted solid residues were used in the incorporation and production of other value-added products, namely in the incorporation of solid soaps and in the production of pellets. Both value-added products may be a viable alternative for the company.

The EOs and Hs of *C. lusitanica* and *C. ladanifer* are the products of greatest commercial interest for Silvapor. This may be promoted either for a local market or for a wider industrial market and may also be sold as such or incorporated into other products, as soaps. In case of solid soaps more studies have to be performed in order to create a validated production method which grants that all normative specifications are met while the commercialization is carried out with due safety and quality.

Also, the ESRs used in the production of pellets have shown to be a viable alternative to these wastes that, as a rule and unlike the EO, have no direct valorisation. The preliminary results obtained in the present study, showed that pellets produced from *C. lusitanica* and *C. ladanifer* ESRs, showed good characteristics, according to the parameters analyzed, with the exception of the ash content, considering the wood and non-woody pellets standards. As for soaps, more testing will have to be done, so that all parameters are in conformity with pellet standards. Certification of this product could also be a possibility, not only because it increases consumer confidence, but also strengthens the pellet market in Europe, in line with international requirements recognised by the whole industry.

According to the simplified economic viability study, this project will cover the initial investment, being viable for the company if all the conditions presented are executed, bringing a profit of 4229.35 € after 6 years, considering only two months of production *per* year. This process of forest biomass valorisation to obtain new value-added products, proved to be a viable alternative to complement other activities developed by Silvapor. In addition to being a process with environmental and economic benefits, it also contributes to the circular bioeconomy.

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Chapter V

Conclusions and Final considerations

1. Conclusions

The large amounts of biomass that are wasted worldwhile, in particular industrial and agroindustrial residues, constitute a dramatic environmental problem. Forest biomass is an example of such residues since large quantities are available and left to abandonment on the ground while they feed potential forest fires.

In the present thesis, different possibilities for the valorisation of underutilized endogenous forest biomass were studied, within a local small-scale biorefinery concept. *Cupressus lusitanica* Mill and *Cistus ladanifer* L., resulting from Silvapor - Ambiente e Inovação, Lda., a company dedicated to cleaning and maintenance of the forest, were the tested species. Applying the steam-distillation methodology to each of these biomasses, four distinct fractions were obtained: essential oils, hydrolates, distiller condensation waters and extracted solid residues. Each fraction was evaluated separately in terms of chemical composition and biological activities. The valorisation of some fractions was tested, namely by producing soaps and pellets using the essential oil and the extracted solid residues fractions, respectively. The profitability of this project for the Silvapor company was also assessed through a simplified economic analysis. Considering all the results obtained, the main conclusions and final considerations are presented below.

1.1. Essential oils

For both *C. lusitanica* and *C. ladanifer*, monoterpenes were dominant in all EOs, namely the monoterpene hydrocarbons fraction, being this independent of the collection date and type of distillation. The main components (≥ 5 %) were the same for *C. lusitanica* EOs, independently of both the time of plant collection and the distillation procedure. For *C. ladanifer*, some differences in the EOs composition were detected, varying according to the moment of collection. While the major component (α-pinene) was the same in all *C. ladanifer* EOs, the relative amount of the second main component (camphene) was lower for the warmer months. Thus, *C. ladanifer* EO showed more chemical variability than *C. lusitanica* EO, which can be important in terms of the stability of production.

From the different biological activities tested for the steam-distillation EOs of both species, the antioxidant activity assays showed the best results. Xanthine oxidase method showed a strong inhibition capacity, i.e. > 90 %, for both *C. lusitanica* and *C. ladanifer* EOs. Moreover, the antioxidant activity was approximately the same in EOs from biomasses collected in different periods. This antioxidant capacity may indicate a potential use in food preservation, protecting against oxidative processes, and thus prolonging its nutritional quality attributes. Opposite to the antioxidant activity, the EOs from both species showed weak antimicrobial activity for the tested microorganisms, i.e. *E. coli*, *S. aureus* and *C. albicans*, with negligible differences between different periods of collection.

In addition to their valorisation *per se*, the EOs may be used in the formulation of other products, namely in cosmetics. In this way, two types of soaps were produced throughout this study: glycerin-based solid and olive oil-based solid soaps, both containing either *C. lusitanica* or *C. ladanifer* EOs. Hence, it was verified that, in a first approach, the conception of the mentioned soaps was possible and well succeeded. Like EOs, also soaps are value added products resulting from the valorisation of the underutilized forest biomass. In the medium/long term, and under specific conditions, this approach may be implemented at the Silvapor company.

1.2. Hydrolates

This study reported, for the first time, the chemical composition of the hydrolate (H) volatiles from *C. lusitanica* and *C. ladanifer* and their corresponding biological properties.

The chemical analysis of the Hs volatiles, obtained by both distillation methods (steam-distillation and hydrodistillation), showed that they were dominated by the oxygen-containing monoterpenes. Although the H volatiles obtained by steam-distillation of *C. lusitanica* were qualitative and quantitatively similar in the two collection moments, they differed from those obtained by hydrodistillation of the same species. For *C. ladanifer*, the H volatiles chemical composition was qualitatively similar, but with some quantitative differences according to different sample collection moments and distillation procedures.

The anti-inflammatory activity assays showed the best results, among the different biological activities tested for the Hs of both species obtained by steam-distillation. In each case no major differences were found between samples harvested in different periods. As mentioned above, Hs showed to be richer in oxygen-containing monoterpenes when compared to the corresponding EOs, which might be the reason for their anti-inflammatory characteristics. The anti-inflammatory capacity observed in Hs may thus indicate a potential use in health area.

For both species, the Hs showed an antioxidant activity lower than the corresponding EOs, being this independent of the method used for antioxidant evaluation. Again, there was no difference in the antioxidant activity observed in the samples harvested in different periods. Xanthine oxidase and chelating metal ions were the methods where higher antioxidant activity was observed.

Although tested, *C. lusitanica* and *C. ladanifer* Hs showed no antimicrobial activity against the microorganisms under study and for the assessed concentration.

In addition to their valorisation *per se*, as aromatizing agents for home or household products, Hs can be used in the co-formulations of other products, such as cosmetics or health-based products.

1.3. Distiller condensation waters

The chemical composition and the biological activities of the distiller condensation waters (DCWs) of both *C. lusitanica* and *C. ladanifer* were reported for the first time in the present study.

This fraction was characterized in terms of its phenolic content (total phenols, tannins and flavonoids) and their corresponding biological properties, namely antioxidant and anti-inflammatory activities.

The *C. lusitanica* and *C. ladanifer* DCWs showed to be rich in phenolic compounds, mostly tannins. *C. ladanifer* extract presented a higher and more complex phenolic content than the *C. lusitanica* extract. Catechins were the major compounds found in the DCWs from both species. It was possible to identify 3-hydroxybenzoic acid and epicatechin in *C. lusitanica* DCW and quercetins, gallocatechins, hydroxycinnamic acid derivatives and gallic acid in *C. ladanifer* DCW.

Both *C. lusitanica* and *C. ladanifer* DCWs presented antioxidant and anti-inflammatory properties. Among the different methods used to evaluate the antioxidant activity, the inhibition of superoxide anion radical formation assay was the one that showed the best results. *C. ladanifer* DCW showed higher antioxidant activity than the ones of *C. lusitanica*.

DCWs are thus an abundant source of natural phenolic compounds that can be exploited as building blocks in different industries. Besides working as natural antioxidants for different industries, they can be used as raw materials for producing medicinal drugs or other chemicals such as polymers.

1.4. Extracted solid residues

The extracted solid residues fraction (ESRs) remaining from these biomasses after steam-distillation were also assessed as value-added by-products. The ESR of *C. lusitanica* was here characterized for the first time in terms of the chemical composition. The results showed that these residues were mainly rich in Klason lignin, followed by carbohydrates, namely hemicellulose. Although the chemical composition of *C. ladanifer* ESR has already been reported, the results from the present study showed that the Klason lignin was higher than the carbohydrate content, and well above the Klason lignin content determined in other published studies. Thus, it was possible to conclude that, in addition to the differences in the starting material (extracted by different procedures), the place and time of harvest and the age of the plant are factors that can influence the lignocellulosic composition of these biomasses, in particular of *C. ladanifer*.

The results showed that the yield of phenolic-rich extract for *C. ladanifer* was higher than the yield obtained for *C. lusitanica*, being independent of the extraction solvent. The extracts from 70 % acetone presented higher total phenolics, flavonoids and tannins content when

compared to ethanol-related ones. Thus, extraction with 70 % acetone was more efficient in removing phenolic compounds from both species. *C. ladanifer* ESR presented a higher and more complex phenolic content than *C. lusitanica* ESR. In the ESR extracts of both species, catechins were the major compounds found, while 3-hydroxybenzoic acid was common to both species.

Among the different methods used to evaluate antioxidant activity, the inhibition of superoxide anion radical formation assay was the one that showed the best results. It was also found that ESR with 70 % acetone showed higher antioxidant activity than the ethanolic extracts. Results for the anti-inflammatory activity were particulary interesting for the ethanolic extracts in both species.

In addition to DCWs, valorisation of *C. lusitanica* and *C. ladanifer* ESRs extractives as a source of natural phenolic compounds could also be exploited as building blocks in different industries such as natural antioxidants and also as raw materials for producing medicinal drugs or other chemicals such as polymers.

The present study also reports for the first time the production of pellets from the ESRs of both *C. lusitanica* and *C. ladanifer* steam-distillation. The production of pellets from both *C. lusitanica* and *C. ladanifer* ESRs could be a viable pathway towards zero waste from steam-distillation. Besides their comercialization, these pellets may be also used by the respective company to feed its own distillator, with the inherent economic advantage.

According to the intended aim and as demonstrated in present study, the full valorisation of underutilized forest biomass is possible and can be done under a local small-scalle biorefinery involving a circular bioeconomy concept (*Proceedings of 27th European Biomass Conference and Exhibition (EUBCE) IBO12.5:1899-1901* in Annex B).

This valorisation has many advantages, namely: i) environmental, as it allows for a reduction in the amount of underused wastes, often left to abandonment on the ground increasing the risk of forest fires, ii) economical, because it enables new value-added products to be obtained, contributing to the development of local small and medium-sized enterprises, iii) social, because it allows the creation of new jobs.

As mentioned, all fractions resulting from the steam-distillation process of *C. lusitanica* and *C. ladanifer* biomasses could be valued. This valorisation could be made:

i) *Per se*, through direct commercialization, namely for EOs, Hs and DCWs;
ii) By the use of the EOs and Hs in the formulation of other products, such as personal hygiene products (i.e. soaps), detergents and cosmetics;
iii) By extracting bioactive compounds, such as phenolics from DCWs and ESRs, with high interest to various industries;
iv) Through the fractionation of the lenhocelulosic material to obtain lignin for different industrial applications;

v) Through direct transformation of ESRs into value-added products (i.e. pellets).

This study also showed, through a simplified economic analysis, that valorisation of the four fractions derived from steam-distillation of forest biomass to obtain new value-added products, may be a viable alternative to complement other activities developed by Silvapor, bringing a estimated profit of 4229.35 € after 6 years.

2. Future work

This study reported for the first time the possibility of full recovery of underused forest biomass by a regional company, within the framework of a local small-scale biorefinery and within the concept of circular bioeconomy. To complement the work developed, it is suggested the following future work:

- In addition to the different beneficial properties observed in all fractions under study, including anti-inflammatory and antioxidante properties, an assessment of the possible toxicity of these same fractions is suggested;

- Being Silvapor a company linked to agriculture, and given the previous knowledge from literature on the potential of componds such as phenolics and terpenoids in this field, it is suggested to carry out tests of both insecticide and herbicide activities to the various fractions;

- Implementation and validation of soap production assays at Silvapor and absence of toxicity tests to the final product;

- It is suggested to test other types of formulations, namely incorporation of other biomasses, in order to optimize the pellets prodution, as well as implementation and validation of pellets production assays at Silvapor.

Annex A

Additional information supporting

Chapter I

Table A.1. Percentage composition of the 282 hydrolates and the corresponding essential oils from different family/plant species, with reference to the country of origin, plant part used, isolation and analysis procedure.

Family / Plant species	Country of Origin	Plant Part	EO IP	EO AP	EO Main components (≥ 5%)*	HV IP	HV AP	Main components HV (≥ 5%)*	Reference
Amaranthaceae									
Chenopodium ambrosioides (= *Dysphania ambrosioides*) ♀	Brazil	L	SD**	GC, GC-MS	Ascaridole 50, *p*--cymene 42	LLE Dcm	GC, GC-MS	*p*-Cymene and other unidentified main compounds	Degenhardt *et al*., 2016
						LLE ethyl acetate	GC, GC-MS	*p*-Cymene and other unidentified main compounds	Degenhardt *et al*., 2016
Amaryllidaceae									
Allium ampeloprasum	Iran	L	dnp	dnp	dnp	LLE Peth	GC-MS	Thymol 57, carvacrol 26	Hamedi *et al*., 2017a
Allium sativum	Iran	Bu	dnp	dnp	dnp	LLE Peth	GC-MS	Thymol 32, carvacrol 24, dill apiole 6, *cis*-dihydro carvone 5, menthol 5	Hamedi *et al*., 2017c
Apiaceae / Umbelliferae									
Anethum graveolens	Iran	L	dnp	dnp	dnp	LLE Peth	GC-MS	Dill ether 41, thymol 19, carvacrol 12, carvone 10, dill apiole 6	Hamedi *et al*., 2017a
Anthriscus sylvestris	Canada	Rt	HDF	GC, GC-MS	β-Phellandrene 24-65, myrcene 9-48, limonene 2-6, *trans*-sabinyl acetate	LLE Dcm	GC, GC-MS	§ Elemicin 0-6, tiglic acid 1-3, 3--methyl-2(*5H*)-furanone 1-2, trans-sabinyl acetate 1-2, carvacrol 1-2, terpinen-4-ol 0-2, cryptone, 1-1	St-Gelais *et al*., 2015
Carum carvi	France	Fr	HD	GC-MS	Carvone 60-80, limonene 20-40	LLE Cy	GC-MS	Carvone 70-98, limonene 0-12	Rivera *et al*., 2010
Cuminum cyminum	Iran	S	SD	dnp	dnp	LLE Peth	GC-MS	*trans*-Caryophyllene 58, 3,4-dimethoxytoluene 19, caryophyllene oxide 12, α--humulene 5	Hamedi *et al*., 2017b
Daucus carota	Algeria	AP	SD**	GC, GC-MS	Alismol 15, α-humulene 10, β--ionone 8, *cis*-β-caryophyllene 6	LLE DE	GC, GC-MS	Caryophyllene oxide 10, *p*--cymen-8-ol 9, α-bisabolol 8, γ--decalactone 7, γ-decalactone 7, humulene epoxide II 7, geranyl acetone 6, geranyl linalool 6, octanal 6	Zatla *et al*., 2017
		Rt	SD**	GC, GC-MS	Geranyl linalool 50, pentacosane 7	LLE DE	GC, GC-MS	Myristicine 18, *cis*-methyl-*iso*-eugenol 17, methyl eugenol 12, eugenol 8, γ-decalactone 8, octadecanoic acid 6	Zatla *et al*., 2017
Daucus muricatus	Algeria	Rt	HD	dnp	dnp	LLE DE	GC, GC-MS	Terpinen-4-ol 16, isochavicol 8, copaborneol 8, isochavicol isobutyrate 7, isochavicol 2-methyl butyrate 6, myristicine 5	Djabou *et al*., 2014
		AP	HD	dnp	dnp	LLE DE	GC, GC-MS	Thymol 12, myristicine 8, isochavicol 7, copaborneol 7, isochavicol 2-methyl butyrate 6	Djabou *et al*., 2014
		St	HD	dnp	dnp	LLE DE	GC, GC-MS	Terpinen-4-ol 10, α-terpineol 8, myristicine 8, *trans*-pinocarveol 6, borneol 5	Djabou *et al*., 2014
		L	HD	dnp	dnp	LLE DE	GC, GC-MS	*trans*-Pinocarveol 8, cryptone 8, undecane-2-one 8, isophytol 8, hexadecanoic acid 8, *cis*--verbenol 7, methyl heptadecanoate 6, thymol 5, isochavico 2-methyl butyrate 5	Djabou *et al*., 2014
		F	HD	dnp	dnp	LLE DE	GC, GC-MS	Thymol 25, copaborneol 16, isochavicol 13, myristicine 9, isochavicol 2-methyl butyrate 9, isocalamendiol 9, isochavicol isobutyrate 5	Djabou *et al*., 2014
Foeniculum vulgare	Iran	S	dnp	dnp	dnp	LLE Peth	GC-MS	Thymol 42, carvone 15, *cis*--anethole 12, fenchone 13	Hamedi *et al*., 2017a
Petroselinum crispum	Iran	L	dnp	dnp	dnp	LLE Peth	GC-MS	Thymol 57	Hamedi *et al*., 2017a

Family / Plant species	Country of Origin	Plant Part	EO IP	EO AP	EO Main components (≥ 5%)*	HV IP	HV AP	Main components HV (≥ 5%)*	Reference
		I	HD	dnp	dnp	LLE Peth	GC-MS	Thymol 57, myristicin 34	Hamedi *et al*., 2017c
Arecaceae / Palmae									
Areca catechu	China	F	HD	dnp	dnp	⁑ Fibers	GC-MS	1-Heptanol 14, benzyl alcohol 14, ethyl-2-(5-methyl-5-vinyltetradyfrofuran-2-yl)propan-2-yl carbonate 13, acetic acid phenylmethyl ester 9, 3,7--dimethyl-1,6-octadien-3-ol 8, 6-ethernyltetrahydro-2,2,6-trimethyl-2H-pyran-3-ol 8, α--methyl-α-[4-methyl-3-pentenyl]oxiranemethanol 5	Shen *et al*., 2017
		FAx	HD	dnp	dnp	⁑ Fibers	GC-MS	Benzyl alcohol 21, ethyl-2-(5-methyl-5-vinyltetradyfrofuran-2-yl)propan-2-yl carbonate 18, 3,7--dimethyl-1,6-octadien-3-ol 11, α--methyl-α-[4-methyl-3-pentenyl]oxiranemethanol 6, 6--ethernyltetrahydro-2,2,6-trimethyl-2H-pyran-3-ol 6, 2,4-bis(1,1-dimethylethyl)-phenol 5, 2-propyl-1-pentanol 5	Shen *et al*., 2017
		Rt	HD	dnp	dnp	⁑ Fibers	GC-MS	3,7-Dimethyl-1,6-octadien-3-ol 20, 5-methyl-2-(1-methylethenyl)-4-hexen-1-ol 13, ethyl-2-(5-methyl-5-vinyltetradyfrofuran-2-yl)propan-2-yl carbonate 6, acetic acid phenylmethyl ester 5	Shen *et al*., 2017
Cocos nucifera	China	F	HD	dnp	dnp	⁑ Fibers	GC-MS	3,7-Dimethyl-1,6-octadien-3-ol 13, ethyl-2-(5-methyl-5-vinyltetradyfrofuran-2-yl)propan-2-yl carbonate 12, benzyl alcohol 10, (*R*)-5-methyl-2-(1-methylethenyl)4-hexen-1-ol 7, nonanal 7, 1-nonanol 5, 6-ethernyltetrahydro-2,2,6-trimethyl-2H-pyran-3-ol 5	Shen *et al*., 2017
		FAx	HD	dnp	dnp	⁑ Fibers	GC-MS	Benzyl alcohol 21, ethyl-2-(5-methyl-5-vinyltetradyfrofuran-2-yl)propan-2-yl carbonate 17, 3,7-dimethyl-1,6-octadien-3-ol 12, 6-ethernyltetrahydro-2,2,6-trimethyl-2H-pyran-3-ol 9, 2,4-bis(1,1-dimethylethyl)-phenol 9, α-methyl-α-[4-methyl-3-pentenyl]oxiranemethanol 6	Shen *et al*., 2017
		Rt	HD	dnp	dnp	⁑ Fibers	GC-MS	1,1-Oxybis-octane 17, 3,7-Dimethyl-1,6-octadien-3-ol 15, ethyl-2-(5-methyl-5-vinyltetradyfrofuran-2-yl)propan-2-yl carbonate 10, benzyl alcohol 8, 5-methyl-2-(1-methylethenyl)-4-hexen-1-ol 8, acetic acid phenylmethyl ester 5	Shen *et al*., 2017
Phoenix dactylifera	Iran	Sp	HD	¥ GC-MS	¥ Carvacrol 37, linalool 24, thymol 10, spathulenol 8	LLE Peth	GC-MS	Menthol 44, methyl hexadecanoate 10, carvone 8, *neo*-menthol 8, *cis*--dihydrocarvone 7, carvacrol 6, pulegone 6, thymol 6	Hamedi *et al*., 2017b; ¥ Hamedi *et al*., 2013
Aristolochiaceae									
Aristolochia trilobata	Brazil	St	SD**	GC, GC-MS	6-Methyl-5-hepten-2-yl acetate 23, limonene 15, linalool 9, *p*-cymene 8	LLE DE	GC, GC-MS	Linalool 30, 6-methyl-5-hepten-2-ol 20, 6-methyl-5-hepten-2-yl acetate 9	Santos *et al*., 2014
Asarum canadense	Canada	Rt	dnp	GC, GC-MS	Methyl eugenol 39, linalool 19, α-terpineol 6	LLE Chl	GC, GC-MS	✕ Linalool 41-184, methyl eugenol 30-180, α-terpineol 108-123, 2,6-dimethyl-7-octene-2,6-diol 2-39,	Garneau *et al*., 2014

Family / Plant species	Country of Origin	Plant Part	EO IP	EO AP	EO Main components (≥ 5%)*	HV IP	HV AP	Main components HV (≥ 5%)*	Reference
								nerol 4-11, geraniol 7-34, elemicin 6-10, terpin-1-en-4-ol 5-8, *trans*-isoelemicine 3-6	
Asteraceae / Compositae									
Achillea millefolium	Iran	AP	HD	dnp	dnp	LLE Peth	GC-MS	Camphor 42, yomogi alcohol 19, 1,8-cineole 8, artemisia alcohol 7	Hamedi *et al*., 2017a
			dnp	dnp	dnp	LLE Peth	GC-MS	Camphor 42, yamogi alcohol 21, 1,8-cineole 8, artemisia alcohol 8	Hamedi *et al*., 2017b
Anthemis nobilis (= *Chamaemelum nobile*) ♀	Belgium	dnp	dnp	GC-MS	Isobutyl angelate 35, isoamyl angelate, methallyl angelate 8, isobutyl isobutylate 5	LLE EAc	GC-MS	Pinocarveol 25, isobutyl hydroxyangelate 11, hydroxyisobutyl angelate 11, pinocarvone 8, isoamylalcohol 8, isobutyl angelate 5	Inouye *et al*., 2008
Anthemis tinctoria (= *Cota tinctoria*) ♀	Japan	AP	SD	GC-MS	Cubenol 11, germacrene D 8, guaiol 8, germacrene D 4-ol 7, β--caryophyllene 7, isoledene 6, cadina--1,4-diene 6, α-cadinol 5	LLE EAc	GC-MS	1,8-Cineole 34, *p*-mentha-1-en-8-ol 21, terpinen-4-ol 8, cubenol 6	Inouye *et al*., 2008
Artemisia princeps	Japan	AP	SD	GC-MS	β-Caryophyllene 26, germacrene D 20, α--humulene 11	LLE EAc	GC-MS	1,8-Cineole 27, camphor 16, borneol 16, *cis*-verbenol 8, yomogi alcohol 5	Inouye *et al*., 2008
Artemisia sieberi	Iran	AP	dnp	dnp	dnp	LLE Peth	GC-MS	Camphor 24, 1,8-cineole 18, terpinene-4-ol 8, *trans*-thujone 7	Hamedi *et al*., 2017b
			HD	¥ GC, GC-MS	¥ Artemisia ketone 48, 1,8-cineole 20	LLE Peth	GC-MS	Camphor 23, 1,8-cineole 17, terpinen-4-ol 6, *trans*-thujone 6	Hamedi *et al*., 2017c; ¥ Behmanesh *et al*., 2007
Balsamita suaveolens (= Tanacetum balsamita) ♀	Italy	AP	HD	GC-MS	Carvone 44, α-thujone 16	LLE *n*-Hex	GC-MS	Carvone 75, α-thujone 6	Gallori *et al*., 2001
						SPME	GC-MS	Carvone 41, α-thijone 16, 1,8-cineole 15, α-terpinene 6	Gallori *et al*., 2001
Calendula arvensis	Algeria	AP	HD	GC, GC-MS	Zingiberenol 1 9-30, *cis*,*trans*-farnesol 4--23, zingiberenol 2 5-20, eremoligenol 4--13, *cis*-phytol 0-12, τ-cadinol 0-9, γ--curcumene 0-8, zingeberene 0-8, β--curcumene 2-3, τ--muurolol 0-8,	LLE DE	GC, GC-MS	Zingiberenol 1 33, *cis*,*trans*--farnesol 24, zingiberenol 2 21, eremoligenol 11	Belabbes *et al*., 2017
Echinacea purpurea	Japan	AP	SD	GC-MS	Germacrene D 54, β--myrcene 11, β--pinene 6, limonene 5	LLE EAc	GC-MS	Terpinen-4-ol 15, *cis*-verbenol 8, phenylacetaldehyde 7, 2-pinen-2-ol 5	Inouye *et al*., 2008
Eupatorium japonicum	Japan	F_L	SD	GC-MS	Thymylmethylether 32, dimethoxydurene 32, α-thujene 6	LLE EAc	GC-MS	Thymylmethylether 13, coumarin 10, nerol 8, angelic acid 8, 2--hexanal 8, α-terpineol 6, α--methylbutyric acid 5	Inouye *et al*., 2008
Eupatorium laciniatum	Japan	F_L	SD	GC-MS	Dimethoxydurene 23, thymyl methyl ether 20, α-thujene 12	LLE EAc	GC-MS	Coumarin 33, 4-propylbenzoic acid 23, angelic acid 10, *p*--acetylanisole 9, thymol 5	Inouye *et al*., 2008
Helichrysum italicum	France	FH	HD	GC, GC-MS	Neryl acetate 34, 2,4,6,9-tetramethyldec-8-en-3,5-dione 10, limonene 6	LLE DE	GC, GC-MS	2,6-Dimethyloctan-3,5-dione 17, α-terpineol 16, 2,4-dimethylheptan-3,5-dione 10, linalool 7	Paolini *et al*., 2008
				dnp	dnp	HS-SPME	GC, GC-MS	2,6-Dimethyloctan-3,5-dione 18-33, α-terpineol 20-30, 2,4,6,9-tetramethyldec-8-en-3,5-dione 1-11, linalool 5-10, 2,4-	Paolini *et al*., 2008

Family / Plant species	Country of Origin	Plant Part	EO IP	EO AP	EO Main components (≥ 5%)*	HV IP	HV AP	Main components HV (≥ 5%)*	Reference
								dimethylheptan-3,5-dione 3-10, 1,8-cineol 3-8	
				dnp	dnp	P&T-ATD	GC, GC-MS	Pentan-3-on 33, 2-methylpentan-3-one 18, 1,8-cineol 13, 4--methylhept-3-one 12	Paolini *et al*., 2008
Inula graveolens (= *Dittrichia graveolens*) ♀	France	FHe	HD	GC, GC-MS	Bornyl acetate 47, borneol 17	LLE DE	GC, GC-MS	Borneol 87	Paolini *et al*., 2008
				dnp	dnp	HS-SPME	GC, GC-MS	Borneol 61-90, bornyl acetate 1-15	Paolini *et al*., 2008
				dnp	dnp	P&T-ATD	GC, GC-MS	Borneol 58, bornyl acetate 12, 3--methylbut-2-enol 9	Paolini *et al*., 2008
Matricaria chamomilla	Iran	F	HD	dnp	dnp	LLE Peth	GC-MS	Thynol 34, α-bisabolol oxide A 19, α-bisabolone oxide A 9, *cis*--anethole 6, piperitenone 6	Hamedi *et al*., 2017a
Matricaria recutita (= *Matricaria chamomilla*) ♀	Japan	Fr	SD	GC-MS	Bisabolol oxide A 31, β-farnesene 25, bisabolene oxide 11, *cis*-en-in-bicycloether 10, chamazulene 6	LLE EAc	GC-MS	Bisabolol oxide A 75, bisabolol oxide B 6, 4-methylangelicin 6	Inouye *et al*., 2008
Santolina chamaecyparissus	Japan	AP	SD	GC-MS	Heptadien-4-one 16, vulgarone A 11, vulgarone B 9, spathulenol 7, β--thujene 5	LLE EAc	GC-MS	Artemisia ketone 25, camphor 17, yomogi alcohol 9, borneol 9, carveol 6	Inouye *et al*., 2008
Solidago puberula	Canada	AP	SD	GC, GC-MS	Limonene 12, germacrene D 12, myrcene 11, α-pinene 7	LLE Chl	GC, GC-MS	Benzyl alcohol *73-74, cis*-3--hexen-1-ol 6-9	Garneau *et al*., 2014
Tanacetum vulgare	Japan	F_L	SD	GC-MS	Chrysanthenyl acetate 55, epoxy-α--terpinyl acetate 30	LLE EAc	GC-MS	Chrysanthenyl acetate 25, epoxy-α-terpinyl acetate 18, piperitone 12, phellandral 8, linalool oxide pyranoside 5	Inouye *et al*., 2008
Berberidaceae									
Berberis vulgaris	Iran	Fr	dnp	dnp	dnp	LLE Peth	GC-MS	α-Bisabolol oxide A 40, thymol 24, bisabolone oxide 17, methyl hexadecanoate 8, carvacrol 7	Hamedi *et al*., 2017c
Boraginaceae									
Cynoglossum cheirifolium	Algeria	AP	HD	GC, GC-MS	*cis*-Phytol 29, *n*--tetradecane 10, hexadecanoic acid 9, *n*-pentadecane 8, geranyl acetone 7, α--copaene 5	LLE DE	GC, GC-MS	2-Pentyl-furan 46, carvone 24, hexadeanoic acid 15	Boussalah, 2020
Echium amoenum	Iran	F	SD	dnp	dnp	LLE Peth	GC-MS	Phenethyl alcohol 59, eugenol 23, citronellol 7	Hamedi *et al*., 2017b
Brassicaceae / Cruciferae									
Brassica rapa	Iran	Rt	dnp	dnp	dnp	LLE Peth	GC-MS	Thymol 49, carvacrol 22, pulegone 6, carvone 5	Hamedi *et al*., 2017c
Bromeliaceae									
Ananas comosus	Malaysia	Fr	HD	dnp	dnp	dnp	GC-MS	Acetic acid 6	Mohamad *et al*., 2019
Burseraceae									
Boswellia sp.	Iran	⚱ OGR	dnp	dnp	dnp	LLE Peth	GC-MS	Thymol 32, *trans*-verbenone 16, carvacrol 11, *trans*-pinocarveol 8, verbenone 6	Hamedi *et al*., 2017b
Caprifoliaceae									
Valeriana officinalis	Iran	AP	dnp	dnp	dnp	LLE Peth	GC-MS	Thymol 8	Hamedi *et al*., 2017a
		AP	dnp	dnp	dnp	LLE Peth	GC-MS	Thymol 10, carvacrol 7, methyl hexadecanoate 5	Hamedi *et al*., 2017b
Cistaceae									
Cistus ladanifer	Portugal	AP	HD	GC, GC-MS	α-Pinene 13, camphene 5, *trans*--pinocarveol 5	LLE *n*-Pen	GC, GC-MS	*trans*-Pinocarveol 8, verbenone 8	Tavares *et al*., 2020
			SD	GC, GC-MS	α-Pinene 28, camphene 25,	LLE *n*-Pen	GC, GC-MS	*trans*-Pinocarveol 5-13, 2,6,6--trimethyl cyclohexanone 9-	Tavares *et al*., 2020

Family / Plant species	Country of Origin	Plant Part	EO IP	EO AP	EO Main components (≥ 5%)*	HV IP	HV AP	Main components HV (≥ 5%)*	Reference
					limonene 7			12, borneol 7-8, terpinen-4-ol 2-6, 1-methyl cycloheptanol 3-5	
Cupressaceae									
Chamaecyparis obtusa	Japan	L	SD	GC-MS	Sabinene 40, *p*--menth-1--methylethenyl-2-ethenyl-8-ol 7, terpinyl acetate 7, bornyl acetate 6, limonene 6, β-pinene 5	LLE EAc	GC-MS	Terpinen-4-ol 66, cubenol 6	Inouye *et al*., 2008
Cryptomeria japonica	Japan	L	dnp	GC-MS	α-Pinene 13, thujopsene 9, kaurene 9, β-pinene 8, limonene 5	HS-SPME	GC-MS	Terpinen-4-ol 37, 3-hexen-1-ol 18, hexanol 6	Nakagawa *et al*., 2016
		Br	dnp	GC-MS	δ-Cadinene 12, thujopsene 8, α--eudesmol 7, α-pinene 6, β-cedrene 6, elemol 6	HS-SPME	GC-MS	Terpinen-4-ol 21, elemol 11, α--terpineol 10, α-eudesmol 9, γ--eudesmol 8, camphor 7	Nakagawa *et al*., 2016
		L_Br	dnp	GC-MS	α-Pinene 23, sabinene 17, thujopsene 10, limonene 6, β--myrcene 5	HS-SPME	GC-MS	Terpinen-4-ol 32, 3-hexen-1-ol 16, hexanol 9, octen-3-ol 6	Nakagawa *et al*., 2016
		St	dnp	GC-MS	β-Cedrene 12, δ--cadinene 12, thujopsene 10, elemol 7, α-eudesmol 7	HS-SPME	GC-MS	Elemol 33, α-eudesmol 21, γ--eudesmol 15	Nakagawa *et al*., 2016
Cupressus lusitanica	Portugal	AP	HD	GC, GC-MS	Camphene 24-32, δ--3-carene 15-21, sabinene 6-11, terpinen-4-ol 3-6	LLE *n*-Pen	GC, GC-MS	Terpinen-4-ol 21-31, *p*-cymen-8-ol 10-16, *cis*-3-henen-1-ol 2-13, camphor 1-11, α-terpineol 6-10, citronellol 3-6	Tavares *et al*., 2020
			SD	GC, GC-MS	α-Pinene 14-36, limonene 8-20, sabinene 13-18, δ-3--carene 8-17	LLE *n*-Pen	GC, GC-MS	Umbellulone 47-48, terpinen-4-ol 23-24, camphor 4-5	Tavares *et al*., 2020
Thuja occidentalis	dnp	dnp	HD	dnp	dnp	PET	dnp	α-Thujone 34, fenchone 26, terpinen-4-ol 12, camphor 7	Fleisher and Fleisher, 1991
Fabaceae / Leguminosae									
Trigonella foenum-graecum	Iran	L	HD	dnp	dnp	LLE Peth	GC-MS	Thymol 20, carvone 13, carvacrol 5, *cis*-dihydro carvone 5	Hamedi *et al*., 2017c
Geraniaceae									
Pelargonium x *asperum*	Japan	F_L	SD	GC-MS	β-Citronellol 28, isomenthone 17, citronellic acid 13, citronellyl tyglate 7, capric acid 6	LLE EAc	GC-MS	β-Citronellol 28, isomenthone 19, citronellic acid 18, *trans*-*p*-menthane-3,8-diol 7, linalool 5	Inouye *et al*., 2008
Pelargonium graveolens	dnp	dnp	HD	dnp	dnp	PET	dnp	Citronellol 42, geraniol 19, isomenthone 11, linalool 9	Fleisher and Fleisher, 1991
	India	dnp	SD	GC, GC-MS	Citronellol 28-28, geraniol 19-22, linalool 7-8, citronellyl formate 6-8, isomenthone 5-7, 10--*epi*-γ-eudesmol 5-6	◊ LLE n-Hex	GC, GC-MS	Geraniol 21-38, citronellol 27-33, linalool 15-20, isomenthone 5-6, sabinene 0-6	Rao *et al*., 2002
Grossulariaceae									
Ribes nigrum	Poland	Fr	SD	dnp	dnp	LLE MeCl/ SPME	GC-MS	Terpinen-4-ol 15	Dawiec-Liśniewska *et al*., 2018b
Hypericaceae									
Hypericum perforatum	Japan	AP	SD	GC-MS	Germacrene D 43, *cis*-4-hexen-1-ol 9, α--pinene 8	LLE EAc	GC-MS	Terpinen-4-ol 22, *cis*-4-hexen-1-ol 19, α-terpineol 13, α-cadinol 11, *trans*-muurolol 7	Inouye *et al*., 2008
Lamiaceae / Labiatae									

Family / Plant species	Country of Origin	Plant Part	EO IP	EO AP	EO Main components (≥ 5%)*	HV IP	HV AP	Main components HV (≥ 5%)*	Reference
Dracocephalum kotschyi	Iran	AP	HD	GC-MS	α-Pinene 12, *trans*-β--ocimene 9, limonene 7	LLE *n*-Pen	GC-MS	*trans*-Verbenol 12, terpinen-4-ol 11, verbenone 9, neral 6	Monsef-Esfahani *et al*., 2007
Glechoma hederacea	Japan	AP	SD	GC-MS	*trans*-Ocimene 15, 14, pinocamphone 13, aromadendrone 11, germacrene D 10, *cis*--ocimene 8, germacrene D-4-ol 6	LLE EAc	GC-MS	Pinocamphone 59, camphor 11, 1,8-cineole 6	Inouye *et al*., 2008
Lavandula allardii (= *Lavandula* x *heterophylla*) ♀	Australia	dnp	dnp	GC-MS	1,8-Cineole 34-39, camphor 13-17, β--phellandrene 8-9, β-pinene 5-6, α--pinene 5-5	dnp	GC-MS	Camphor 39, β-phellandrene 23, linalool 12, α-terpineol 6	Moon *et al*., 2007
Lavandula angustifolia	dnp	dnp	dnp	dnp	dnp	LLE DE	GC-MS	Linalool 69-83, 4-terpineol 6-20	Yohalem and Passey, 2011
	Japan	F	SD	GC-MS	Linalyl acetate 48, linalool 15, β-caryophyllene 6, *cis*--ocimene 6	LLE EAc	GC-MS	Linalool 39, α-terpineol 15, coumarin 7, terpinen-4-ol 7	Inouye *et al*., 2008
	Poland	F	HD	GC, GC-MS	α-Linalool 25, linalyl acetate 14, borneol 6, caryophyllene oxide 5	LLE *n*-Pen	GC, GC-MS	α-Linalool 27, borneol 9, *cis*--linalool oxide 7, *trans*-linalool oxide 5, α-terpineol 5	Śmigielski *et al*., 2013
		He	HD	dnp	dnp	LLE *n*-Pen	GC-MS	Linalool 48-53, α-terpineol 8-9, borneol 5-6, geraniol 4-5	Kunicka-Styczyńska *et al*., 2014
		F	HD	dnp	dnp	LLE *n*-Pen	GC-MS	Linalool 39-44, *trans*-linalool oxide 10, *cis*-linalool oxide 8, α--terpineol 7-8, borneol 5-7, terpinen-4-ol 5-6	Kunicka-Styczyńska *et al*., 2014
		F	HD	dnp	dnp	DLLME	GC, GC-MS	Linalool 26-45, α-terpineol 4-9, octan-3-one 0-8, borneol 4-7, δ--cadinol 1-7, terpinen-4-ol 4-6	Prusinowska and Smigielski, 2015
		F	HD	dnp	dnp	LLE *n*-Pen	GC-MS	Linalool 24-39, linalool oxide 18-25, borneol 6-14, α-terpineol 7-13, terpinen-4-ol 3-7	Prusinowska *et al*., 2016
Lavandula x intermedia	dnp	dnp	dnp	dnp	dnp	LLE DE	GC-MS	Linalool 42-50, camphor 22-24, cineole 8-22, borneol 2-6, 4-terpineol 2-5	Yohalem and Passey, 2011
	Italy	L_St	SD	GC-MS	Linalool 36, 1,8-cineole 20, α-pinene 9, linalyl acetate 8	HSE	GC-MS	1,8-Cineole 53, camphor 20, linalool 13	Garzoli *et al*., 2020
	Spain	F_L	SD	dnp	dnp	LLE Dcm	GC-MS	Linalool 15, 1-α-terpineol 15, camphor 10, 1-borneol 9, *cis*--linalool oxide 8, *trans*-linalool oxide 7, 1,8-cineole 5	Andrés *et al*., 2018
	Turkey	dnp	SD	GC-MS	Linalyl acetate 48, linalool 34	dnp	GC-MS	Linalool 56, borneol 14, camphor 13, 1,8-cineole 10, linalool oxide 6	Baydar and Kineci, 2009
Lavandula x *intermedia* "Miss Donnington"	Australia	dnp	dnp	GC-MS	Camphor 20, 1,8--cineole 12, linalool 12, linalyl acetate 9, borneol 6	dnp	GC-MS	Borneol 32, linalool 20, camphor 18, α-terpineol 10, cryptone + *p*--cymene-8-ol 8	Moon *et al*., 2007
Lavandula x *intermedia* "Seal"	Australia	dnp	dnp	GC-MS	Linalool 36, 1,8--cineole 15, β-phellandrene 8, *trans*--β-ocimene 7, linalyl acetate 6	dnp	GC-MS	α-Terpineol 24, linalool 19, terpinen-4-ol 14, cryptone + p--cymen-8-ol 7, borneol 5	Moon *et al*., 2007
Lavandula luisieri ♯	Spain	AP	HD	GC-MS	Camphor 60, 2,3,4,4--tetramethyl-5-methylidenecyclopent-2-en-1-one 9	LLE Dcm	GC-MS	Camphor 49, 2,3,4,4-tetramethyl-5-methylidenecyclopent-2-en-1-one 20, 5-hydroxymethyl-2,3,4,4-tetramethylcyclopent-2-en-1-one 10, (2,2,3,4-tetramethyl-5-oxocyclopent-3-en-1-yl)-methyl acetate 8	Julio *et al*., 2017
		F_L	SD	dnp	dnp	LLE Dcm	GC-MS	Camphor 49, 2,3,4,4-tetramethyl-	Andrés *et*

Family / Plant species	Country of Origin	Plant Part	EO IP	EO AP	EO Main components (≥ 5%)*	HV IP	HV AP	Main components HV (≥ 5%)*	Reference
								5-methylidenecyclopent-2-en-1-one 20, 5-hydroxymethyl-2,3,4,4-tetramethylcyclopent-2-en-1-one 10, (2,2,3,4-tetramethyl-5-oxocyclopent-3-en-1-yl)-methyl acetate 8	*al*., 2018
Lavandula officinalis	Morocco	AP	SD***	dnp	dnp	dnp	GC, GC-MS	Linalool 45, camphor 16, 1,8--cineole 15, α-terpineol 12, borneol 11	Aazza *et al*., 2011
Lavandula stoechas	France	L	HD	GC, GC-MS	Camphor 27, fenchone 22, 1,8--cineol 9, camphene 7	LLE DE	GC, GC-MS	Camphor 49, fenchone 23, 1,8--cineole 7	Paolini *et al*., 2008
				dnp	dnp	HS-SPME	GC, GC-MS	Camphor 46-61, fenchone 22-31, 1,8-cineole 5-9	Paolini *et al*., 2008
				dnp	dnp	P&T-ATD	GC, GC-MS	Camphor 34, 1,8-cineole 20	Paolini *et al*., 2008
Marrubium vulgare	Iran	AP	HD	dnp	dnp	LLE Peth	GC-MS	Menthol 36, menthone 16, thymol 15, *p*-xylene 6, carvacrol 5, *iso*--menthone 6	Hamedi *et al*., 2017a
Melissa officinalis	Canada	Rt	dnp	GC, GC-MS	Geranial 24, neral 18, β-caryophyllene 12, geraniol 9	LLE Chl	GC, GC-MS	✕ Geraniol 9-73, *cis*-3-hexen-1-ol 6-38, geranial 6-33, nerol 2-33, linalool 7-26, α-terpineol 3-23, *trans*-*p*-menth-2-ene-1,8-diol 6-14, 6-methyl-5-hepten-2-one 2-10, *cis*-*p*-menth-2-ene-1,8-diol 6-9, *p*-cymen-8-ol 1-8, β-phellandren-8-oll 3-7, *p*-mentha-1,5-dien-8-ol 2-5	Garneau *et al*., 2014
	Greece	L	HD	dnp	dnp	LLE DE	GC-MS	Carvacrol 35, neral 17, geranial 13	Petrakis *et al*., 2015
	Iran	L	dnp	dnp	dnp	LLE Peth	GC-MS	Thymol 47, carvacrol 30	Hamedi *et al*., 2017a
			dnp	dnp	dnp	LLE Peth	GC-MS	Thymol 45, carvacrol 32	Hamedi *et al*., 2017b
	Japan	L	SD	GC-MS	Citral 28, β--caryopyllene 12, citronellal 10	LLE EAc	GC-MS	Citral 43, *cis*-*p*-mentha-1-en-3-acetoxy-8-ol 15, *trans*-*p*-mentha-1-en-3-acetoxy-8-ol 13, geraniol 5	Inouye *et al*., 2008
Mentha arvensis	Japan	AP	SD	GC-MS	Menthol 69, menthone 10, menthyl acetate 8	LLE EAc	GC-MS	Menthol 86, menthone 5	Inouye *et al*., 2008
Mentha citrata (= *Mentha aquatica*) ♀	Japan	L	SD	GC-MS	Linalyl acetate 34, linalool 26, 1,8--cineole 10	LLE EAc	GC-MS	Linalool 44, α-terpineol 14, 1,8-cineole 12, menthol 8	Inouye *et al*., 2008
Mentha longifolia	Senegal	AP	SD**	GC, GC-MS	Pulegone 42-52, menthone 14-21, 1,8--cineole 11-13, isomenthone 9-13	dnp	GC, GC-MS	Pulegone 47-60, 1,8-cineole 8-20, isomenthone 7-11, menthone 6-9, chrysanthenone 3-6	Diop *et al*., 2016
Mentha x *piperita*	dnp	dnp	HD	dnp	dnp	PET	dnp	Menthol 37, menthone 17, 1,8-cineole 16	Fleisher and Fleisher, 1991
	Canada	AP	SD	GC, GC-MS	Menthol 52, menthone 16, menthyl acetate 5	LLE Chl	GC, GC-MS	Menthol 62-69, *cis*-3-hexen-1-ol 2-6, menthone 2-6	Garneau *et al*., 2014
	Egypt	dnp	SD	GC, GC-MS	Menthol 49, menthone 20, 1,8--cineole 5	LLE MeCl	GC, GC-MS	Menthol 22, menthone 14, 1,8--cineole 7	Edris, 2009
	Japan	AP	SD	GC-MS	Menthol 32, menthone 30, 1,8--cineole 6, isomenthon 6, neomenthol 6	LLE EAc	GC-MS	Menthol 50, menthone 23, 1,8--cineole 11, isomenthone 5	Inouye *et al*., 2008
Mentha pulegium	Greece	AP	HD	dnp	dnp	LLE DE	GC-MS	Piperitone 98	Petrakis *et al*., 2015
	Morocco	AP	SD**	dnp	dnp	LLE DE	GC, GC-MS	Carvacrol 39, piperitenone 10	Zekri *et al*., 2016
Mentha spicata	Egypt	dnp	SD	GC, GC-MS	Carvone 75	LLE MeCl	GC, GC-MS	Carvone 56, limonene 7	Edris, 2009

Family / Plant species	Country of Origin	Plant Part	EO IP	EO AP	EO Main components (≥ 5%)*	HV IP	HV AP	Main components HV (≥ 5%)*	Reference
	Japan	F_L	SD	GC-MS	Carvone 36, limonene 24, germacrene D 8	LLE EAc	GC-MS	Carvone 89	Inouye *et al*., 2008
Mentha suaveolens	Corsica	AP	HD	GC, GC-MS	Pulegone 44, *cis*-*cis*-*para*-menthenolide	LLE DE	GC, GC-MS	*cis*-*cis*-*para*-Menthenolide 67, pulegone 15	Sutour *et al*., 2008
	Morocco	L	SD**	dnp	dnp	LLE Et	GC, GC-MS	Piperitenone oxide 69	Zekri *et al*., 2016
Monarda citriodora	Italy	F_L_St	HD	GC-MS	Thymol 20, *p*-cymene 16, γ-terpinene 14, α--terpinene 9, carvacrol 9, myrcene 6	LLE *n*-Hep	GC-MS	Thymol 66, carvacrol 29	Di Vito *et al*., 2019
Monarda fistulosa	Japan	S	SD	GC-MS	Thymoquinone 33, *p*--cymene 25, carvacrylmethylether 9, 1-octen-3-ol 6, thymol 5	LLE EAc	GC-MS	Carvacrol 79, 1-octen-3-ol 10	Inouye *et al*., 2008
Ocimum basilicum	Egypt	dnp	SD	GC, GC-MS	Linalool 59, eugenol 26, 1,8-cineol 7	LLE MeCl	GC, GC-MS	Linalool 66, eugenol 7, 1,8-cineol 5	Edris, 2009
	Greece	L	HD	dnp	dnp	LLE DE	GC-MS	Linalool 67, eugenol 19, eucalyptol 7	Traka *et al*., 2018
	Iran	AP	HD	dnp	dnp	LLE Peth	GC-MS	Carvacrol 24, eugenol 23, thymol 22	Hamedi *et al*., 2017b
	Poland	AP	HD	GC-MS	Methyleugenol 45-46, eugenol 9-13, linalool 10-10, 1,8-cineole 6-8, α-bergamotene 4-6	LLE *n*-Pen	GC-MS	Methyleugenol 33-51, eugenol 6-26, linalool 10-11, 1,8-cineole 4-6	Śmigielski *et al*., 2016
Origanum compactum	Morocco	dnp	HD	GC, GC-MS	Carvacrol 20-40, rhymol 15-30, γ--terpinene + *trans*-β--ocimene 10-25, *p*-cymene 4-21	LLE DE	GC, GC-MS	Carvacrol 55-77, thymol 20-41	Jeannot *et al*., 2003
Origanum majorana	Greece	AP	HD	dnp	dnp	LLE DE	GC-MS	Carvacrol 78, terpinen-4-ol 11	Petrakis *et al*., 2015
	Morocco	AP	SD***	dnp	dnp	dnp	GC, GC-MS	Terpinen-4-ol 71, α-terpineol 9	Aazza *et al*., 2011
Origanum onites	Turkey	L_S	HD	dnp	dnp	ϫ Fibers	GC-MS	Carvacrol 30, thymol 28, borneol 6	Sagdic *et al*., 2013
		L	HD	dnp	dnp	ϫ Fibers	GC-MS	Thymol 51, carvacrol 44, *p*--cymene 6	Ozturk *et al*., 2016
Origanum vulgare	Saudi Arabia	AP	HD	GC-MS	Carvacrol 70, γ--terpinene 6	LLE EAc	GC-MS	Carvacrol 93	Khan *et al*., 2018
Perilla frutescens	Japan	AP	SD	GC-MS	Perilla aldehyde 47, limonene 19, α--bergamotene 15, β--caryophyllene	LLE EAc	GC-MS	Perilla aldehyde 78, shisool 8	Inouye *et al*., 2008
		L	SD	GC-MS	Myristicin 60, β--caryophyllene 24, dimethyl--methylpentenyl-2-norpinene 8, 1--furanyl-hexanone 6	LLE EAc	GC-MS	Myristicin 58	Inouye *et al*., 2008
Rosmarinus officinalis	Colombia	F_L	HD	GC-MS	Camphor 29, 1,8--cineole 21, α--pinene 10, camphene 9, β--pinene 7	dnp	GC-MS	Camphor 52, 1,8-cineole 38	Hay *et al*., 2018
	France	FT	HD	GC, GC-MS	Borneol 12, camphor 12, verbenone 12, bornyl acetate 12, camphene 7	LLE DE	GC, GC-MS	Verbenone 52, camphor 15	Paolini *et al*., 2008
				dnp	dnp	HS-SPME	GC, GC-MS	Verbenone 13-36, camphor 14-29, borneol 16-24, filifolone 3-11, 1,8-cineole 3-6, a-terpineol 0-6	Paolini *et al*., 2008
				dnp	dnp	P&T-ATD	GC, GC-MS	Camphor 25, 1,8-cineole 20, filifolone 17, borneol 8, linalool 6	Paolini *et al*., 2008
	Israel	L_St	SD	GC-MS	Camphor 2-50, 1,8--cineole 8-29, α--pinene 12-25,	dnp	GC-MS	Camphor 4-84, verbenone 1-66, 1,8-cineole 5-43, borneol 1-29, α--terpineol 2-10, linalool 1-5	Sadeh et al., 2019

Family / Plant species	Country of Origin	Plant Part	EO IP	EO AP	EO Main components (≥ 5%)*	HV IP	HV AP	Main components HV (≥ 5%)*	Reference
					bornyl acetate 4-16, verbenone 1-10, camphene 5-9, β--pinene 1-6, limonene 5-5, borneol 3-5				
	Japan	dnp	SD	GC-MS	α-Pinene 25, 1,8--cineole 18, camphor 9, camphene 8, β--pinene 6, sabinene 6	LLE EAc	GC-MS	Verbenone 23, camphor 22, 1,8--cineole 19, borneol 13, α-terpineol 6	Inouye *et al*., 2008
		L	HD	GC-MS	Eucalyptol 17-20, α--pinene 12-15, camphor 10-13, borneol 5-10, bornyl acetate 4-9, β-pinene 6-8, camphene 5-7, β--myrcene 5-6, limonene 4-5	LLE *n*-Hex	GC-MS	Borneol 20-27, camphor 25-24, eucalyotol 16-23, verbenone 4-8, α-terpineol 4-7	Tomi *et al*., 2016
	Morocco	AP	SD***	dnp	dnp	dnp	GC, GC-MS	1,8-Cineole 44, verbenone 26, camphor 12, borneol 7, α--terpineol 5	Aazza *et al*., 2011
	Turkey	L	HD	dnp	dnp	⊼ Fibers	GC-MS	1,8-Cineole 50, borneol 7	Ozturk *et al*., 2016
Salvia officinalis	Morocco	AP	SD***	dnp	dnp	dnp	GC, GC-MS	Camphor 51, 1,8-cineole 24, β--thujone 13	Aazza *et al*., 2011
		dnp	HD	dnp	dnp	⊼ Fibers	GC-MS	Linalool 13, δ-cadinene 10, carvacrol 9	Tornuk *et al*., 2011
		He	SD	GC, GC-MS	*cis*-Thujone 20, camphor 20, 1,8--cineole 18, *trans*--thujone 9	LLE EtOH	GC, GC-MS	Camphor 43, 1,8-cineole 24, *cis*--thujone 15, borneol 8	Baydar et al., 2013
		L	HD	dnp	dnp	⊼ Fibers	GC-MS	*o*-Cymene 10	Ozturk *et al*., 2016
Satureja hellenica	Greece	Leaves and Flowers	HD	GC-MS	p-Cymene 28, carvacrol 23, borneol 7, carvacrol methylether	LLE DE	GC-MS	Carvacrol 50, borneol 20, 4--terpineol 7	Pardavella *et al*., 2020
Satureja hortensis	Turkey	L	HD	dnp	dnp	⊼ Fibers	GC-MS	Carvacrol 25, thymol 15, *o*--cymene 11, linalool 6, borneol 5	Sagdic *et al*., 2013
								Thymol 30, α-terpineol acetate 19, *o*-cymene 14, γ-terpinene 6, 1,8-cineole 5	Ozturk *et al*., 2016
Satureja parvifolia (= *Clinopodium gilliesii*) ♀	Argentina	AP	HD	dnp	dnp	HS-SPME	GC-MS	Piperitenone oxide 69, piperitenone 7	Cabana *et al*., 2013
Sideritis canariensis	Turkey	L	HD	dnp	dnp	⊼ Fibers	GC-MS	1,8-Cineole 25, α-thujone 9, α--terpineol acetate 7, camphor 7	Ozturk *et al*., 2016
Teucrium polium	Iran	AP	HD	dnp	dnp	LLE Peth	GC-MS	Carvacrol 37, thymol 26, methyl hexadecanoate 8, eugenol 5	Hamedi *et al*., 2017c
Thymbra capitata	Greece	dnp	dnp	GC-MS	γ-Terpinene 35, carvacrol 20	HS-SPME	GC-MS	✕ Carvacrol 946, 1-octen-3-ol 10.8, terpinen-4-ol-9, borneol 7	Karampoula *et al*., 2016
Thymus capitatus	Poland	F	HD	GC, GC-MS	Carvacrol 70, *p*--cymene 12	LLE DE	GC, GC-MS	Carvacrol 95	Tabti *et al*., 2014
Thymus linearis	India	He	SD	GC, GC-MS	Thymol 44, γ--terpinene 25, *p*--cymene 13	LLE *n*-Hex	GC, GC-MS	Thymol 92	Verma *et al*., 2016
Thymus mastichina	Japan	AP	SD	GC-MS	1,8-Cineole 54, α--terpinyl acetate 7, β-caryophyllene 6, β--pinene 6	LLE EAc	GC-MS	1,8-Cineole 75	Inouye *et al*., 2008
Thymus serpyllum	India	He	SD	GC, GC-MS	Thymol 35, γ--terpinene 23, *p*--cymene 8, thymol methyl ether 7	LLE *n*-Hex	GC, GC-MS	Thymol 91	Verma *et al*., 2016
Thymus vulgaris	Colombia	AP	HD	GC-MS	Thymol 36, *p*-cymene	dnp	GC-MS	Thymol 98	Hay *et al*.,

Family / Plant species	Country of Origin	Plant Part	EO IP	EO AP	EO Main components (≥ 5%)*	HV IP	HV AP	Main components HV (≥ 5%)*	Reference
					28, γ-terpinene 12				2018
	Japan	AP	SD	GC-MS	Thymol 37, *p*-cymene 33, γ-terpinene 8	LLE EAc	GC-MS	Thymol 79, α-hydroxylthymol 10	Inouye *et al*., 2008
	Morocco	AP	SD***	dnp	dnp	dnp	GC, GC-MS	Carvacrol 94	Aazza *et al*., 2011
	Spain	F_L	SD	dnp	dnp	LLE Dcm	GC-MS	Thymol 77, linalool 7	Andrés *et al*., 2018
	Turkey	dnp	HD	dnp	dnp	⧖ Fibers	GC-MS	Carvacrol 48, thymol 18, *(-)*-spathulenol 10	Tornuk et al., 2011
	Turkey	L	HD	dnp	dnp	⧖ Fibers	GC-MS	Carvacrol 36, *o*-cymene 8, thymol 7, linalool 5, carvacrol methyl ether 5	Sagdic et al., 2013
								Thymol 57, *o*-cymene 13, γ--terpinene 6	Ozturk *et al*., 2016
Thymus zygis	Spain	F_L	SD	dnp	dnp	LLE Dcm	GC-MS	Thymol 62, 1-borneol 8, carvacrol 6	Andrés *et al*., 2018
Lauraceae									
Benzoin praecox (= *Lindera praecox*) ♀	Japan	L_Br	SD	GC-MS	Camphor 12, 1,8--cineole 11, *cis*--ocimene 9, *p*--mentha-3-isopropenul-1--ethenyl-3-en 7, limonene 6	LLE EAc	GC-MS	Camphor 42, 1,8-cineole 25, α--terpineol 10, linalool 6	Inouye *et al*., 2008
Cinnamomum cassia	Iran	St_B	dnp	dnp	dnp	LLE Peth	GC-MS	*cis*-Cinnamaldehyde 84	Hamedi *et al*., 2017b
Cinnamomum verum	Morocco	AP	SD***	dnp	dnp	dnp	GC, GC-MS	*trans*-Cinnamaldehyde 92	Aazza *et al*., 2011
Cinnamomum osmophloeum	Taiwan	L	SD	dnp	dnp	dnp	GC-MS	*trans*-Cinnamaldehyde 88, benzaldehyde 7, cinnamyl acetate 5	Ho *et al*., 2019
Laurus nobilis	Argentina	L_St	SD	GC-MS	1,8-Cineole 41-49, linalool 10-15, sabinene 8-10, α--terpinyl acetate 7-8, α-pinene 4-6, methyl eugenol 2-5	LLE *n*-Hex	GC-MS	1,8-Cineole 40-69, methyl eugenol 4-18, α-terpineol 11-13, linalool 6-11, eugenol 2-10, terpinen-4-ol 7-8	Di Leo Lira *et al*., 2009
	France	L	HD	GC, GC-MS	1,8-Cineol 32, α--terpenyl acetate 13, β-pinene 9, linalool 9	LLE DE	GC, GC-MS	1,8-Cineole 59, linalool 9, α--terpineol 7, terpinen-4-ol 6	Paolini *et al*., 2008
						HS-SPME	GC, GC-MS	1,8-Cineole 35-60, methyl eugenol 9-25, linalool 6-18, α--terpineol 6-10, eugenol 3-10, *trans*-hex-3-ol 0-9	Paolini *et al*., 2008
						P&T-ATD	GC, GC-MS	1,8-Cineole 90	Paolini *et al*., 2008
	Turkey	L	HD	dnp	dnp	⧖ Fibers	GC-MS	α-Terpineol acetate 24, 1,8--cineole 10	Ozturk *et al*., 2016
Lindera obtusiloba	Japan	L_Br	SD	GC-MS	Caryophyllene oxide 28, β-caryophyllene 12, limonene 9, *p*--cymene 6, camphene 6, isobornyl acetate 5	LLE EAc	GC-MS	Camphor 22, borneol 11, linalool 10, fenchone 8, hexanol 7, hexenol 6, citronellol 5	Inouye *et al*., 2008
Lindera umbellata	Japan	L_Br	SD	GC-MS	Linalool 42, 1,8--cineole 13, geraniol 5	LLE EAc	GC-MS	Linalool 54, 6-methyl-2-(2-oxiranyl)-5-hepten-2-ol 12, geraniol 11, 6-methyl-2-(2-oxiranyl)-5-hepten-2-ol 6	Inouye *et al*., 2008
Magnoliaceae									
Yulania denudata	China	F	HD	GC, GC-MS	β-Thujene 16, β--pinene 11, germacrene D 8, limonene 7, β--eudesmol 7, caryophyllene 7, α--eudesmol 6	LLE Et_2O	GC, GC-MS	Eucalyptol 67, α-terpineol 16	Lei *et al*., 2015

Family / Plant species	Country of Origin	Plant Part	EO IP	EO AP	EO Main components (≥ 5%)*	HV IP	HV AP	Main components HV (≥ 5%)*	Reference
Myrtaceae									
Astartea leptophylla	Australia	L_St	HD	GC-MS	α-Pinene 52, myrtenal 10	dnp	GC-MS	α-Terpineol 23, myrtenal 13, *trans*-pinocarveol 13, myrtenol 6	Lowe *et al*., 2005
Astartea sp.	Albany	L_St	HD	GC-MS	Myrtenal 26, α-pinene 18, linalool 11, myrtenol 6	dnp	GC-MS	Myrtenal 16, myrtenol 16, linalool 10, α-terpineol 8, verbenone 6	Lowe *et al*., 2005
Campomanesia viatoris (= *Campomanesia ilhoensis*) ♀	Brazil	L	HD	GC-MS	Tasmanone 71, flavesone 13, agglomerone 7	LLE DE	GC-MS	Tasmanone 75, flavesone 12, agglomerone 11	Matos *et al*., 2015
Eucalyptus camaldulensis ♀	Senegal	L	SD**	GC, GC-MS	1,8-Cineole 3-70, *p*--cymene 1-31, β--pinene 0-28, α--eudesmol *t*-21, limonene 4-13, α--pinene 2-12, α--phellandrene 0-9, epiglobulol 7-8, γ--eudesmol 0-7	LLE *n*-Hex	GC, GC-MS	1,8-Cineole 13-77, α-terpineol 4-20, *trans*-pinocarveol 1-10, α--eudesmol 0-10, piperitone 0-10, terpinene-4-ol 2-9, spathunelol 0-7	Ndiaye *et al*., 2018
Eucalyptus cinerea	Italy	L_St	SD	GC-MS	1,8-Cineole 84	HS-SPME	GC-MS	1,8-Cineole 88, α-terpineol 6	Ieri *et al*., 2019
Eucalyptus citriodora (= *Corymbia citriodora*) ♀	Egypt	dnp	SD	GC, GC-MS	Citronellal 34, citronellol 9	LLE MeCl	GC, GC-MS	Citronellal 76, citronellol 10	Edris, 2009
	Japan	L	SD	GC-MS	Citronellal 89, citronellol 8	LLE EAc	GC-MS	*cis-p*-menthane-3,8-diol 37, citronellal 16, citronellol 14, isopulegol 6	Inouye *et al*., 2008
Eucalyptus globulus	France	L_Br	HD	GC, GC-MS	1,8-Cineole 56, α--pinene 18, limonene 6	LLE DE	GC, GC-MS	1,8-Cineole 62, α-terpineol 9	Paolini *et al*., 2008
				dnp	dnp	HS-SPME	GC, GC-MS	1,8-Cineole 39-93, α-terpineol 3-17	Paolini *et al*., 2008
				dnp	dnp	P&T-ATD	GC, GC-MS	1,8-Cineole 93	Paolini *et al*., 2008
	Japan	L	SD	GC-MS	1,8-Cineole 60, α--pinene 19, limonene 8	LLE EAc	GC-MS	1,8-Cineole 72, α-terpineol 13	Inouye *et al*., 2008
Eucalyptus kruseana	Japan	L	SD	GC-MS	1,8-Cineole 67, α--terpinyl acetate 12, limonene 6	LLE EAc	GC-MS	1,8-Cineole 76, α-terpineol 6	Inouye *et al*., 2008
Eucalyptus parvula	Italy	L_St	SD	GC-MS	1,8-Cineole 87-89	HS-SPME	GC-MS	1,8-Cineole 89-90, α-terpineol 5-6	Ieri *et al*., 2019
Eucalyptus pulverulenta	Italy	L_St	SD	GC-MS	1,8-Cineole 85-88	HS-SPME	GC-MS	1,8-Cineole 89-91, α-terpineol 4-5	Ieri *et al*., 2019
Eucalyptus radiata	Japan	L	SD	GC-MS	α-Phellandrene 42, piperitone 20, β--phellandrene 6, α--thujene 5	LLE EAc	GC-MS	Piperitone 84, terpinen-4-ol 5	Inouye *et al*., 2008
Eugenia caryophyllata (= *Syzygium aromaticum*) ♀	Egypt	dnp	SD	GC, GC-MS	Eugenol 97	LLE MeCl	GC, GC-MS	Eugenol 84	Edris, 2009
	Madagascar	Bud	SD	GC-MS	Eugenol 84, acetyleugenol 10, β--caryophyllene 6	LLE EAc	GC-MS	Eugenol 97	Inouye *et al*., 2008
Leptospermum petersonii	Japan	L_Br	SD	GC-MS	Citral 57, citronellar 29	LLE EAc	GC-MS	Citral 40, *cis-p*-menthane-3,8-diol 12, *cis-p*-mentha-1-en-3-acetoxy-8-ol 9, *trans-p*-mentha-1-en-3-acetoxy-8-ol 8, *trans-p*-menthane-3,8-diol 7, geraniol 6	Inouye *et al*., 2008
Leptospermum scoparium	Japan	F_L	SD	GC-MS	dnp	LLE EAc	GC-MS	Methyl cinnamate 24, 1,8-cineole 7, α-terpineol 7, humulane-1,6-dien-3-ol 6, selina-6-en-4-ol 6, terpinen-4-ol 5	Inouye *et al*., 2008
Melaleuca alternifolia	Japan	L_Br	SD	GC-MS	Terpinen-4-ol 34, γ--terpinene 22,	LLE EAc	GC-MS	Terpinen-4-ol 75, α-terpineol 10	Inouye *et al*., 2008

Family / Plant species	Country of Origin	Plant Part	EO IP	EO AP	EO Main components (≥ 5%)*	HV IP	HV AP	Main components HV (≥ 5%)*	Reference
					α--terpinene 11				
Myrtus communis	France	Br	HD	GC, GC-MS	α-Pinene 53, 1,8--cineole 27	LLE DE	GC, GC-MS	1,8-Cineole 63, α-terpineol 9, linalool 7	Paolini *et al*., 2008
						HS-SPME	GC, GC-MS	1,8-Cineole 61-82, α-terpineol 3-13, methyl eugenol 2-7	Paolini *et al*., 2008
						P&T-ATD	GC, GC-MS	1,8-Cineole 76, isobutanal 17, isovaleraldehyde 9, 2,4-dimethyl-3-pentanone 8	Paolini *et al*., 2008
Syzygium aromaticum	Morocco	L_B	SD***	dnp	dnp	dnp	GC, GC-MS	Eugenol 81, 1,8-cineole 8, camphor 7	Aazza *et al*., 2011
Oleaceae									
Olea europaea	Iran	L	HD	dnp	dnp	LLE Peth	GC-MS	Methyl 5-vinylnicotinate 28, thymol 12, dihydroactinidiolide 7	Hamedi *et al*., 2017b
								Methyl 5-vinylnicotinate 30, dihydroactinidiolide 6, thymol ethanoate 6	Hamedi *et al*., 2017c
Osmanthus fragrans	China	F	HD	GC, GC-MS	*cis*,β-Ionone 22, *cis*--linalool oxide 18, dihydro-β-ionone 17, *trans*-linalool oxide 15	LLE DE	GC, GC-MS	*cis*-Linalool oxide 51, *trans*--linalool oxide 38	Lei *et al*., 2016
Paeoniaceae									
Paeonia x *suffruticosa*	China	F	HD	GC, GC-MS	1,3,5--Trimethoxybenzene 0-50, 1,4--dimethoxybenzene 0-15, tricosane 4-14, geraniol 0-13, nonadecane 2-13, heptadecane 2-12, pentadecane 2-11, *trans*-linalool oxide 0-9, linalool 0-9, *trans*--9-tetradecen-1-ol 0-9, pentacosane 2-7, *trans*-3-hexen-1-ol 1-6, germacrene D *t*-6	LLE MeCl	GC, GC-MS	1,3,5-Trimethoxybenzene *t*-65, 2--phenylethanol 0-64, geraniol *t*-20, *trans*-3-hexen-1-ol 1-17, 1,4--dimethoxybenzene *t*-17, linalool *t*-12, cinnamyl alcohol 1-11, 1-hexanol 1-9, geranic acid 1-8, *trans*-linalool oxide 1-7	Lei *et al*., 2018
				dnp	dnp	LLE Dcm	GC, GC-MS	2-Phenylethanol 0-77, β--citronellol 3-57, 1,3,5-trimethoxybenzene 0-50, geraniol 0-16, *trans*-3-hexen-1-ol 3-10, geranic acid 0-10, *trans*-linalool oxide 1-7, linalool 1-6, 1-hexanol 2-5	Lei *et al*., 2020
Papaveraceae									
Fumaria parviflora	Iran	AP	dnp	dnp	dnp	LLE Peth	GC-MS	Methyl hexadecanoate 38, dill apiole 20, phenol-4-ethyl-2-methoxy 8, thymol ethanoate 7, dihydroactinidiolide 7, methyl octadecanoate 6	Hamedi *et al*., 2017c
Pinaceae									
Abies alba	Poland	S	HD	GC, GC-MS	Limonene 83, α--pinene 6	LLE DE	GC, GC-MS	Selin-6-en-4-ol 52, β-himachalol 15, τ-cadinol 11, intermedeol 10	Wajs-Bonikowska *et al*., 2015
Abies balsamea	dnp	dnp	HD	dnp	dnp	PET	dnp	α-Terpineol 52, terpinen-4-ol 10, bornyl acetate 6	Fleisher and Fleisher, 1991
	Canada	Br	SD	GC, GC-MS	β-Pinene 34, δ-carene 14, α-pinene 11, bornyl acetate 10, limonene 7, β--phellandrene 7, camphene 6	LLE Chl	GC, GC-MS	α-Terpineol 42, maltol 9, borneol 6, bornyl acetate 5	Garneau *et al*., 2012
Abies koreana	Poland	S	HD	GC, GC-MS	Limonene 54, α-pinene 12, bornyl acetate 12, camphene 11	LLE DE	GC, GC-MS	Intermedeol 77, borneol 6	Wajs-Bonikowska *et al*., 2015
Picea glauca	Canada	Br	SD	GC, GC-	Camphor 20, bornyl	LLE Chl	GC, GC-	Camphor 65, borneol 11	Garneau *et*

Family / Plant species	Country of Origin	Plant Part	EO IP	EO AP	EO Main components (≥ 5%)*	HV IP	HV AP	Main components HV (≥ 5%)*	Reference
				MS	acetate 12, camphene 8, limonene 8		MS		*al*., 2012
					Camphor 20	LLE Chl	GC, GC-MS	Camphor 66-69, borneol 12-12	Garneau *et al*., 2014
Picea mariana	Canada	Br	SD	GC, GC-MS	Bornyl acetate 34, camphene 16, α-pinene 13, δ-carene 6	LLE Chl	GC, GC-MS	α-Terpineol 15, borneol 14, bornyl acetate 9, *cis*-3-Hexen-1-ol 7, terpinen-4-ol 7, camphene hydrate 5	Garneau *et al*., 2012
					α-Pinene 41, β-pinene 34	LLE *n*-Hex	GC, GC-MS	α-Terpineol 29, *trans*-pinocarveol 5, terpinen-4-ol 5	Francezon and Stevanovic, 2017
					α-Pinene 41, β-pinene 26	LLE *n*-Hex	GC, GC-MS	α-Terpineol 34, terpinen-4-ol 6, 2--methoxy-4-vinylphenol 6, verbenone 5	Francezon and Stevanovic, 2017
Platanaceae									
Platanus orientalis	Iran	L	dnp	dnp	dnp	LLE Peth	GC-MS	Carvone 24, *p*-xylene 12, dihydrocarveol 7, dill apiole 7, thymol 7, pulegone 6	Hamedi *et al*., 2017b
								Carvone 23, *p*-xylene 13, dill apiole 8, dihydro carveol 6, thymol 6, pulegone 5	Hamedi *et al*., 2017c
Poaceae / Gramineae									
Cymbopogon citratus	Egypt	dnp	SD	GC, GC-MS	Geranial 37, neral 35, linalool 9	LLE MeCl	GC, GC-MS	Geranial 44, neral 32	Edris, 2009
	Japan	L	SD	GC-MS	Citral 73, limonene 17	LLE EAc	GC-MS	Citral 43, *cis*-*p*-mentha-1-en-3-acetoxy-8-ol 17, *trans*-*p*-mentha-1-en-3-acetoxy-8-ol 14, geraniol 10	Inouye *et al*., 2008
Cymbopogon martini	Japan	AP	SD	GC-MS	Geraniol 66, geranyl acetate 25	LLE EAc	GC-MS	Geraniol 79, linalool 15	Inouye *et al*., 2008
Ranunculaceae									
Nigella sativa	Turkey	dnp	HD	dnp	dnp	ϫ Fibers	GC-MS	Cuminaldehyde 17, carvacrol 11, *p*-cymene 9	Tornuk *et al*., 2011
Ranunculus repens	Iran	F	dnp	dnp	dnp	LLE Peth	GC-MS	Carvacrol 88	Hamedi *et al*., 2017b
Rosaceae									
Cerasus serrulata ǂ	China	F	HD	GC, GC-MS	Benzaldehyde 42, tricosane 28, pentacosane 19, heptacosane 5	LLE DE	GC, GC-MS	Benzaldehyde 64, mandelonitrile 12	Lei *et al*., 2014
Cerasus subhirtella	China	F	HD	GC, GC-MS	Benzaldehyde 31, tricosane 23, pentacosane 23, heptacosane 9	LLE DE	GC, GC-MS	Benzaldehyde 68, mandelonitrile 13	Lei *et al*., 2014
Crataegus azarolus	Iran	L_Fr	dnp	dnp	dnp	LLE Peth	GC-MS	Thymol 29, *p*-xylene 20, hexadecanoic acid 7	Hamedi *et al*., 2017b
			HD	dnp	dnp	LLE Peth	GC-MS	Thymol 29, *p*-xylene 20, hexadecanoic acid 8	Hamedi *et al*., 2017c
Geum iranicum ǂ	Iran	AP	HD	GC, GC-MS	Palmitic acid 11, linoleic acid 10, tridecanal 6	LLE Peth	GC, GC-MS	Eugenol 46, linalool 7	Shahani *et al*., 2011
		Rt	HD	GC, GC-MS	Eugenol 84	LLE Peth	GC, GC-MS	Eugenol 65, myrtenol 10, chavicol 8	Shahani *et al*., 2011
Malus domestica	Poland	Fr	SD	dnp	dnp	LLE MeCl/SPME	GC-MS	5-Hexyn-1-ol 78	Dawiec-Liśniewska *et al*., 2018a
Malus sp. Ⱥ	dnp	Fr	HD	dnp	dnp	PET	dnp	*n*-Hexanol 41, *trans*-2-hexenal 19, *n*-hexanal 8, 2-methyl-1-butanol 6	Fleisher, 1990
Prunus avium	Poland	Fr	SD	dnp	dnp	LLE MeCl/SPME	GC-MS	Benzaldehyde 56	Dawiec-Liśniewska *et al*., 2018b
Prunus serotina	dnp	L	HD	dnp	dnp	PET	dnp	Benzaldehude 90	Fleisher, 1990
Rosa brunonii	India	F	HD	GC, GC-MS	Eugenol 24, geraniol 19, *n*-heneicosane 8, α-pinene 6,	LLE DE	GC, GC-MS	Eugenol 52, geraniol 13, phenyl ethyl alcohol 9	Verma *et al*., 2016

Family / Plant species	Country of Origin	Plant Part	EO IP	EO AP	EO Main components (≥ 5%)*	HV IP	HV AP	Main components HV (≥ 5%)*	Reference
					n--nonadecane 6				
Rosa canina	Iran	F	dnp	dnp	dnp	LLE Peth	GC-MS	Phenethyl alcohol 47, eugenol 29, citronellol 8	Hamedi *et al*., 2017b
Rosa centifolia	France	F	SD	dnp	dnp	LLE *n*-Hex	GC-MS	2-Phenyl ethanol 46, citronellol 25, geraniol 11	Labadie *et al*., 2015
Rosa damascena	France	F	HD	dnp	dnp	LLE *n*-Hex	GC-MS	2-Phenyl ethanol 25, citronellol 21, geraniol 21, nerol 11	Labadie *et al*., 2015
	India	F	HD	GC, GC-MS	Citronellol 18-32, geraniol 13-17, 2-phenylethanol 13-16, nerol 7-8, linalool 3-8	LLE Dcm	GC, GC-MS	2-Phnylethanol 22-82, citronellol 2-23, geraniol 1-12, nerol 0-12	Agarwal *et al*., 2005
						LLE NaCl/Dcm	GC, GC-MS	2-Phenylethanol 72, citronellol 6	Agarwal *et al*., 2005
						LLE BEN	GC, GC-MS	2-phenylethanol 67, citronellol 7	Agarwal *et al*., 2005
						HYDRO/ BEN	GC, GC-MS	2-Phenylethanol 31, geraniol 17, citronellol 16, nerol 9, linallol 8	Agarwal *et al*., 2005
	Iran	F	dnp	dnp	dnp	LLE Peth	GC-MS	Phenethyl alcohol 12-73, dibutyl phthalate 4-19, eugenol 2-18, curzerene 1-11, β-citronellol 3-10, linalool 1-9	Moein *et al*., 2014
								Phenethyl alcohol 77, citronellol 13, eugenol 5	Hamedi *et al*., 2017b
						LLE DE	GC, GC-MS	Phenethyl alcohol 48, *n*--heptadecane 16	Samani *et al*., 2018
	Japan	F	SD	GC-MS	Citronellol 48, geraniol 20, nonadecane 8	LLE EAc	GC-MS	Phenethyl alcohol 76, citronellol 8, geraniol 7	Inouye *et al*., 2008
	Turkey	F	dnp	GC, GC-MS	Citronellol 35, geraniol 22, *n*--nonadecane 14, nerol 10	dnp	GC, GC-MS	Geraniol 31, citronellol 29, phenylethyl alcohol 24, neral 16	Ulusoy *et al*., 2009
			HD	GC-MS	Citronellol 43, geraniol 12, *n*--heneicosane 7	HS-SPME	GC-MS	Citronellol 40, geraniol 16, benzoic acid 2-hydroxy-3-methyl butyl ester 7, carbamic acid methyl ester 5	Koksal *et al*., 2015
Rosa damascena x *Rosa chinensis*	Japan	Pe	HD	dnp	dnp	HS-SPME	GC, GC-MS	Citronellol 35, geraniol 24, phenylethyl acetate 23, 3,5--dimethoxy toluene 11	Tomi *et al*., 2017
Rosa rugosa	Poland	Pe	HD	GC, GC-MS	*n*-Tricosane 22, *n*--pentacosane 22, citronellol 14, geraniol 9, palmitic acid 6	LLE DE	GC, GC-MS	Geraniol 2-31, β-phenylethanol 4-20, citronellol 0-18, phenylethyl benzoate 2-10, geranial t-10, nerol *t*-8, palmitic acid 0-7	Maciąg and Kalemba, 2015
Rutaceae									
Citrus aurantiifolia	Senegal	Fr	HD	GC, GC-MS	Limonene 35-51, β--pinene 7-14, geraniol 2-13, *p*--cymene 6-12, neral 2-9, γ-terpinene 5-7	LLE *n*-Hex	GC, GC-MS	Geranial 18, nerol 16, neral 15, α-terpineol 15, geraniol 13, terpinen-4-ol 6	Ndiaye *et al*., 2017
Citrus aurantium	Belgium	dnp	dnp	GC-MS	Linalool 35, limonene 18, β-pinene 13	LLE EAc	GC-MS	Linalool 41, α-terpineol 19, phenethyl alcohol 6	Inouye *et al*., 2008
	Brazil	P	HD	GC-MS	Limonene 74-83	LLE EAc	GC-MS	Nootkatone 17, α-terpineol 10, linalool 10, *cis*-linalool oxide 8, *trans*-linalool oxide 5	Wolffenbüttel *et al*., 2015
	Cyprus	F	SD**	GC-MS	Linalool 14, squalene 7, limonene 6	dnp	GC-MS	Linalool 17, neryl acetate 6, nerolidol 6, linalyl acetate 5	Değirmenci and Erkurt, 2020
	Egypt	dnp	SD	GC, GC-MS	Linalool 53, linalyl acetate 10	LLE MeCl	GC, GC-MS	Linalyl acetate 47, linalool 33	Edris, 2009
	France	F	HD	dnp	dnp	LLE *n*-Hex	GC-MS	Linalool 44, α-terpineol 24, *cis*--linalool oxide 6	Labadie *et al*., 2015
	Iran	F	dnp	dnp	dnp	LLE Peth	GC-MS	Linalool 37, α-terpineol 29, methyl anthranilate 11, *cis*--geraniol 9, indole 6	Hamedi *et al*., 2017b
	Morocco	dnp	dnp	dnp	dnp	LLE DE	GC, GC-MS	Linalool 40-60, α-terpineol 15-25, geraniol 1-7, methyl anthranilate 1-6, phenylethyl alcohol 1-5, benzyl	Jeannot *et al*., 2005

Family / Plant species	Country of Origin	Plant Part	EO IP	EO AP	EO Main components (≥ 5%)*	HV IP	HV AP	Main components HV (≥ 5%)*	Reference
								nitrile 1-5	
Citrus clementina ǂ	France	L_Fr	HD	GC, GC-MS	α-Pinene 38, linalool 18, limonene 7, *cis*-β-ocimene 6	LLE DE	GC, GC-MS	Linalool 69, terpinen-4-ol 12, α--terpineol 9	Paolini *et al*., 2008
						HS-SPME	GC, GC-MS	Linalool 58-72, terpinen-4-ol 10-18, α-terpineol 3-11, trans-hex-3-enol 0-7	Paolini *et al*., 2008
						P&T-ATD	GC, GC-MS	Linalool 70, terpinen-4-ol 6, dehydroxylinaloloxyde A 5	Paolini *et al*., 2008
Citrus x *junos*	Japan	Fr	SD	GC-MS	Limonene 79, α--terpinene 11	LLE EAc	GC-MS	Linalool 58, α-terpineol 16, thymol 8	Inouye *et al*., 2008
Citrus limon	Turkey	Fr	SD	dnp	dnp	dnp	GC-MS	Geraniol 48, α-terpineol 30, citral 29, terpinolene 10, terpinen-4-ol 7	Lante and Tinello, 2015
Citrus maxima	Senegal	Fr	HD	GC, GC-MS	Limonene 78-94	LLE *n*-Hex	GC, GC-MS	*trans*-Linalloloxide 21, α-terpineol 13, *cis*-linalooloxide 10, linalool 10, neral 6	Ndiaye *et al*., 2017
Citrus medica	Turkey	Fr	SD	dnp	dnp	dnp	GC-MS	α-Terpineol 17, citral 17, geraniol 15, terpinolene 6	Lante and Tinello, 2015
Citrus reticulata (= *Citrus* x *aurantium*) ♀	Senegal	Fr	HD	GC, GC-MS	Limonene 90-93	LLE *n*-Hex	GC, GC-MS	Linalool 18, citronellol 16, *trans*--carveol 12, α-terpineol 10, *n*-decanal 7	Ndiaye *et al*., 2017
Citrus sinensis (= *Citrus* x *aurantium*) ♀	Senegal	Fr	HD	GC, GC-MS	Limonene 92-94	LLE *n*-Hex	GC, GC-MS	Linalool 35, α-terpineol 10, limonene-10-ol 10, citronellol 8	Ndiaye *et al*., 2017
	Turkey	Fr	SD	dnp	dnp	dnp	GC-MS	Terpinolene 12	Lante and Tinello, 2015
Fortunella japonica (= *Citrus japonica*) ♀	France	L	HD	GC, GC-MS	Germacrene D 15, β--elemol 9, β-eudesmol 8, limonene 7, *cis*-guai-6-en-10β-ol 6, δ-elemene 5	LLE DE	GC, GC-MS	Cryptomeridiol 23, β-eudesmol 21, β-elemol 11, α-eudesmol 11, τ-muurolol 7	Sutour *et al*., 2017
Geijera parviflora	Australia	L	HD	GC-MS	*cis*-Caryophyllene 32, bicyclogermacrene 24, spathulenol 8, β--*cis*-ocimene 6	LLE Dcm	GC-MS	Linalool 24, isopsoralen 22, spathulenol 13, globulol 8, caryophylla-4(12),8(13)-dien-5-ol 6	Sadgrove *et al*., 2014
Ruta chalepensis	Greece	F_L	HD	dnp	dnp	LLE DE	GC-MS	2-Nonanone 77, 2-undecanone 9	Traka *et al*., 2018
Salicaceae									
Salix aegyptiaca	Iran	Ck	dnp	dnp	dnp	LLE Peth	GC-MS	*p*-Cymen-7-ol 29, cumin aldehyde 26, *cis*-anethol 18, methyl anthranilate 7	Hamedi *et al*., 2017b
Saururaceae									
Houttuynia cordata	Japan	F_L	SD	GC-MS	2-Undecanone 26, caprylaldehyde 16, trimethyl.bicyclo(2,2,1)-hept-2-yl-acetate 12, decanol 7, *n*--nonylalcohol 6, *n*--nonenol 6	LLE EAc	GC-MS	Terpinen-4-ol 57, linalool 20, α--terpineol 11	Inouye *et al*., 2008
Siparunaceae									
Siparuna guianensis	Brazil	L	HD	dnp	dnp	LLE Dcm	GC-MS	Siparunone 21-60, viridiflorol 1-26, dihydrocarvyl acetate 1-24, caryophyllene oxide 2-19, spatulenol 3-17, ledol 1-10, phethalic acid 1-6	Valentini *et al*., 2010
Thymelaeaceae									
Aquilaria spp.	Japan	W	HD	GC-MS	8-*epi*-γ-Eudesmol 10	HS-SPME	GC-MS	Benzylacetone 9	Takamatsu and Ito, 2018
Urticaceae									
Urtica dioica	Iran	AP	HD	dnp	dnp	LLE Peth	GC-MS	Thymol 27, hexadecanoic acid 18, carvacrol 12, camphor 6	Hamedi *et al*., 2017a
Verbenaceae									
Aloysia citriodora ♀	Iran	L	dnp	dnp	dnp	LLE Peth	GC-MS	Limonene 21, geranial 14,	Hamedi *et*

Family / Plant species	Country of Origin	Plant Part	EO IP	EO AP	EO Main components (≥ 5%)*	HV IP	HV AP	Main components HV (≥ 5%)*	Reference
								trans--caryophyllene 10, methyl hexadecanoate 8, neral 6, α--terpineol 6, terpinen-4-ol 5	*al*., 2017b
Lippia alba	Brazil	L_Br	HD	dnp	dnp	LLE *n*-Hex	GC-MS	Citral 65, carvone 10	Silva *et al*., 2018
		AP	HD	GC-MS	Geranial 34, neral 24, limonene 7, carvone 7	LLE *n*-Hex	GC-MS	Geranial 33, neral 31	Maia *et al*., 2019
	Colombia	L	HD	GC-MS	Carvone 42, limonene 41, germacrene D 8	dnp	GC-MS	Carvone 93	Hay *et al*., 2018
Lippia citriodora (= *Aloysia citrodora*) ♀	France	L	HD	GC, GC-MS	Geranial 27, neral 19, limonene 9	LLE DE	GC, GC-MS	Geranial 33, neral 29, 6--methylhept-5-en-2-one 6, 1,8--cineole 5	Paolini *et al*., 2008
				dnp	dnp	HS-SPME	GC, GC-MS	Geranial 24-42, neral 23-34, 6--methylhept-5-en-2-one 3-22, 1,8-cineole 4-13, spathulenol 0-7	Paolini *et al*., 2008
						P&T-ATD	GC, GC-MS	1,8-Cineole 62, 6-methylhept-5-en-2-one 8, isovaleraldehyde 5, neral 5	Paolini *et al*., 2008
	Japan	L	SD	GC-MS	Citral 43, limonene 12, α-curcumene + germacrene D 7, germacrene B 5	LLE EAc	GC-MS	Citral 53, terpinen-4-ol 8	Inouye *et al*., 2008
Xanthorrhoeaceae									
Aloe spp.	Iran	L	dnp	dnp	dnp	LLE Peth	GC-MS	Menthol 37, thymol 11, carvacrol 6, *trans*-menthone 5, pulegone 5	Hamedi *et al*., 2017c
Zingiberaceae									
Zingiber officinale	Iran	R	HD	dnp	dnp	LLE Chl	GC-MS	Thymol 41, carvacrol 26	Hamedi *et al*., 2017a

0: Values between 0.05 and 0.5, ǂ Designation from authors, ǂ OGR: Ole-gum-resin, * Unless otherwise stated the data is provided in percentage, ** Steam-distillation using a Clevenger-type apparatus, *** Steam-distillation using a alembic-type apparatus, ¥ Comparison with literature data, not data from the authors, ◊ Hexane was distilled to recovered hydrolate volatiles, § Data given in values ≥ 1 mg/kg, ≠ Species name according to authors but not present in World Checklist of Vascular Plants (WCVP), ♀ Species name according to authors and synonym according World Checklist of Vascular Plants (WCVP), ⁑ Technique not detailed, Ж Data given in values ≥ 5 mg/L, Ѧ Authors denominate as Apple essence, AP: Aerial parts; B: Bark, Br: Branches, Bu: Bulb, Bud: Buds, Ck: Catkins, DLLME: Dispersive liquid-liquid microextraction, dnp: Data not provided, EO AP: Essential oil analysis procedure, EO IP: Essential oil isolation procedure, F: Flowers, F_L: Flowers and leaves, F_L_S: Flowers, leaves and stems, FAx: Floral axis, FH: Flower heads, FHe: Flowering herb, Fr: Fruits, FT: Flowering tops, GC: Gas chromatography, GC-MS: Gas chromatography-Mass spectrometry, HD: Hydrodistillation, HDF: Hidrodiffusion, He: Herbs, HSE: Headspace extraction, HS-SPME: Headspace solid-phase microextraction, HV AP: Hydrolate volatiles analysis procedure, HV IP: Hydrolate volatiles isolation procedure, HYDRO/ Ben: Hidrodistillation using benzene, L: Leaves, L_B: Leaves and bark, L_Br: Leaves and branches, L_Fr: Leaves and fruits, L_S: Leaves and seeds, L_St: Leaves and stems, LLE Ben: Liquid-liquid extraction with benzene, LLE Chl: Liquid-liquid extraction with chloroform, LLE Cy: Liquid-liquid extraction with cyclohexane, LLE Dcm: Liquid-liquid extraction with dichloromethane, LLE DE: Liquid-liquid extraction with diethyl ether, LLE EAc: Liquid-liquid extraction with ethyl acetate, LLE Et: Liquid-liquid extraction with ether, LLE EtOH: Liquid-liquid extraction with ethanol, LLE MeCl: Liquid-liquid extraction with methylene chloride; LLE NaCl/Dcm: Liquid-liquid extraction with sodium chloride/dichloromethane, LLE *n*-Hep: Liquid-liquid extraction with *n*-heptane, LLE *n*-Hex: Liquid-liquid extraction with *n*-hexane, LLE *n*-Pen: Liquid-liquid extraction with *n*-pentane, LLE Peth: Liquid-liquid extraction with petroleum ether, P: Pericarp, P&T-ATD: Purge-and-trap-automatic thermal desorption, Pe: Petals, PET: Poroplast extraction technique, R: Rhizome, Rt: Roots, S: Seeds, SD: Steam-distillation, Sp: Spathe, St: Stems, St_B: Stems and Bark, W: Wood,WD: Water distillation.

Annex B

Regional Micro-biorefineries: A dream turning real?

Oral communication presented by Tavares *et al*., 2019 at the 27th European Biomass Conference

Paper of the 27th European Biomass Conference – setting the course for a biobased economy

Extracted from the Proceedings of the International Conference held in Lisbon, Portugal 27-30 May 2019

Regional Micro-biorefineries: A dream turning real?

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1. Abstract

Silvapor is a Portuguese SME based in Idanha-a-Nova that provides services across the country integrated in the promotion, protection and rural and environmental conservation, facing annually huge amounts of waste removed during thinning of forest. Into the scope of industrial-academic projects and within the need for an integrated forest policy that promotes the sustainability of the forest and its management, and also the prevention of forest fires, the present study aims at develop environmentally sustainable operations towards a micro-scale integrated biorefinery unit at Silvapor premises, capable of processing different types of biomass into bioproducts and energy, as an alternative to open burning.

2. Introduction

The Portuguese forest occupies ca. 40 % of the continental territory, existing therefore a considerable potential for waste coming from the forest. This biomass availability is the basis for making renewable bioenergy, biofuels and other bio-products that are increasingly replacing fossil-fuel based products thus, it should be recovered with environmental, economic and social benefits, as an alternative to open burning (Han *et al*., 2018). The concept of biorefinery is defined as an approach for the generation of value-added products such as biochemical, biofuels, heat and electricity from renewable energy sources such as forest biomass (Kehili *et al*., 2016). However, the concept of biorefinery is still in early stages at most places in the world. Problems like raw material availability, feasibility in product supply chain, scalability of the model are hampering its development at commercial-scales.

Within the need for an integrated forest policy that promotes the sustainability of the forest and its management, and also the prevention of forest fires for defending the integrity of the populations and the preservation of their means of subsistence and patrimonial assets, it is

intended that the development of advanced biorefineries using endogenous resources sustainably generate new value chains around biomass (Figure B.1), in the so-called bioeconomy and circular economy (PCM, 2017).

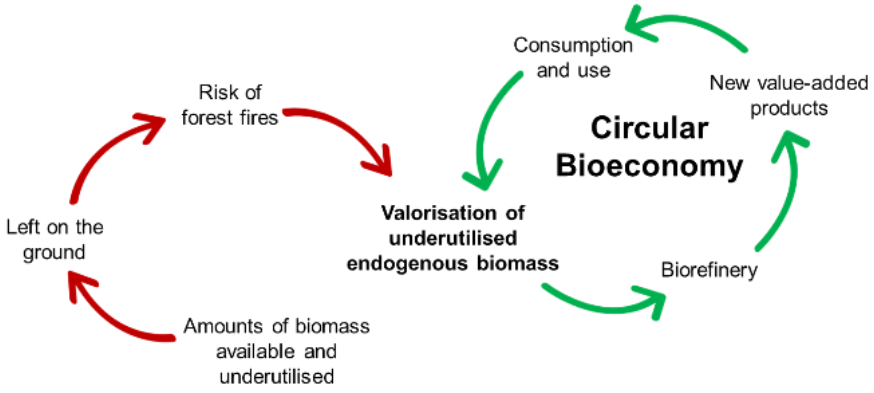

Figure B.1. Integrated forest policy.

Biomass samples were studied for bio-products and energy, aiming at the possibility of installing a small-scale biorefinery within regional SMEs premises. Examples of available biomass, namely *Cupressus lusitanica* Mill. aerial parts and *Cistus ladanifer* L. shrubs, were collected at Silvapor, a Portuguese SME that provides services across the country related to the activities of agriculture, forestry, green spaces and landscaping, as well as others integrated in the promotion, protection and rural and environmental conservation. Essential oils and their co-product hydrolates are examples of bio-products that can be obtained from these biomasses, as they are high value commodities. Therefore, a semi-industrial scale steam-distillation was set up and used with these biomasses as feedstocks, resulting four distinct products thereof: essential oils, hydrolates, distiller condensation waters and the remaining extracted solid fractions (Figure B.2).

Figure B.2. Distiller and the resulting four co-products.

All products were characterized for their composition and potential use. Condensation waters and the remaining extracted solid fractions were further scrutinized.

3. Material and methods

3.1. Plant biomass

Waste samples of *Cupressus lusitanica* Mill. and *Cistus ladanifer* L. aerial parts were collected and obtained from Silvapor (Idanha-a-Nova, Portugal), The dried biomass samples composed mainly by leaves and small branches, but also by flowers for *Cistus* and cones for *Cupressus*, were collected in the Beira-Baixa region of Portugal.

3.2. Distillation

Essential oils (EO) were obtained by steam-distillation at a semi-industrial scale performed at Silvapor premises using a 1100 L stainless-steel distiller (Vieirinox®, Aveiro, Portugal). In the steam-distillation process, additional aromatic waters called hydrolates (also referred as hydrolats, hydrosols, floral waters, herbal waters, condensate waters), were obtained as co-products. Circa 100 Kg of dried biomass residues from each species was used and steam was generated in a boiler and injected. The distillate contained a mix of water vapour and essential oils which returned to their liquid form in the condensing recipient and were separated using a Florentine separator. Both the essential oil and the hydrolate were retained. Oil was then separated from hydrolate using a separation funnel, stored in dark flasks and both oil and hydrolates were kept refrigerated for further analysis. The remaining condensation waters and extracted solid fractions were also collected for further characterisation.

3.3. Characterization of essential oils and hydrolates

Essential oils and hydrolates composition were characterised by GC and GC-MS, according to methodologies described elsewhere (Tavares *et al*., 2019).

3.4. Preparation of condensation waters and extracted solid residues

Condensation waters, resulting from either *Cupressus lusitanica* Mill. and *Cistus ladanifer* L. essential oils extraction, were freeze-dried and further characterized for bioactive compounds. Resulting steam-distillation extracted solid fractions were used for further extraction with other solvents in order to obtain bioactive compounds, namely for phenolic compounds with potential biological activities. Each were mixed with ethanol at a solid:liquid ratio of 1:20 and subjected to ultrasound-assisted extraction (UAE) at 30 °C for 30 min, using a Transsonic T700 sonifier (320 W, 35 kHz) (Elma GmbH & Co, Germany). Extracts were then

filtered through filter paper (Whatman nº. 1), concentrated under vacuum to obtain the ethanol extract. This procedure was done in triplicate.

The remaining UAE solids were further extracted with 70 % acetone (1:20) using the same procedure, and the acetone extracts were collected, concentrated under vacuum and freeze-dried. Remaining solids from both UAE solvent systems of each biomass were dried and stored for further treatments, namely, using auto-hydrolysis and ionic liquids (data not shown), in order to get other components, such as sugars for bio-ethanol.

3.5. Determination of biological activities and phenolic content

Essential oils, hydrolates, condensation waters and the ethanolic and 70 % acetone fractions obtained from UAEs of the solid residue were tested for different biological activities, namely, antimicrobial, antioxidant activity determined using different methodologies and anti-inflammatory activity using the albumin denaturation assay (Tavares *et al*., 2019). Biological essays were performed in four replicates.

Condensation waters and the ethanolic and 70 % acetone extracts obtained from UAEs of the solid fraction from steam-distillation were also tested for the phenolic and tannin content by the Folin-Ciocalteau method and also for their flavonoid content.

The phenolic profile for all samples was also evaluated by capillary electrophoresis, and compounds tentatively identified by electrophoretic comparison of UV spectra and migration times with authentic standards.

4. Results and Discussion

4.1. Essential oils and hydrolates

Results for the essential oils and hydrolates are disclosed elsewhere (Tavares *et al*., 2019). Biomass examples such as the ones shown here, revealed to contain essential oils with the same characteristics and biological activities as the ones obtained from the fresh plants. Their hydrolates also showed interesting composition and properties, suggesting that both products can be directly commercialized by the producer to potentially interested industries, such as perfumery, which is highly interested in fragances of endogenous origin, and also for the production of cosmetics and detergents.

4.2. Condensation waters and extracted solid fractions

Tables B.1 and B.2 show the results for the total phenolic, tannins and flavonoids content for condensation waters and the ethanolic and 70 % acetone extracts obtained from UAEs of the extracted solid fractions for the two biomasses studied, and their respective antioxidant activity. It can be observed that all of them revealed to be good sources of phenolic compounds,

with particular attention to the extracts from *Cistus ladanifer*. The later are especially richer in tannins and also flavonoids. All presented antioxidant activity, but *Cistus ladanifer* showed to have higher amount than *Cupressus lusitanica* extracts, being these also related with their phenolic content. The electropherograms of these samples revealed a complex matrix with a catechin-rich phenolic profile (data not shown). Results for the anti-inflammatory activity (Table B.3), were particularly interesting for the ethanolic extracts of the extracted solid fractions for both biomasses studied.

These results suggest that forest wastes are rich sources of natural bioactive compounds, which can be explored, for example, as alternatives for synthetic antioxidants in the food industry, that have been reported as toxic.

Table B.1. Total phenolic, tannins and flavonoids content.

	Samples	**Total Phenolics** mg GAE/g ext.	**Tannins** mg GAE/g ext.	**Flavonoids** mg QE/g ext.
Cupressus lusitanica	**Condensation water**	**146.0**	**95.1**	1.3
	EtOH_Extract	61.2	23.0	4.4
	70 % Acet_Extract	**165.8**	**82.2**	2.9
Cistus ladanifer	**Condensation water**	**221.1**	**134.7**	11.9
	EtOH_Extract	177.5	108.4	12.2
	70 % Acet_Extract	**275.6**	**115.1**	29.6

GAE: Gallic acid equivalentes; QE: Quercetin equivalentes.

Table B.2. Antioxidant activity by different methods.

	Samples	**ABTS** % I	**Superoxide** % I	**Chelating** % I	**Xantine Oxidade** % I
Cupressus lusitanica	Condensation water	24.9	89.7	16.6	59.8
	EtOH_Extract	8.7	82.8	18.0	76.3
	70 % Acet_Extract	33.7	87.3	7.5	72.1
Cistus ladanifer	**Condensation water**	44.4	**97.5**	16.8	68.4
	EtOH_Extract	28.6	79.8	24.1	80.3
	70 % Acet_Extract	75.7	97.2	10.5	73.1

Table B.3. Anti-inflammatory activity.

	Samples	**Anti-inflammatory activity**, % I
Cupressus lusitanica	Condensation water	29.3
	EtOH_Extract	**77.4**
	70 % Acet_Extract	61.5
Cistus ladanifer	Condensation water	44.8
	EtOH_Extract	**78.2**
	70 % Acet_Extract	72.3

It is not expected that the local and regional SMEs premises have the necessary facilities to carry on with further treatments of the resulting extracted solid fraction, not even the preparation of these phenolic-rich extracts, particularly the ones obtained by UAE, and condensation waters need to be properly preserved, either frozen or freeze-dried, before further purification. Nevertheless, the results here presented show that these are worth considering by some intermediary stakeholder for further valorisation into bio-products that could be of interest for other industries, such as food, cosmetic and pharmaceutical.

However, not all forest wastes need to be processed in the way here described, as most do not have essential oils. Those forest residues, together with the remaining solids from both essential oil production and /or UAEs solvent systems, could be used as a renewable source of energy, by locally producing biochar and/or pellets, using a domestic pelletizer, for example. This energy could then be used for self-consumption, also as energy source for the steam-distillation, or even sold, within the circular economy concept (Figure B.3).

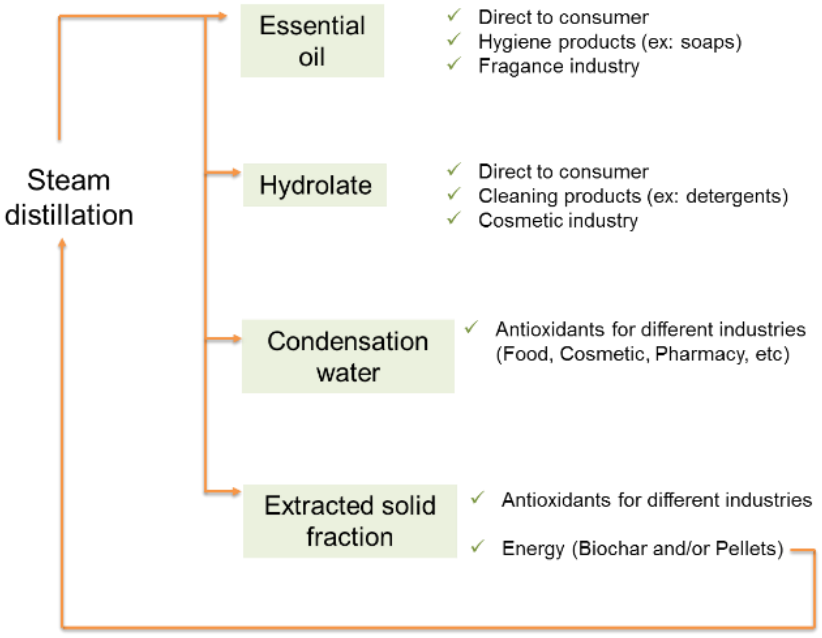

Figure B.3. Biorefinery/Circular economy concept.

5. Conclusion

The present work discloses that it is possible to produce value and reduce waste at local and regional forest areas, with a small investment in a distiller and a domestic mobile pelletizer, aiming at local micro-biorefineries and zero waste, with all the inherent advantages thereof and within a circular economy.

Results from this study demonstrate that forest wastes may still have some additional value. Both essential oils and hydrolates obtained from steam-distillation of available biomasses can be immediately commercialized. Condensation waters and the remaining extracted solid

fractions also revealed to have interesting bioactive compounds that could be further valorised and used for different industries, namely as natural antioxidants. Although these still need further treatments which require more sophisticated equipments and methodologies, they can be sold as building-blocks, provided they are adequately preserved. Finally, the possibility of local bioenergy production using the remaining extracted solid wastes added to other forest biomasses, namely pellet production using a domestic mobile pelletizer, is under study.

6. Acknowledgements

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Logo space