Universidade de Aveiro Departamento de Química
2015

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Caracterização química e avaliação da atividade biológica de componentes extratáveis da *Cynara cardunculus*

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Química, realizada sob a orientação científica do Doutor Armando Jorge Domingues Silvestre, Professor Associado com Agregação do Departamento de Química da Universidade de Aveiro, e da Doutora Maria de Fátima Pereira Duarte, Investigadora Auxiliar do Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo (CEBAL).

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Aos meus pais e Tiago.

o júri

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palavras-chave

Cynara cardunculus L. var. *altilis* (DC), cardo cultivado, GC-MS, HPLC-MS, fração lipofílica, lactonas sesquiterpénicas, fração fenólica, ácidos hidroxicinâmicos, atividade antioxidante, atividade antiproliferativa, cancro da mama de fenótipo triplo-negativo, atividade antibacteriana, bactérias multirresistentes.

resumo

A espécie mediterrânica *Cynara cardunculus* L. é reconhecida na medicina tradicional, pelos seus efeitos hepatoprotetor e colerético. A biomassa de *C. cardunculus* L. var. *altilis* (DC), ou cardo cultivado, poderá ser explorada não só para a produção de energia e fibras de pasta de papel, mas também para a extração de compostos bioativos. A caracterização química dos componentes extratáveis, nomeadamente os compostos terpénicos e fenólicos, poderá valorizar a plantação de cardo cultivado, dadas as suas atividades antioxidante, antitumoral e antimicrobiana.

Neste estudo, a composição química das frações lipofílica e fenólica de *C. cardunculus* L. var. *altilis* (DC), cultivado no sul de Portugal (região do Baixo Alentejo), foi caracterizada em detalhe, com vista à valorização integral da sua biomassa. A atividade biológica dos extratos de cardo cultivado foi avaliada em termos de efeitos antioxidante, antiproliferativa em células humanas tumorais, e antibacteriano.

A análise química dos compostos lipofílicos foi realizada por cromatografia em fase gasosa acoplada à espectrometria de massa (GC-MS). Identificaram-se 65 compostos lipofílicos, dos quais 1 lactona sesquiterpénica e 4 triterpenos pentacíclicos foram descritos, pela primeira vez, como componentes do cardo cultivado, tais como: desacilcinaropicrina, acetatos de *β*- e *α*-amirina, acetato de lupenilo e acetato de *ψ*-taraxasterilo. As lactonas sesquiterpénicas foram a principal família de compostos lipofílicos das folhas (≈94,5 g/kg), maioritariamente representadas pela cinaropicrina (≈87,4 g/kg). Os triterpenos pentacíclicos foram também detetados, em teores consideravelmente elevados, nas restantes partes do cardo cultivado, em especial nos floretos (≈27,5 g/kg). O acetato de taraxasterilo foi o triterpeno pentacíclico maioritário (≈8,9 g/kg nos floretos).

Para a análise química dos compostos fenólicos foi utilizada a cromatografia líquida de alta resolução acoplada à espectrometria de massa (HPLC-MS). Entre os 28 compostos fenólicos identificados, o erioditiol hexósido foi descrito pela primeira vez como componente de *C. cardunculus* L., e 6 como componentes do cardo cultivado, nomeadamente ácido 1,4-di-*O*-cafeoilquínico, naringenina 7-*O*-glucósido, naringenina rutinósido, naringenina, luteolina acetil-hexósido e apigenina acetil-hexósido. A concentração mais alta de compostos fenólicos identificados foi observada nos floretos (≈12,6 g/kg). A parte externa dos caules continha o maior teor em ácidos hidroxicinâmicos (≈10,3 g/kg), e os floretos apresentaram o maior teor em flavonoides (≈10,3 g/kg).

A atividade antioxidante da fração fenólica foi examinada face ao radical livre 2,2-difenil-1-picril-hidrazilo (DPPH). Os extratos da parte externa do caule, e dos recetáculos e brácteas demonstraram o maior efeito antioxidante, face ao DPPH (IC_{50} de 34,35 µg/mL e 35,25 µg/mL, respetivamente). (cont.)

resumo (cont.)

A atividade antioxidante face ao DPPH foi correlacionada linearmente com a concentração total de ácidos hidroxicinâmicos (*r* = -0.990).
A atividade antiproliferativa *in vitro* dos extratos lipofílicos e fenólicos de cardo cultivado foi avaliada numa linha de células tumorais humanas de cancro da mama de fenótipo triplo-negativo (MDA-MB-231), um dos tipos de cancro humano mais refratários às terapêuticas convencionais. Após 48 h de exposição, o efeito inibitório do extrato lipofílico das folhas (IC_{50} = 10,39 µg/mL) foi superior ao do extrato lipofílico dos floretos (IC_{50} = 315,22 µg/mL), sobre a viabilidade celular de MDA-MB-231. O composto puro da cinaropicrina, representativo do composto maioritário identificado no extrato lipofílico das folhas, também inibiu a proliferação das células MDA-MB-231 (IC_{50} = 17,86 µM). As células MDA-MB-231 foram muito mais resistentes, ao tratamento de 48 h, com os extratos fenólicos da parte externa dos caules (IC_{50} = 3341,20 µg/mL) e dos floretos (IC_{50} > 4500 µg/mL), e também com o composto puro do ácido 1,5-di-*O*-cafeoilquínico (IC_{50} = 1741,69 µM).
As células MDA-MB-231 foram expostas, durante 48 h, às respetivas concentrações de IC_{50} do extrato lipofílico das folhas e do composto puro da cinaropicrina, de modo a perceber a sua capacidade em modelar respostas celulares, e consequentemente potenciais vias de sinalização importantes para o decréscimo da viabilidade celular. O extrato lipofílico das folhas aumentou a atividade enzimática da caspase-3, ao contrário do composto puro da cinaropicrina. Além disso, o extrato lipofílico das folhas e o composto puro da cinaropicrina causaram paragem do ciclo celular na fase G2, possivelmente através do aumento da expressão proteica de $p21^{Waf1/Cip1}$ e da acumulação das proteínas fosfo-Tyr15-CDK1 e ciclina B1. Os efeitos inibitórios do extrato lipofílico das folhas e do composto puro da cinaropicrina, contra a proliferação das células MDA-MB-231, poderão também estar relacionados com a diminuição da expressão proteica da fosfo-Ser473-Akt.
A atividade antibacteriana dos extratos lipofílicos e fenólicos de cardo cultivado foi avaliada, pela primeira vez, sobre duas bactérias multirresistentes, tais como a bactéria Gram-negativa *Pseudomonas aeruginosa* PAO1 e a bactéria Gram-positiva *Staphylococcus aureus* resistente à meticilina (MRSA), duas das principais bactérias responsáveis pelas infeções associadas aos cuidados de saúde. Para tal, determinaram-se as concentrações inibitórias mínimas (MIC). Os extratos lipofílicos e fenólicos dos floretos não revelaram atividade antibacteriana contra *P. aeruginosa* PAO1 e MRSA (MIC > 2048 µg/mL). O extrato lipofílico das folhas não inibiu o crescimento de *P. aeruginosa* PAO1, mas o composto puro da cinaropicrina foi ligeiramente ativo (CIM = 2048 µg/mL). O extrato lipofílico das folhas e o padrão puro da cinaropicrina bloquearam o crescimento de MRSA (MIC de 1024 e 256 µg/mL, respetivamente).
O conhecimento científico revelado nesta tese, quer do ponto de vista químico, quer do ponto de vista biológico, contribui para a valorização da biomassa de *C. cardunculus* L. var. *altilis* (DC). O cardo cultivado tem potencial para ser explorado como fonte de compostos bioativos, em conciliação com outras vias de valorização, e a produção de queijos tradicionais portugueses.

keywords

Cynara cardunculus L. var. *altilis* (DC), cultivated cardoon, GC-MS, HPLC-MS, lipophilic fraction, sesquiterpene lactones, phenolic fraction, hydroxycinnamic acids, antioxidant activity, antiproliferative activity, human triple-negative breast cancer, antibacterial activity, multidrug resistant bacteria.

abstract

The Mediterranean species *Cynara cardunculus* L. is recognized in the traditional medicine, for their hepatoprotective and choleretic effects. Biomass of *C. cardunculus* L. var. *altilis* (DC), or cultivated cardoon, may be explored not only for the production of energy and pulp fibers, but also for the extraction of bioactive compounds. The chemical characterization of extractable components, namely terpenic and phenolic compounds, may valorize the cultivated cardoon plantation, due to their antioxidant, antitumoral and antimicrobial activities.

In this study, the chemical composition of lipophilic and phenolic fractions of *C. cardunculus* L. var. *altilis* (DC), cultivated in the south of Portugal (Baixo Alentejo region) was characterized in detail, intending the integral valorization of its biomass. The biological activity of cultivated cardoon extracts was evaluated in terms of antioxidant, human tumor cell antiproliferative and antibacterial effects.

Gas chromatography-mass spectrometry (GC-MS) was used for the chemical analysis of lipophilic compounds. Sixty-five lipophilic compounds were identified, from which 1 sesquiterpene lactone and 4 pentacyclic triterpenes were described, for the first time, as cultivated cardoon components, such as: deacylcynaropicrin, acetates of *β*- and *α*-amyrin, lupenyl acetate and *ψ*-taraxasteryl acetate. Sesquiterpene lactones were the major family of lipophilic components of leaves (≈94.5 g/kg), mostly represented by cynaropicrin (≈87.4 g/kg). Pentacyclic triterpenes were also detected, in considerably high contents, in the remaining parts of cultivated cardoon, especially in the florets (≈27.5 g/kg). Taraxasteryl acetate was the main pentacyclic triterpene (≈8.9 g/kg in florets).

High pressure liquid chromatography-mass spectrometry (HPLC-MS) was utilized for the chemical analysis of phenolic compounds. Among the identified 28 phenolic compounds, eriodictyol hexoside was reported for the first time as *C. cardunculus* L. component, and 6 as cultivated cardoon components, namely 1,4-di-*O*-caffeoylquinic acid, naringenin 7-*O*-glucoside, naringenin rutinoside, naringenin, luteolin acetylhexoside and apigenin acetylhexoside. The highest content of the identified phenolic compounds was observed in the florets (≈12.6 g/kg). Stalks outer part contained the highest hydroxycinnamic acids abundance (≈10.3 g/kg), and florets presented the highest flavonoids content (≈10.3 g/kg).

The antioxidant activity of phenolic fraction was examined through 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. Stalks outer part, and receptacles and bracts extracts demonstrated the highest antioxidant effect on DPPH (IC_{50} of 34.35 µg/mL and 35.25 µg/mL, respectively). (cont.)

abstract (cont.)

The DPPH scavenging effect was linearly correlated with the total contents of hydroxycinnamic acids (r = -0.990).
The *in vitro* antiproliferative activity of cultivated cardoon lipophilic and phenolic extracts was evaluated on a human tumor cells line of triple-negative breast cancer (MDA-MB-231), one of the most refractory human cancers to conventional therapeutics. After 48 h of exposition, leaves lipophilic extract showed higher inhibitory effect (IC_{50} = 10.39 µg/mL) than florets lipophilic extract (IC_{50} = 315.22 µg/mL), upon MDA-MB-231 cellular viability. Pure compound of cynaropicrin, representative of the main compound identified in leaves lipophilic extract, also prevented the cell proliferation of MDA-MB-231 (IC_{50} = 17.86 µM). MDA-MB-231 cells were much more resistant to the 48 h-treatment with phenolic extracts of stalks outer part (IC_{50} = 3341.20 µg/mL) and florets (IC_{50} > 4500 µg/mL), and also with the pure compound of 1,5-di-*O*-caffeoylquinic acid (IC_{50} = 1741.69 µM).
MDA-MB-231 cells were exposed, for 48 h, to the respective IC_{50} concentrations of leaves lipophilic extract and pure compound of cynaropicrin, in order to understand their ability in modelling cellular responses, and consequently important potentially signaling pathways for the cellular viability decrease. Leaves lipophilic extract increased the caspase-3 enzymatic activity, contrarily to pure compound of cynaropicrin. Additionally, leaves lipophilic extract and pure compound of cynaropicrin caused G2 cell cycle arrest, possibly by upregulating the $p21^{Waf1/Cip1}$ and the accumulation of phospho-Tyr15-CDK1 and cyclin B1. The inhibitory effects of leaves lipophilic extract and cynaropicrin pure compound, against the MDA-MB-231 cell proliferation, may also be related to the downregulation of phospho-Ser473-Akt.
The antibacterial activity of cultivated cardoon lipophilic and phenolic extracts was assessed, for the first time, on two multidrug-resistant bacteria, such as the Gram-negative *Pseudomonas aeruginosa* PAO1 and the Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA), two of the main bacteria responsible for health care-associated infections. Accordingly, the minimum inhibitory concentrations (MIC) were determined. Lipophilic and phenolic extracts of florets did not have antibacterial activity on *P. aeruginosa* PAO1 and MRSA (MIC > 2048 µg/mL). Leaves lipophilic extract did not prevent the *P. aeruginosa* PAO1 growth, but pure compound of cynaropicrin was slightly active (MIC = 2048 µg/mL). Leaves lipophilic extract and pure compound of cynaropicrin blocked MRSA growth (MIC of 1024 and 256 µg/mL, respectively).
The scientific knowledge revealed in this thesis, either by the chemical viewpoint, or by the biological viewpoint, contributes for the valorization of *C. cardunculus* L. var. *altilis* (DC) biomass. Cultivated cardoon has potential to be exploited as source of bioactive compounds, in conciliation with other valorization pathways, and Portuguese traditional cheeses manufacturing.

Table of contents

Notation

Abbreviations and symbols

A	antioxidant compound
AAI	antioxidant activity index
ABTS	2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
AH	after alkaline hydrolysis
ArO^-	phenoxide ion
ATM	ataxia-telangiectasia-mutated
ATR	ataxia and rad3 related
AUC	areas under the fluorescence decay curves
BC	breast cancer
BH	before alkaline hydrolysis
BHT	3,5-di-*tert*-4-butylhydroxytoluene
BSA	bovine serum albumin
^{13}C NMR	carbon-13 nuclear magnetic resonance
cfu	colony forming unit
CAE	chlorogenic acid equivalents
CAK	CDK-activating kinase
Cdc	cell division cycle
CDK	cyclin-dependent kinase
CID	collision ionization dissociation
CKI	cyclin dependent kinase inhibitor
COSY	correlated spectroscopy
d	doublet
dd	doublet of doublets
ddd	doublet of doublet of doublets
dt	doublet of triplets
DAD	diode array detector
dc	direct current
DEPT	distortionless enhancement by polarization transfer
DISC	death-inducing signaling complex
DMBA	7,12-dimethylbenz[*a*]anthracene
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl

dw	dry weight
EDTA	ethylenediaminetetraacetic acid
EI	electron ionization
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
ESI	electrospray ionization
FBS	fetal bovine serum
FDA	food and drug administration
FRAP	ferric reducing antioxidant power
GAE	gallic acid equivalent
GC	gas chromatography
GSH	reduced glutathione
^{1}H NMR	proton nuclear magnetic resonance
HAT	hydrogen atom transfer
HCAI	health care-associated infections
HCC	hepatocellular carcinoma
HER	human epidermal growth factor receptor
HMBC	heteronuclear multiple bond coherence
HMQC	heteronuclear multiple quantum coherence
HPLC	high-performance liquid chromatography
HSQC	heteronuclear single quantum coherence
HT-UHPLC	high temperature-ultra HPLC
IC_{50}	inhibitory concentration at 50%
IU	international unit
J	coupling constant
LDL	low-density lipoprotein
ox*A*	oxidized antioxidant
$[M]^{+\cdot}$	molecular ion
$[M-H]^{-}$	deprotonated molecular ion
m	multiplet
MBC	minimum bactericidal concentration
MDA	malonaldehyde
MDR	multidrug-resistance
MIC	minimum inhibitory concentration
MFC	minimum fungicidal concentration
MOMP	mitochondrial outer membrane permeabilization

MPF	mitosis promoting factor
mRNA	messenger ribonucleic acid
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectrometry
MS^n	tandem MS
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
m/*z*	mass-to-charge ratio
NADH	nicotinamide adenine dinucleotide, in the reduced form
NME	new molecular entity
NMR	nuclear magnetic resonance
NOESY	nuclear overhauser effect spectroscopy
ORAC	oxygen radical absorbance capacity
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PMS	phenazine methosulfate
PR	progesterone receptor
PROC GLM	procedure general linear model
PVDF	polyvinylidene difluoride
q	quartet
r	correlation coeficient
Rb	retinoblastoma
R_f	retention factor
rf	radio-frequency voltage
ROS	reactive oxygen species
rpm	rotations per minute
RNA	ribonucleic acid
RT	retention time
s	singlet
SET	single electron transfer
t	triplet
t-BHP	*tert*-butylhydroperoxide
TCM	traditional and complementary medicine
TEAC	Trolox equivalent antioxidant capacity
TMSOH	trimethylsilanol
Thr	threonine
TLC	thin-layer chromatography
TMS	trimethylsilyl

TNBC	triple-negative breast cancer
TNF	tumor necrosis factor
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TPC	total phenolic content
tt	triplet of triplets
Tyr	tyrosine
UHPLC	ultra high-performance liquid chromatography
UV	ultraviolet spectroscopy
UV-Vis	UV-visible spectroscopy
X/XO	xanthine/xanthine oxidase system
w/w	weight of extract to dry weight of biomass ratio

Greek letters

λ_{max}	wavelength at which ultraviolet/visible absorbance is maximum
δ	chemical shift

Part A

Chapter I

General introduction

1. The context

1.1 Natural products within the pharmaceutical field

The empiric use of medicinal plants is as old as humanity's history. The interest of pharmaceutical industry for the natural products field was boosted by the isolation of morphine from *Papaver somnifera*, in 1804, by Friedrich Serturner. Due to its analgesic action, morphine started to be commercialized in 1827 by Heinrich Merck, long before the creation of the modern Food and Drug Administration (FDA) in 1938.[1]

In the 1930-1970s, natural-based derivatives represented more than 50% of the total annually FDA-approved new molecular entities (NMEs). Nevertheless, there was a decrease of natural-based NMEs percentage, with FDA approval, to ≈30% in the 1990s, which was ascribed to a higher production of synthetic compounds "libraries".[1] Due to a "crisis" in finding new active principles, pharmaceutical industry has been turning to the research of natural compounds, in an effort to synthesize novel, biologically active, "natural product-like" molecules, owing to: (i) chemical diversity; (ii) "privileged structures", resulting from selected evolution to bind to biological macromolecules; and (iii) pleiotropic mechanisms of action.[1–3] In this manner, the natural-based drugs fraction, in the 2000s, increased to 38% of the annually FDA-approved NMEs, and it reached more than 20% in 2011-2013.[1]

Between 1935 and 2013, a huge search for anticancer and antibacterial drugs contributed to respectively 13% and 20% of the total natural-based NMEs approved by FDA.[1] Secondary metabolites of plants origin have been a source of natural-based NMEs, approved by FDA, accounting for 25%, in the same period. Among natural-based NMEs, 16% and 2% corresponded to plant-based NMEs for the therapeutic of cancer and bacterial infections, respectively.[1]

There has been a growing interest in the consumption of herbal products, as a complementary approach to the treatment with synthetic drugs, to manage in a gentler way, some of the dominant chronic diseases in the 21st century, namely cardiovascular diseases, cancer, diabetes and mental disorders.[4] The scientific knowledge, about the chemical composition and physiological effects of herbal products, has contributed to enhance their effectiveness and safe usage.[5] Veregen, an aqueous extract of green tea leaves containing catechins, was the first botanical prescription drug approved by FDA, in 2006. The FDA-approval of Veregen represents an important step in the botanical drug development.[6]

1.2 Biorefinery concept

The term biorefinery was recently redefined within the project Biorefinery Euroview: "Biorefineries could be described as integrated bio-based industries using a variety of technologies to make products such as chemicals, biofuels, food and feed ingredients,

biomaterials, fibers, heat and power, aiming at maximizing the added value along the three pillars of sustainability (Environment, Economy and Society)".[7] Nowadays, the production of chemicals, including pharmaceuticals, materials and energy, are highly dependent on non-renewable fossil fuels, implicating serious global issues, namely climate change, petroleum depletion, energy and food supply, biodiversity, etc.[8,9] For reducing production costs and environmental negative impact, the pharmaceutical industry should integrate alternative raw materials, based on renewable resources as plant biomass, which perfectly fulfills the biorefinery concept. Additionally, the application of clean processes with reduced solvent consumption, lower energy input, renewable catalysts, and mild conditions for reaction and separation should also be considered.[8]

1.3 Industrial perspectives for *Cynara cardunculus* L.

Cynara cardunculus L. is a Mediterannean species, englobing three varieties, namely var. *sylvestris* (Lamk) (wild cardoon), var. *scolymus* (L.) (globe artichoke) and var. *altilis* (DC) (cultivated cardoon). Growing conditions of cultivated cardoon fit well with the clay soils and hot summers of the Mediterranean countries. Cultivated cardoon plantation is, until now, located in Spain, Italy, France, Greece,[10,11] and south of Portugal.[12] Experimental plantations of cultivated cardoon could afford a biomass productivity from 7.5 t dw/ha (Beja, Portugal)[12] to 33.4 t dw/ha (Tebas, Greece),[11] after the second growing season. Wild cardoon naturally develops in the Mediterranean basin countries and Macaronesia (Madeira and Canary Islands), while the artichoke cultivar is spread worldwide.[10]

Cultivated cardoon is much appreciated in regional dishes in Spain, Italy and France,[13] due to its fleshy stems and leaf petioles. Traditionally, capitula of wild cardoon and cultivated cardoon are used for producing several traditional ewe's cheeses, namely "Serra da Estrela", "Serpa", "Nisa" and "Évora" in Portugal,[14,15] and "La Serena" and "Guía" in Spain.[14] Furthermore, infusions of *C. cardunculus* L. leaves have been known in the folk medicine, given their hepatoprotective[16] and choleretic actions,[17] mainly ascribed to phenolic compounds, such as caffeoylquinic acids (e.g., cynarin)[16] and luteolin derivatives (e.g., luteolin 7-*O*-glucoside and luteolin).[17] Besides the traditional uses, various prospective industrial applications have also been considered for cultivated cardoon, owing to its high contents of cellulose and hemicelluloses,[11,18–21] as follows: pulp fibers production,[11,18] solid biofuel,[19] biogas[20] and bioethanol production.[21] Moreover, cultivated cardoon seed oil has shown to be adequate for the biodiesel production.[11]

Several reports have shown valuable biological properties of artichoke extracts, namely rat serum-triglycerides elevation inhibition,[22] antioxidant,[23] anti-inflammatory,[24] antitumoral[24,25] and antimicrobial.[26] These have been associated with the secondary metabolites composition, namely sesquiterpene lactones[22] (e.g. cynaropicrin), pentacyclic

triterpenes[24] (e.g. taraxasterol) and hydroxycinnamic acids (e.g. 5-*O*-caffeoylquinic acid).[23,25,26] Few studies have also revealed some of those biological activities, in what concerns to extracts of both wild and cultivated varieties of cardoon, namely antioxidant[27,28] and antitumoral,[27] mainly attributed to phenolic compounds.

Taking in account the increasing interest on the production of cultivated cardoon in the Mediterranean countries,[12,11,27,29] a detailed chemical characterization of the bioactive extractable components is still missing. This would open several possibilities for the valorization of cultivated cardoon biomass, integrated in a sustainable biorefinery concept,[30] through the extraction of added-value bioactive compounds, conciliated with the manufacturing of Iberian traditional ewe's cheeses, as well as with the energy production.

2. Objectives and outline of the thesis

The detailed chemical characterization of extractable compounds from *C. cardunculus* L. var. *altilis* (DC), as well as evaluation of their associated biological properties, would contribute for the valorization of its plantation. Some reports evidenced antioxidant[27,28] and antitumoral[27] effects of cultivated cardoon hydroalcoholic extracts, being mainly attributed to phenolic compounds. Nevertheless, it was not carried out a complete study describing, in detail, the chemical composition and the biological activity of different extractable classes of cultivated cardoon, principally regarding the lipophilic fraction. Moreover, no study reported, so far, the inhibitory functions of the lipophilic components of cultivated cardoon in important signaling pathways, in what concerns to tumor cells proliferation. Finally, at the current knowledge, no study has demonstrated the antibacterial activity of different extractable fractions of cultivated cardoon against multidrug-resistant (MDR) bacteria.

In this context, several objectives were traced for this thesis:

- to determine the chemical composition of lipophilic and phenolic-rich fractions of stalks (outer and inner parts), capitula (receptacles, bracts and florets) and leaves of *C. cardunculus* L. var. *altilis* (DC), by employing respectively gas chromatography-mass spectrometry and high performance liquid chromatography-mass spectrometry;
- to evaluate the antioxidant activity of cultivated cardoon phenolic-rich extracts, through an *in vitro* scavenging assay;
- to assess the *in vitro* inhibitory effects of lipophilic and phenolic-rich fractions of cultivated cardoon, as well as pure compounds regarding the most abundant identified compounds, on the cellular viability of MDA-MB-231, a human triple-negative breast cancer (TNBC) cell line;
- to study the suppressive actions of lipophilic and phenolic-rich extracts of cultivated cardoon, on two MDR bacteria, namely *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA).

The present thesis is thus organized in eight chapters.

In **Part A**, introduction and objectives of the thesis are indicated in the **Chapter I**. **Chapter II** is also included in **Part A**, which reviews the most relevant literature data about the chemical composition of *C. cardunculus* L. varieties, with more emphasis on extractable compounds, and the biological activity of their derived extracts and pure compounds, namely antioxidant, antitumoral and antibacterial properties.

The **Part B** is devoted to the chemical characterization of *C. cardunculus* L. var. *altilis* (DC).

Chapter III highlights the identification and quantification of lipophilic compounds in stalks (outer and inner parts), capitula (receptacles, bracts and florets) and leaves of *C. cardunculus* L. var. *altilis* (DC), by applying gas chromatography-mass spectrometry. Nuclear magnetic resonance spectroscopic techniques were also applied for the chemical identification of some specific compunds.

Chapter IV addresses the identification and quantification of phenolic compounds in the several morphological parts of *C. cardunculus* L. var. *altilis* (DC), by utilizing high-performance liquid chromatography-ultraviolet detection-mass spectrometry.

The **Part C** is focused on the evaluation of biological activity of lipophilic and phenolic-rich extracts derived from *C. cardunculus* L. var. *altilis* (DC).

Chapter V describes the antioxidant activity of cultivated cardoon phenolic-rich extracts, by assessing the *in vitro* scavenging effect on 2,2-diphenyl-1-picrylhydrazyl free radicals.

Chapter VI presents the inhibitory effects of cultivated cardoon lipophilic and phenolic-rich extracts, as well as of pure compounds, on TNBC MDA-MB-231 cellular viability. Moreover, downstream mechanisms involved in the cellular inhibition of the most potent extract(s) and pure compound(s) were further evaluated, in terms of other cellular assays. Furthermore, the molecular signaling pathways were investigated in what concerns to cell cycle regulation and Akt pathway.

Chapter VII comprehends the antibacterial activity of cultivated cardoon lipophilic and phenolic-rich extracts on the growth of two MDR bacteria, namely Gram-negative *P. aeruginosa* and Gram-positive MRSA.

Finally, **Part D** comprises the most relevant conclusions obtained in this thesis, as well as suggestions for future work (**Chapter VIII**).

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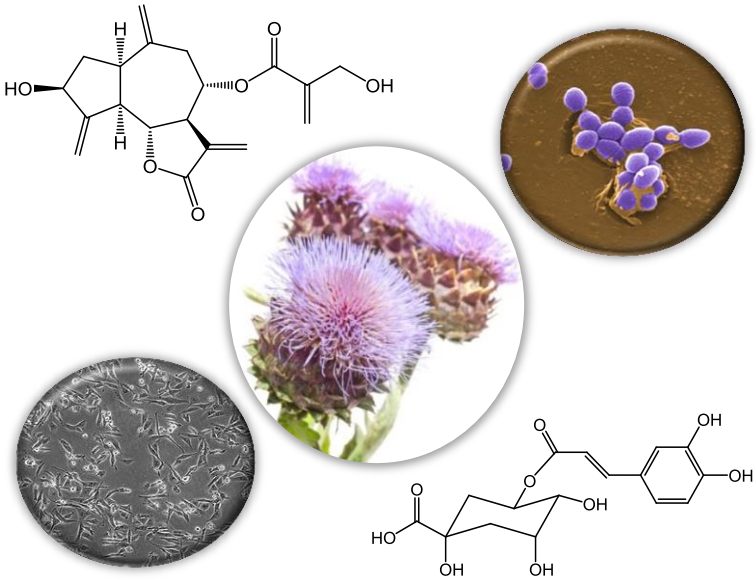
Chapter II

Bibliographic review



1. General considerations about *Cynara cardunculus* L.

1.1 Morphological characteristics

Cynara cardunculus L. belongs to the Asteraceae family,[1,2] being subclassified in three varieties (Figure 1): *sylvestris* (Lamk) Fiori (wild cardoon), *scolymus* (L.) Fiori (globe artichoke) and *altilis* DC (cultivated cardoon).

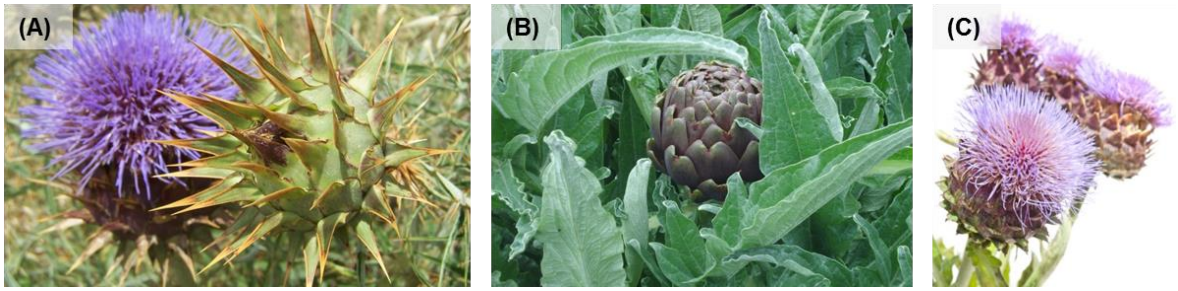


Figure 1: Illustrations of plants belonging to the three *C. cardunculus* L. varieties: (A) *sylvestris* (Lamk) (wild cardoon); (B) *scolymus* (L.) Fiori (artichoke); (C) *altilis* DC (cultivated cardoon).

Wild cardoon (Figure 1A) has a characteristic rosette of large leaves and branched stalks, with violet-blue capitula, all with long spines.[2,3] Several cytogenetic and isozyme studies[4–7] suggested that wild cardoon is the ancestor of both artichoke and cultivated cardoon, showing completely different morphological characteristics, as a result of agricultural selection criteria. Contrary to wild cardoon, *C. cardunculus* L. cultivated forms do not have any spines in stalks, capitula and leaves.[3,4,8] Artichoke (Figure 1B) was domesticated through the enlargement of capitula (immature inflorescences), displaying bracts notched at the tip. Cultivated cardoon (Figure 1C) was developed for the enlargement of the main leaf rib,[2] showing a globous violet-blue capitula with bracts tapering to a stout point.[3,9,10]

1.2 Geographical distribution

Wild cardoon is native from west and central parts of the Mediterranean basin (north and south Portugal, south Spain (Andalusia)), and it is spread over Macaronesia (Madeira and Canary Islands), North Africa, Cyprus and Turkey. This feral type is also a colonizer in Mexico, California, Argentina and Australia.[2,3] Regarding artichoke, its production is disseminated for all over the world, bringing an important economic impact, specially in Italy, Spain, France and Turkey. This crop is also produced in North Africa (Egypt, Morocco and Tunisia) and near East (Israel, Lebanon, Syrian and United Arab Republic). Apart from Mediterranean countries, artichoke is also cultivated in United States of America (California), South America (Argentina, Brazil, Chile and Peru) and China.[3,4,8,11] Finally, the production of cultivated cardoon is, until now, restricted to Spain, Italy, France, Greece[3,9,12] and south of Portugal.[13,14]

1.3 Life cycle

Cultivated and wild varieties of cardoon are herbaceous perennial plants (10-15 years) that grow under the xerothermic conditions of Mediterranean summers, marked by high temperatures and water stress. These plants have a robust root system (5 m in depth), which contributes greatly for their resistance to water-limited conditions, and for their regrowth of aboveground part after the summer.[12,15]

Figure 2 illustrates the main stages of life cycle of cultivated cardoon and wild cardoon, under the Mediterranean climatic conditions, namely cool humid winters and dry summers.[16]

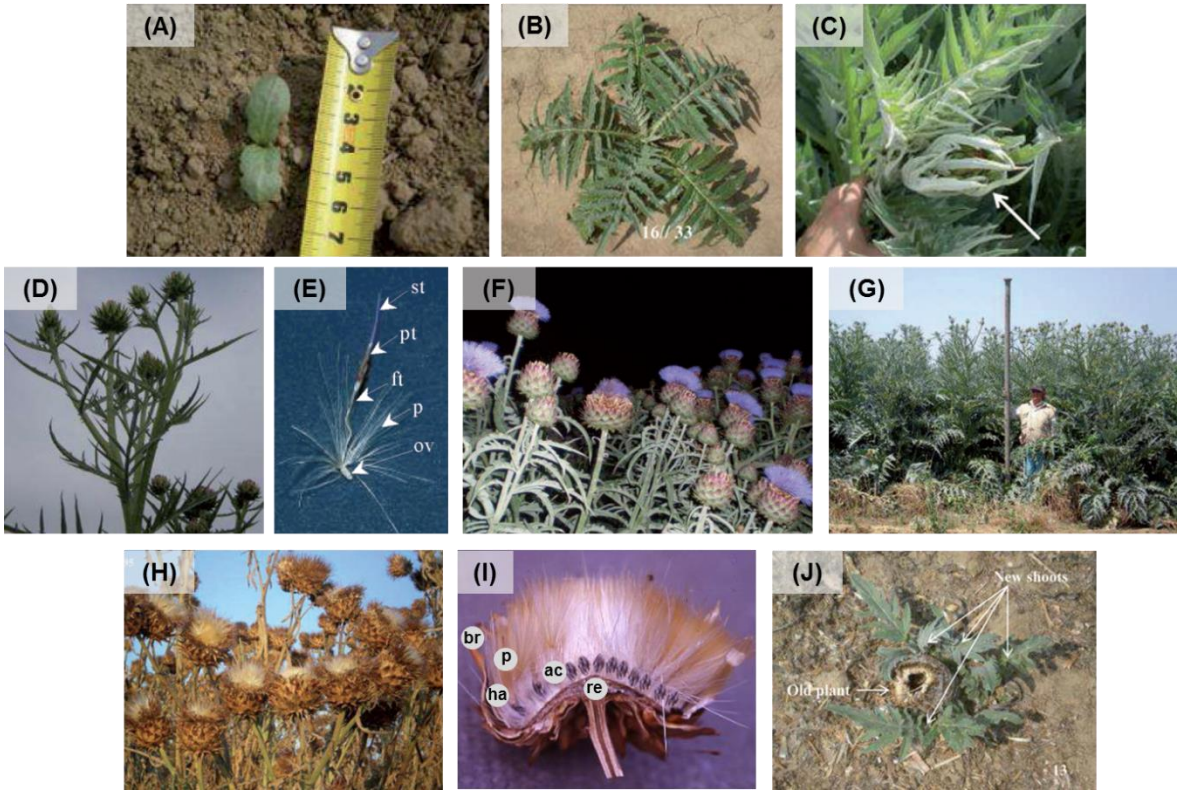


Figure 2: Illustrations of the main life cycle steps of cardoon (cultivated and wild varieties): (A) cotyledons emergence; (B) leaf rosette state; (C) first buds; (D) corymb group; (E) macroscopic view of floret: st, stigma; pt, petals; ft, floral tube; p, pappus; ov, ovary; (F) blossoming phase; (G) general perspective of adult wild cardoon; (H) senescence phase; (I) longitudinal cut of the capitulum at harvest time: br, bract; p, pappus; ha, hairs; ac, achene; re, receptacle; (J) first leaves burst in the beginning of the second cycle (adapted from [16–18]).

The first growth cycle of cardoon plants begins with the seed germination in early autumn (September-October). The two first fresh cotyledons then emerge through the soil surface (Figure 2A), followed by the growing of leaves in a rosette arrangement (November) (Figure 2B). Cardoon plants hold in the rosette stage during winter and early spring. Stalk

starts to elongate in April-May, whereas buds are already formed, though initially surrounded by green leaves (Figure 2C). The normal plant development goes on with the formation of higher branches (new buds) and elongation of older ones (enlarged buds), giving rise to a corymb group (Figure 2D). The inflorescence is formed by the capitulum (head), in which the florets (flowers) are located on a flattened surface (receptacle), surrounded by an ovoid-globose involucre with bracts. Mature florets (Figure 2E) have lilac petals and a calyx modified into a pappus, required for seed (achene) dispersal. Inflorescence emergence usually starts in the end of April, followed by the flowering (blossoming) (Figure 2F) in June. An adult plant can reach a height of 3 m (Figure 2G) and spread over an area of 1.5 m in diameter.[12] The plant senescence (Figure 2H) begins in mid-July and continues during August, with the dryness of stalks and leaves, as well as the opening and dryness of the heads (Figure 2I), and further seed dispersal. The underground part enters in dormancy (Figure 2J), until a new cycle begins with the leaf burst in the early autumn.[12,15,16,19]

1.4 Traditional and industrial applications

1.4.1 Traditional uses

Artichoke capitula make part of the Mediterranean diet since the 15th century.[20] Artichoke capitula are worldwide consumed as vegetables, either in fresh, frozen or canned form.[3,4,11] The artichoke leaf extract is also used to prepare the popular bitter liqueur “Cynar”.[2]

Fleshy inner stalks and leaf petioles of cultivated cardoon are collected in late autumn-early winter, and submitted to a blanching process before cooking, i.e. they are wrapped with straw to be buried underground for three weeks, in order to intensify their flavor and tenderness.[21] This “delicacy” is much appreciated in regional soups and stews in Spain, Italy, France.[3,9,10,21]

Traditionally, wild cardoon capitula have been used for centuries, in the Iberian Peninsula, for the manufacturing of valuable ewe’s cheeses,[3] such as “Serra da Estrela”, “Serpa”, “Nisa” and “Évora” in Portugal,[11,22] and “La Serena” and “Guía” in Spain.[11] The clotting activity of wild cardoon stigmas and styles extracts results from the high abundance of aspartic proteinases, namely cardosins A, B,[18,23] E, F, G and H.[24]

Artichoke and wild cardoon leaves infusions have been used since the 4th century B.C.,[25] owing to their healthy benefices, in protecting liver[26] and stimulating bile flow from the gallbladder (choleretic action).[25,27–29] Artichoke leaves and seeds extracts are also consumed to protect towards atherosclerosis, arterial hypertension and hyperuricemia.[2,30] Wild cardoon leaves are also popular in folk medicine, given their cardiotonic, antihemorrhodial, and antidiabetic actions.[31] Many of these physiological effects are related to the secondary metabolites composition, mainly terpenic and phenolic compounds, as it will be further discussed in this chapter.

1.4.2 Industrial applications

Cultivated cardoon experimental plantations have been implemented, under several European research and development projects, in some Mediterranean countries, such as Spain, Greece and Italy.[12] Depending on the edapho-climatic conditions, the annual biomass yield of cultivated cardoon could reach, at maximum, 33.4 t dw/ha in Greece, after the second growing season.[12] Ierna, *et al.*[32] also demonstrated that plantations of cultivated cardoon, in Italy, gave rise to higher biomass yields (24.8-31.0 t dw/ha) compared to the one of wild cardoon (8.9-19.4 t dw/ha), after the second growing season. A cultivated cardoon field was also implemented in the south of Portugal, producing about 7.5 t dw/ha, after the second growing season, which is a value considerably lower than the previous cultivated cardoon samples. This may be associated with, on the one hand, the soil and climatic conditions, particularly the rainfall periodicity, and, on the other hand, the biomass estimation procedure.[13] Recently, Lourenço *et al.*[14] described that a plantation of cultivated cardoon in Spain could yield, at maximum, 2804.7 g dw/plant; capitula, stalks and leaves represented 49.2%, 31.4% and 19.4%, respectively.

The lignocellulosic fraction of cultivated cardoon may be used as a solid biofuel,[12] as well as for the production of biogas[33] and bioethanol,[34] being a potential alternative to fossil materials in European countries. Stalks and capitula of cultivated cardoon may be also used to produce pulp fibers.[12,15,17] Moreover, the fatty acid composition of seed oil has revealed to be suitable for the production of biodiesel.[12,35]

Artichoke extracts make part of commercial dietary juices and capsules for the treatment of digestion perturbations, being related with the bioactive extractives composition, as explained bellow.[36–38] Finally, leaf rosettes of cultivated cardoon can be a good substitute for green forage during wintertime.[12]

2. General chemical composition of *C. cardunculus* L.

The proximate and elemental analyzes, as well as the heat value of cultivated cardoon biomass are listed in Table 1. *C. cardunculus* L. var. *altilis* (DC) biomass has high volatile matter content (73%), which is adequate to a good regulation of combustion or gasification processes in large-scale.[39] However, some environmental questions may occur, due the emission of nitrogen oxides during combustion, due to the relatively high nitrogen levels (1.5%). The study of Angelini, *et al.*[39] also indicated that the calorific value of cultivated cardoon was 14.9 MJ/kg. Moreover, Fernández, *et al.*[12] highlighted that the calorific values, in the different morphological parts of cultivated cardoon, can vary between 15.3 MJ/kg (basal leaves) and 23.3 MJ/kg (seeds). Recently, Lourenço, et al.[14] noticed a calorific value of 17.6 MJ/kg for cultivated cardoon stalks.

Table 1: Proximate analysis, elemental analysis and heat value of cultivated cardoon.[39]

Proximate analysis **(% dw)**	**Cultivated cardoon**
Moisture	10
Volatile matter	73
Fixed carbon	13.1
Ash	13.1
Elemental analysis **(% dw)**	
C	39
H	6.6
N	1.5
S	0.2
O	52.7
Cl	18.7
Calorific value **(MJ/kg)**	14.9

2.1 Monosaccharides

Table 2 depicts the monosaccharides composition of several morphological parts from cultivated cardoon. Glucose is the major monosaccharide of cultivated cardoon biomass, ranging from 36.3% dw in stalks[33] to 48.9% dw in capitula hairs.[17] Moreover, glucose represented the most abundant of the monosaccharides of cultivated cardoon (64.9-67.33% dw, regarding to the total monosaccharides content).[34,40] Xylose was also found in cultivated cardoon, at considerable contents (Table 2), varying between 16.3% in stalks[33] and 29.2% dw in capitula pappi.[17]

Table 2: Monosaccharides composition of cultivated cardoon.

Cultivated cardoon	**Monosaccharide content (% dw)**						**Source**
	Glucose	Galactose	Mannose	Rhamnose	Xylose	Arabinose	
Stalks	65.5 [(I)]	1.4 [(I)]	3.7 [(I)]	0.9 [(I)]	26.0 [(I)]	2.5 [(I)]	15
	67.33 [(I)]	1.74 [(I)]	1.42 [(I)]	0.96 [(I)]	27.07 [(I)]	1.48 [(I)]	40
	36.3	1.4	1.4	0.6	16.3	1.3	33
Stalks and leaves	64.9 [(I)]	2.5 [(I)]	1.8 [(I)]	1.1 [(I)]	27.0 [(I)]	2.7 [(I)]	34
Capitula hairs	48.9	0.8	1.0	–	24.4	0.9	17
Capitula pappi	40.5	1.4	1.0	0.7	29.2	1.7	

[(I)] Percent of total monosaccharides content.

Figure 3 elucidates the chemical structures of the most common monosaccharides found in cultivated cardoon biomass.

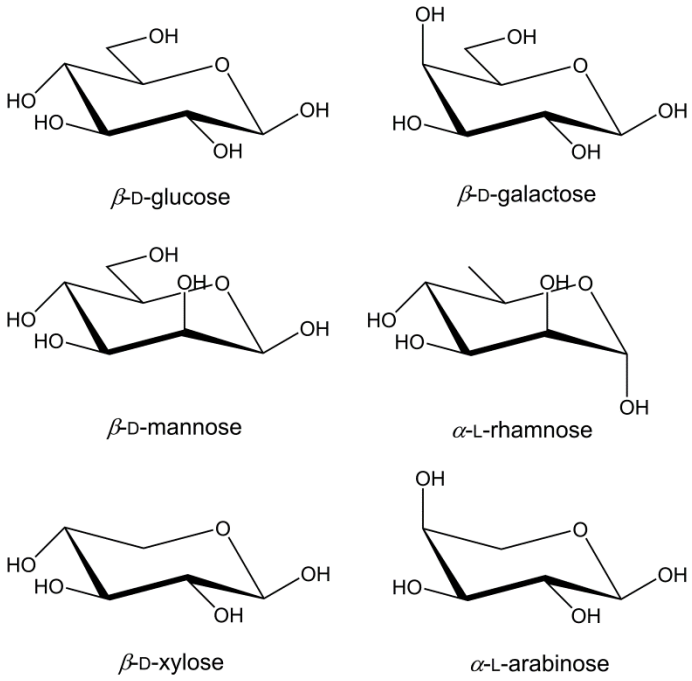


Figure 3: Structures of the most common monosaccharides in cultivated cardoon.

2.2 Polysaccharides

Polysaccharides are constituted by a large number of monosaccharide units, ranging from hundreds to thousands, connected by glycosidic bonds. These macromolecules are used, in plants, as energy storage forms or as structural materials.[41] Various works[17,33,34,40] have studied the polysaccharide composition of cultivated cardoon, as summarized in Table 3.

Table 3: Polysaccharide composition of cultivated cardoon.

Cultivated cardoon	Polysaccharides (% dw)			Source
	Cellulose	α-Cellulose	Hemicellulose	
Stalks	–	39.28	30.59	[40]
	–	36.3	16.3	[33]
Stalks and leaves	41.9	–	12.8	[34]
Capitula hairs	–	55.2	–	[17]
Capitula pappi	–	46.8	–	

Cellulose (Figure 4) consists of a homopolysaccharide composed by D-glucose units, linked to each other via *β*-1,4-glucosidic bonds,[41] and it is the most important structural polysaccharide in plants.[41] Stalks and leaves of cultivated cardoon (Table 3) exhibited 41.9% dw of cellulose.[34] More specifically, *α*-cellulose was detected in considerably high amounts in cultivated cardoon stalks (Table 3), ranging from 36.3 to 39.28% dw,[33,40] and even at higher levels in capitula hairs and pappi, accounting for 55.2 and 46.8% dw, respectively.[17]

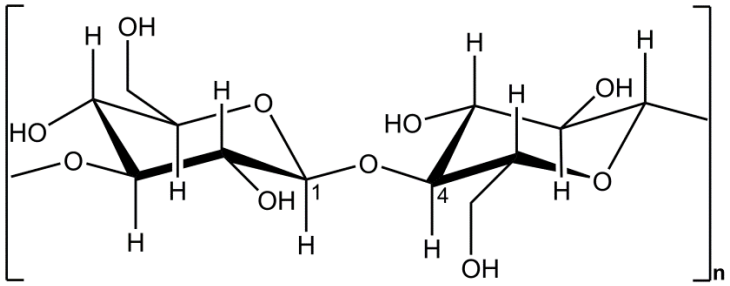


Figure 4: Structure of cellulose.

Hemicellulose comprises different heteropolysaccharides, formed by various monosaccharide units linked through *β*-1,4- and, occasionally, *β*-1,3-glycosidic bonds: D-xylose, D-mannose, D-galactose, D-glucose and L-arabinose (Figure 3), 4-*O*-methyl-glucuronic, D-galacturonic and D-glucuronic acids.[42] Depending on the predominant monosaccharide units, hemicelluloses can be separated in xylans, mannans, *β*-glucans and xyloglucans. Hemicelluloses are found associated with cellulose in the cell walls of higher plants.[43] Studies of Shatalov and Pereira,[40] and Oliveira, *et al.*[33] demonstrated that hemicellulose content of cultivated cardoon stalks can vary from 16.3 to 30.59% dw (Table 3). Nevertheless, Fernandes, *et al*.[34] indicated lower hemicellulose contents (12.8% dw in stalks and leaves of cultivated cardoon).

Among the hemicelluloses present in stalks, capitula and leaves of cultivated cardoon, the majority were probably xylans, based on the high xylose concentration.[33,34,40] Mannans and galactanans are also thought to be part of cultivated cardoon biomass,[33,34,40] owing to the detection of mannose and galactose (Table 2).

Inulin (Figure 5) is a linear polysaccharide, constituted by *β*-(2→1) linked fructofuranosyl units, with a terminal glucose residue. Being a reserve carbohydrate in Asteraceae plants, inulin is retained in roots of wild cardoon, artichoke and cultivated cardoon, at considerably high contents, accounting for 71.5%, 50.8% and 71.8% dw, respectively.[44] This is an important polysaccharide for the *C. cardunculus* L. development, since the rapid growth of aerial organs, and the subsequent sprouting of buds, demand high amounts of energy.[44]

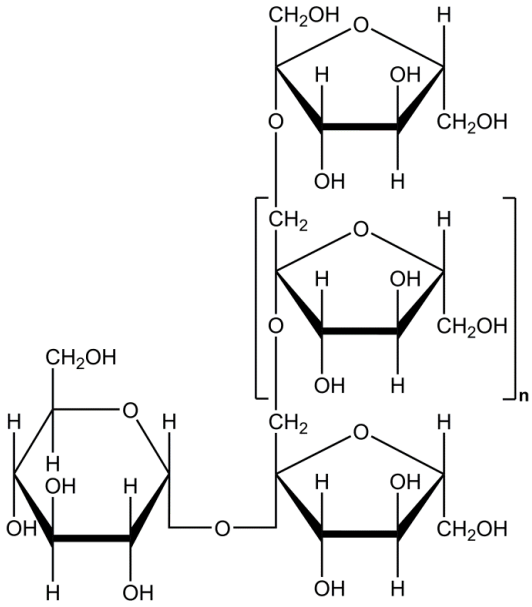


Figure 5: Structure of inulin.

2.3 Lignin

Lignin is characterized by an amorphous crosslinked and highly irregular macromolecular structure. Lignins can be considered as resulting from the polymerization of hydroxycinnamyl alcohol monomers, with different structures depending on the plant type.[45] The most important monomers are 4-hydroxycinnamyl alcohol (*p*-coumaryl alcohol), coniferyl alcohol and sinapyl alcohol[46] (Figure 6). Lignin carries an import strength function in the plant cell wall, as a matrix for cellulose microfibrils.

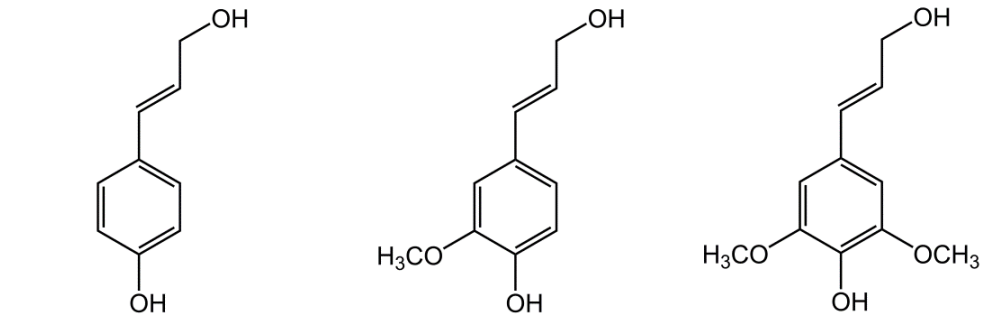


Figure 6: Structures of the most common hydroxycinnamyl alcohol monomers detected in lignin.

Table 4 indicates the lignin composition of stalks, capitula and leaves of cultivated cardoon. Low lignin content was observed in whole stalks (17.0-19.8% dw).[15,33] More specifically, low lignin abundances were detected in the outer (13.6% dw) and in the inner

(20.3% dw) parts of stalks[15] (Table 4). Hairs and pappi of capitula also presented low lignin proportions (10.6% dw and 17.8% dw, respectively).[17] In all morphological parts, the greatest part of lignin was acid-insoluble, ranging from 6.9% dw in capitula hairs[17] to 18.8% dw in stalks inner part.[15]

Table 4: Lignin composition of cultivated cardoon.

Cultivated cardoon	Lignin content (% dw)			Source
	Total	Klason (acid-insoluble)	Acid soluble	
Stalks	17.0	15.0	1.8	[15]
	–	15.46	–	[40]
	19.8	–	–	[33]
Stalks outer part	13.6	11.3	2.3	[15]
Stalks inner part	20.3	18.8	1.5	
Stalks and leaves	–	14.9	–	[34]
Capitula hairs	10.6	6.9	3.7	[17]
Capitula pappi	17.8	14.0	3.7	

Lourenço, *et al.*[14] have recently revealed the structure of lignin pyrolysis products, derived from stalks outer and inner parts of cultivated cardoon. This group justified that syringyl-units, including sinapyl alcohol (Figure 6), represented the major lignin component, being mostly retained in the inner part of stalks (64% of total lignin), compared to the outer part (53% of total lignin). Guaiacyl-units, like coniferyl alcohol (Figure 6), were mainly found in the stalks outer part (40% of total lignin), relatively to the inner part (29% of total lignin). Lourenço, *et al.*[14] also reported minor amounts of hydroxyphenyl-units (7% dw of total lignin in both parts), e.g. 4-hydroxycinnamyl alcohol (Figure 6). In this manner, outer and inner parts of stalks showed high syringyl-units/guaiacyl-units ratios (1.3 and 2.1, respectively), which means that the lignin of both components of cultivated cardoon stalks may have a more open matrix, being easier to remove it during the production of pulp fibers.[14]

3. Extractives composition of *C. cardunculus* L.

Extractives are the nonstructural components of plants, which can be removed with organic solvents and water.[47] These compounds are mainly present in plant cell lumens, albeit they may also be found in cell walls.[48] Most of the plant extractives are secondary metabolites.[47] Certain classes of secondary metabolites can be restricted to a particular taxonomic group (species, genus, family, or closely related group of families).[49] For example, sesquiterpene lactones are mostly exclusive from Asteraceae plants.[50]

Plants biosynthesize secondary metabolites with specific tasks for their survival, namely: (i) chemical defense against microorganisms, insects and herbivores, and even other plants (allelochemicals); (ii) attraction of animals for pollination and seed dispersal; and (iii) chemical adaptations to environmental stresses, for instance ultraviolet electromagnetic radiation, evaporation and cold.[49,51] Although frequently present in small amounts, secondary metabolites have shown to be highly valuable products, being commercially used as, for instance, pharmaceuticals, flavors, fragrances and pesticides.[49]

According to their biosynthesis pathways (Figure 7), plant secondary metabolites can be classified in three main groups: terpenes, phenolic compounds and alkaloids.[52,53] Terpenes are biosynthesized from dimethylallyl pyrophosphate and isopentenyl pyrophosphate. Sterols are also afforded by this pathway.[46,54] Phenolic compounds are derived from the shikimic acid pathway or the malonate/acetate pathway. Finally, alkaloids are mainly generated within the amino acids pathway.[46,49,52] Other plant compounds can be assigned as primary or secondary metabolites. For example, in the case of fatty acids and glucosides, the largest part is better described as primary metabolites, whilst some of them are extremely rare, being referred as secondary metabolites.[46] As indicated in the Figure 7, fatty acids are formed in the acetate pathway.[46]

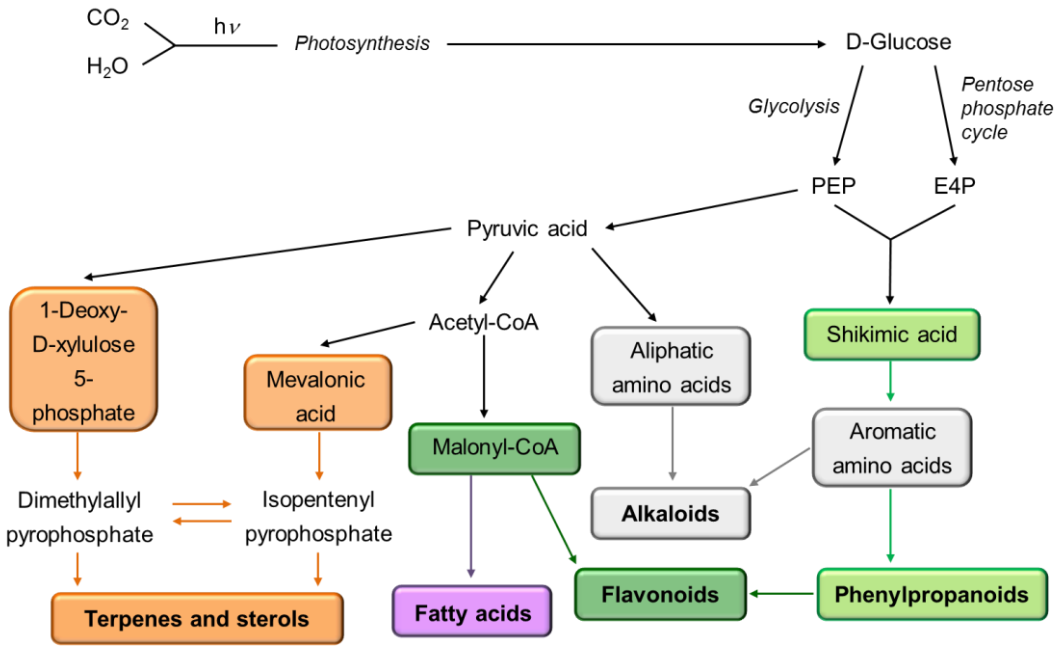


Figure 7: Secondary metabolism pathways for the biosynthesis of terpenes, phenolic compounds and alkaloids. Abbreviations: PEP, phosphoenolpyruvate; E4P, D-erythrose 4-phosphate; CoA, coenzyme A (adapted from [46,49,55]).

Table 5 outlines the extractives yields from stalks, capitula and leaves of cultivated cardoon[15,17,34,40] and wild cardoon,[56] by using organic solvents of different polarities, and water.

Table 5: Extractives yield (% dw) of stalks, capitula and leaves of cultivated and wild varieties of cardoon, by using organic solvents and water.

Cardoon	Extractives yield (% dw)							
	Hexane	Cyclohexane	DCM	Acetone	MeOH	EtOH	Water	Source
Cultivated cardoon								
Stalks (whole)	–	1.7	–	–	–	7.2	5.7	[15]
Stalks (whole)	–	–	0.41	–	–	3.25	3.70	[40]
Stalks outer part	–	1.6	–	–	–	5.4	6.8	[15]
Stalks inner part	–	1.4	–	–	–	6.4	5.1	
Stalks and leaves	–	–	–	1.4	–	–	–	[34]
Capitula hairs	–	–	0.5	–	–	2.8	2.1	[17]
Capitula pappi	–	–	0.8	–	–	3.9	1.2	
Wild cardoon								
Stalks	2.24	–	0.91	0.5	14.20	–	–	[56]
Leaves	2.77	–	4.44	3.15	5.77	–	–	

Abbreviations: DCM, dicholoromethane; EtOH, ethanol; MeOH, methanol.

Extractives yields (Table 5), obtained from cultivated cardoon stalks, were higher with ethanol (3.25-7.2% dw)[15,40] and water (3.70-6.8% dw)[15,40] than with non-polar organic solvents, such as cyclohexane (1.4-1.7% dw)[15] and dichloromethane (0.41% dw).[40] The extractives yields of cultivated cardoon capitula parts (Table 5) were also higher with ethanol and water, compared to those with dichloromethane.[17] These results may be associated with the extraction of more polar components, such as carbohydrates and phenolic compounds. Regarding to wild cardoon, Kammoun, *et al.*[56] highlighted much higher extractives yield of stalks with methanol (14.20% dw) than those obtained with less polar organic solvents, like hexane, dichloromethane and acetone. The extractives yields of wild cardoon leaves were similar between each other, being also higher with methanol (5.77% dw).[56]

The chemical composition of *C. cardunculus* L. varieties, in terms of lipophilic extractives, namely fatty acids, *syn*-alkane-6,8-diols, terpenes and sterols, as well as of phenolic compounds, will be discussed in detail.

3.1 Fatty acids

Fatty acids are biosynthesized through the acetate pathway. These plant metabolites are linear chains, formed by an even number of carbon atoms, ranging from 4 to 30, albeit the

commonest have 16 or 18 carbon atoms. Odd-chain fatty acids can arise either through the incorporation of other biosynthesis starting unit (e.g. propionic acid), or by loss of one carbon from an even-numbered fatty acid. Fatty acids can be classified as saturated, monounsaturated or polyunsaturated, depending on the number of double bonds. Among the unsaturated fatty acids, the *Z*-isomers are the most commonly present in plants.[46]

Fatty acids are mainly found esterified with glycerol, forming triglycerides, as in the case of vegetal oils, being an important energy reserve of seeds.[46] Maccarone, *et al.*[57] referred that the seed oil yields corresponded to 21.80-24.25% for wild cardoon, 23.50-24.55% for artichoke and 24.6-26.6% for cultivated cardoon. They also observed that the seed oils from *C. cardunculus* L. varieties presented 9*Z*,12*Z*-octadeca-9,12-dienoic acid (linoleic acid) (Figure 8), followed by 9*Z*-octadec-9-enoic acid (oleic acid) (Figure 8), either in the free or in the esterified form. Moreover, hexadecanoic acid (palmitic acid) and octadecanoic acid (stearic acid) (Figure 8) were the main saturated fatty acids found. Unsaturated fatty acids were prevalent over the saturated ones, in *C. cardunculus* L. seed oils.[57] Moreover, Curt, *et al*[19] also detected odd-numbered fatty acids, namely heptadecanoic and heptadecenoic acids, in cultivated cardoon seed oil (20.6-32.47% oil yield).

Farag, *et al.*[58] discovered 9*Z*,12*Z*,15*Z*-octadeca-9,12,15-trienoic acid (linolenic acid) in artichoke leaves, in addition to isomers of hydroxyoctadecadienoic, hydroxyoctadecatrienoic, dihydroxyoctadecadienoic, dihydroxyoctadecatrienoic acids.

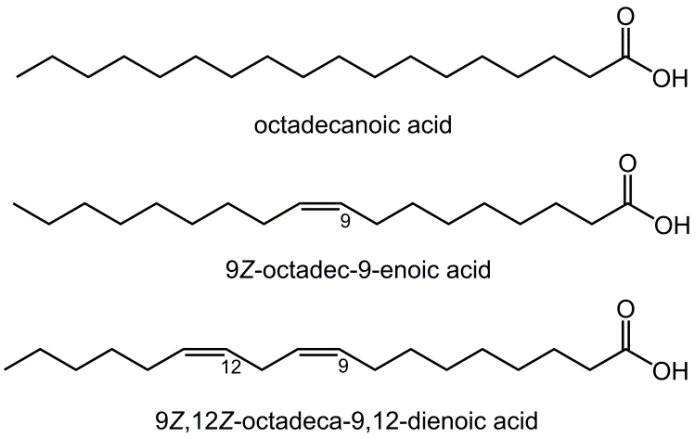
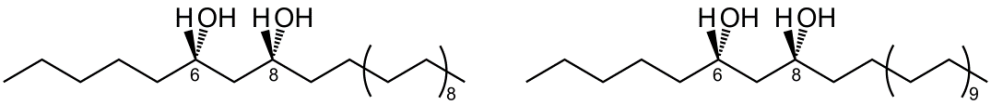


Figure 8: Structure of the most common fatty acids in seed oil of the three varieties of *C. cardunculus* L.

3.2 *syn*-alkane-6,8-diols

Akihisa, *et al.*[59] isolated ten *syn*-alkane-6,8-diols from capitula of cardoon, without referring the variety, namely *syn*(*R*,*S* and/or *S*,*R*)-C23-, C25-, C27-C33, and C35-alkane-6,8-diols. The odd chain *syn*-alkane-6,8-diols were clearly prevalent in the non-saponifiable lipid

fraction of methanol extract (91.3%). Later, Ukiya, *et al.*[60] indicated the 6*S*,8*R*-stereochemistry of C27- and C29-alkane-6,8-diols (Figure 9).



syn-6*S*,8*R*-heptacosanediol *syn*-6*S*,8*R*-nonacosanediol

Figure 9: Structures of two syn-alkane-6,8-diols isolated from cardoon capitula.

3.3 Terpenes

Terpenes can be considered as isoprene (2-methylbuta-1,3-diene) units-based structures, albeit their biosynthetic precursors are isopentenyl pyrophosphate and dimethylallyl pyrophosphate.[46] The number of isoprene units defines the different terpenic classes, such as: hemiterpenes (1 unit), monoterpenes (2 units), sesquiterpenes (3 units), diterpenes (4 units), sesterterpenes (5 units), triterpenes (6 units) and tetraterpenes (8 units) and polyterpenes (> 8 units).[46] Mono-, di-, tetraterpenes and some sesquiterpenes are biosynthesized through the 1-deoxy-D-xylulose 5-phosphate pathway, whereas the most of the sesquiterpenes and triterpenes are produced in the mevalonate pathway.[61]

Regarding isoprene structure (Figure 10), the isopropylene moiety is considered as the "head", while the ethylene moiety as the "tail". The isoprene units are bonded with each other from "head-to-tail" in mono-, sesqui-, di- and sesterterpenes, and from "tail-to-tail" in the center of tri- and tetraterpenes.[54]

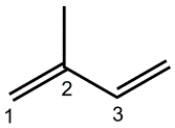


Figure 10: Structure of isoprene.

A great diversity of mono-, sesqui- and triterpenes were identified in the three varieties of *C. cardunculus* L., as next explained.

3.3.1 Monoterpenes

The biosynthetic precursor of monoterpenes (C_{10}) is the geranyl pyrophosphate which is originated by the combination of isopentenyl pyrophosphate and dimethylallyl pyrophosphate.[46] Monoterpenes are generally present in essential oils, which can be selectively removed from plants by hydrodistillation.

Four monoterpenes were found in the artichoke volatile oil, namely linalool, linalool oxide C, *α*-terpineol[62] and limonene[63] (Figure 11).

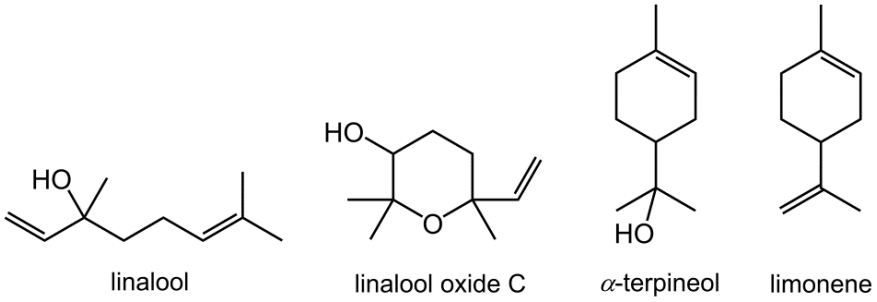


Figure 11: Structures of monoterpenes identified in artichoke volatile oil.

3.3.2 Sesquiterpenes

The farnesyl pyrophosphate is the direct precursor of sesquiterpenes (C_{15}), resulting from the addition of isopentenyl pyrophosphate to geranyl pyrophosphate.

Three sesquiterpene hydrocarbons were found in the artichoke volatile oil: *β*-selinene, caryophyllene and *α*-humulene (Figure 12).[62,63] Other members of this family were also detected in the artichoke volatile oil, namely *β*-elemene, *α*-cedrene and longifolene.[63]

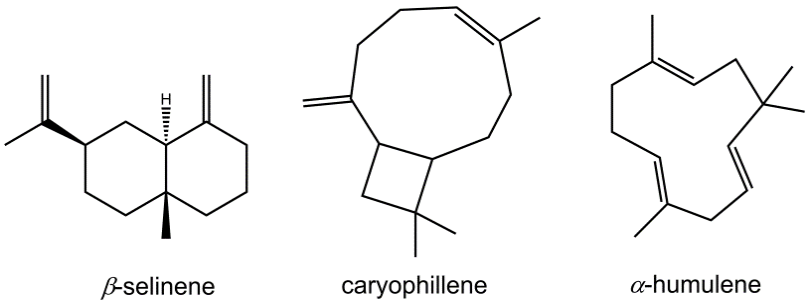


Figure 12: Structures of sesquiterpene hydrocarbons identified in artichoke volatile oil.

Sesquiterpene lactones are a specific class of sesquiterpenes, containing a lactone ring. This sesquiterpenic subclass can be organized in four main groups, according to their carbon-cyclic skeleton: (i) germacranolides (ten-membered ring); (ii) eudesmanolides and eremophilanolides (six/six-bicyclic compounds); (iii) guaianolides and pseudoguaianolides (all five/seven-bicyclic compounds)[50] and (iv) elemanolides (six-membered ring).[64]

The bitter taste of artichoke has been attributed to four guaianolides, namely dehydrocynaropicrin, cynaratriol, grosheimin and cynaropicrin[36,65] (Figure 13). These compounds were already isolated from capitula[66] and leaves[36,65–69] of artichoke.

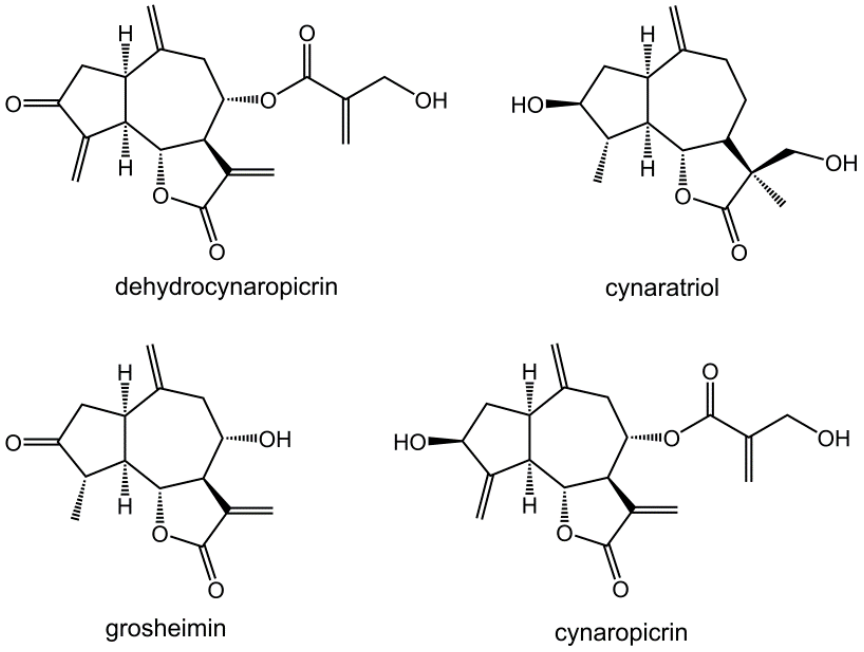


Figure 13: Structures of sesquiterpene lactones that contribute for the artichoke bitter taste.

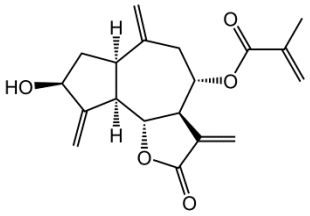
Cynaropicrin (Figure 13) is regarded as the main artichoke bitter principle, accounting for 80% of the total bitter artichoke taste.[36] This guaianolide is quite important to the organoleptic characteristics of the fresh artichoke capitula, as well as to the preparation of bitter liqueurs from artichoke leaf.[65] Schneider and Thiele[70] quantified the bitter principle, represented as cynaropicrin in artichoke leaves (<0.5-4.5% dw). Moreover, this group realized that the bitter principle in leaves achieved two maxima during the vegetative period, namely in early summer (July), and the in September until October.[70] Analyzing the distribution of cynaropicrin in the all morphological parts of artichoke, Eljounaidi, *et al.*[71] recently observed that this compound was mainly retained in leaves, at the mature stage (6 weeks old), accounting for 9.6 mg/g dw. They also noticed that the content of this compound decreased in the receptacles up to 8-fold, during the capitula development, until they reached the commercial stage (0.05 mg/g dw). This probably resulted from the domestication of artichoke.[72] Traces were registered in stalks and roots, while cynaropicrin was absent in external bracts.[71] Furthermore, Fritsche, *et al.*[36] noticed that the cynaropicrin content varied from <0.06 to 22.6 mg/g in commercial artichoke leaves extracts, available in the European market. In addition to cynaropicrin, 8-deoxy-11,13-dihydroxygrosheimin and grosheimin were also discovered in the commercial artichoke leaves extracts.[36] Several other sesquiterpene lactones, isolated from capitula[66,68,73] and leaves[36,65–68,74,75] of artichoke, are given in Table 6.

Table 6: Sesquiterpene lactones isolated from the three varieties of *C. cardunculus* L.

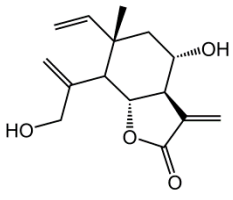
Sesquiterpene lactones	WC	Artichoke	CC
Aguerin A		c, l[66]	
Aguerin B	l[76]	c, l[66]	
Cynarascoloside A		l[68]	
Cynarascoloside B		l[68]	
Cynarascoloside C		c,[66] l[58,66,68]	
Cynaropicrin	l[76]	c,[66] l[65–69]	
Cynaratriol		c, l[66]	
Cynarinin A		c, l[66]	
Cynarinin B		c, l[66]	
Cynaroside A		c[73]	
Deacylcynaropicrin		[77]	
Dehydrocynaropicrin		c, l[66]	
Dehydromelitensin	l[76]		
11*β*,13-Dihydrodesacylcynaropicrin-8-*β*-$_D$-glucoside		l[68]	c[73]
11,13-Dihydroxi-8-desoxigrosheimin	l[76]		
11-H-13-Methylsulfonylgrosheimin (grosulfeimin)		l[74]	
8*α*-Acetoxyzaluzanin C	l[76]		
8-Deoxy-11,13-dihydroxygrosheimin		l[74]	
8-Deoxy-11-hydroxy-13-chlorogrosheimin		l[74]	
8-Epigrosheimin		l[74]	
Grosheimin	l[76]	c,[66] l[65–68]	
3*β*,8*α*,11*β*,13-Tetrahydroxy-10(14)-guaien-1*α*,4*β*,5*α*,6*β*H-6*α*,12-olide		l[75]	

Abbreviations: A, artichoke; c, capitula; CC, cultivated cardoon; l, leaves; WC, wild cardoon.

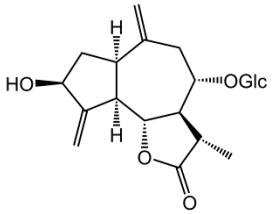
Súchy, *et al.*[69] earlier isolated cynaropicrin from cardoon, though without referring the variety. Besides cynaropicrin, Rial, *et al.*[76] isolated five guaianolides, including aguerin B (Figure 14), and an elemanolide named as dehydromelitensin (Figure 14) from wild cardoon leaves (Table 6). Moreover, Shimizu and colleagues[73] isolated 11*β*,13-dihydrodesacylcynaropicrin-8-*β*-D-glucoside (Table 6) from cultivated cardoon capitula.



aguerin B



dehydromelitensin



11*β*,13-dihydrodesacylcynaropicrin-8-*β*-D-glucoside

Figure 14: Structures of sesquiterpene lactones isolated from wild cardoon leaves and cultivated cardoon capitula. Abbreviation: Glc, glucosyl.

3.3.3 Triterpenes

Squalene, the biosynthetic intermediary of triterpenes (C_{30}), is constituted by two farnesane units bonded in the tail-to-tail manner.[54] Then, squalene is oxidized to 2,3-oxidosqualene. When the cyclization of 2,3-oxidosqualene occurs in the chair-chair-chair-boat conformation, the precursor of cyclic triterpenes, known as dammarenyl cation, is formed.[46,78] Dammarenyl cation can lead to the formation of a diversity of tetracyclic and pentacyclic triterpenic cations, direct precursors of tetracyclic (6-6-6-5) and pentacyclic (6-6-6-6-5 or 6-6-6-6-6) triterpenes, respectively.[46,79] The backbone structures of pentacyclic triterpenes comprise baccharanes, lupanes, oleananes, taraxastanes and ursanes.[54]

Table 7 describes the pentacyclic triterpenes isolated from capitula[80] and leaves[67,81] of artichoke.

Table 7: Pentacyclic triterpenes isolated from artichoke.

Triterpenes	Artichoke
α-Amyrin	c[80]
α-Amyrin acetate	c[80]
β-Amyrin	c[80]
β-Amyrin acetate	c[80]
Lupeol	l[67]
Ψ-Taraxasterol	c,[80,81] l[81]
Taraxasterol	c,[80,81] l[81]
Ψ-Taraxasteryl acetate	c[80]
Taraxasteryl acetate	c[80]

Abbreviations: c, capitula; l, leaves.

Two ursanes (*α*-amyrin and its acetate derivative), two oleananes (*β*-amyrin (Figure 15) and its acetate derivative), one lupane (lupeol) and four taraxastanes (*ψ*-taraxasterol and taraxasterol (Figure 15), and their acetate derivates) were isolated from artichoke capitula[80,81] and leaves (Table 7).[81] Lupeol was also isolated from artichoke leaves.[67]

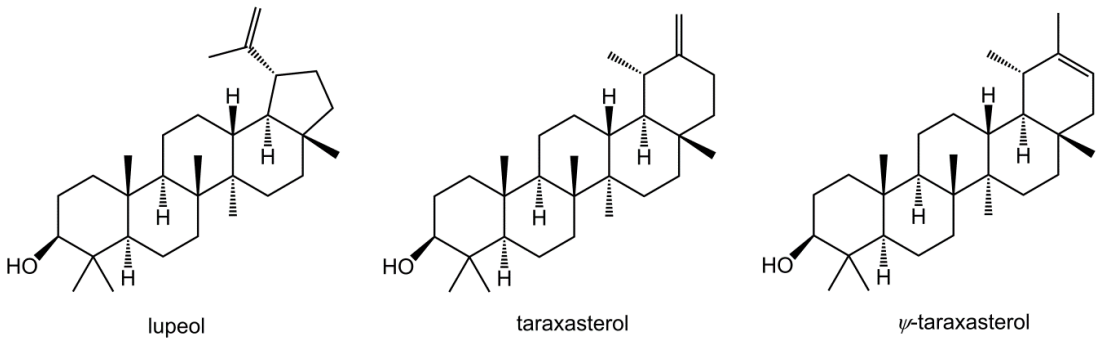


Figure 15: Structures of pentacyclic triterpenes isolated from capitula and leaves of artichoke.

The pentacyclic triterpenic composition, regarding to wild or cultivated variety, has been less explored. Grancai, *et al.*[82] isolated taraxasterol (Figure 15) and its acetate derivative from roots of cardoon, although without citing the variety. Moreover, Akihisa, *et al.*[83] also indicated the isolation of five pentacyclic triterpenes from cardoon capitula (without explaining the variety wild or cultivated), namely: *α*- and *β*-amyrin, lupeol, *ψ*-taraxasterol (Figure 15) and taraxasterol.

3.4 Sterols

The protosteryl cation, resulting from the cyclization of 2,3-squalene oxide in a chair-boat-chair-boat conformation, is the biosynthetic precursor of sterols.[46]

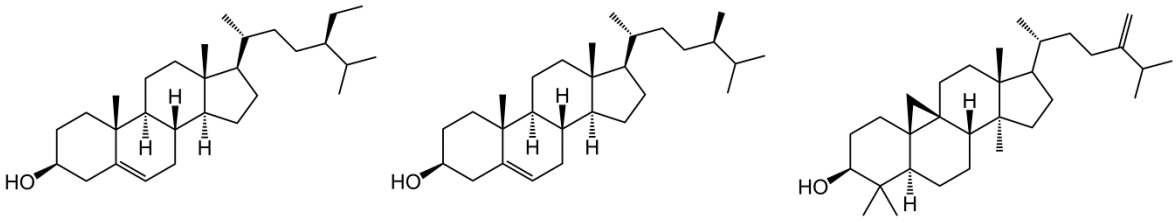
Table 8 lists the sterols detected in the three varieties of *C. cardunculus* L.

Table 8: Sterols identified in several morphological parts of *C. cardunculus* L. varieties.

Sterols	Wild cardoon	Artichoke	Cultivated cardoon
β-Sitosterol	s[57]	c,[81] s[57]	s[57]
Campesterol	s[57]	s[57]	s[57]
Δ^7-Stigmastenol	s[57]	s[57]	s[57]
Stigmasterol	s[57]	s[57]	s[57]
24-Methylenecycloartanol		c[83]	

Abbreviations: c, capitula; s, seed oil.

Maccarone, *et al.*[57] identified three stigmastanes, namely *β*-sitosterol, stigmasterol (Figure 16) and Δ^7-stigmastenol, and a campestane, known as campesterol, in the seed oil of the three varieties of *C. cardunculus* L. (Table 8). Furthermore, a cycloartane, named as 24-methylenecycloartanol (Figure 16), was isolated from cultivated cardoon capitula.[57]



β-sitosterol campesterol 24-methylenecycloartanol

Figure 16: Structures of sterols identified in the three varieties of *C. cardunculus* L.

A study of Grancai, *et al.*[82] indicated the isolation of *β*-sitosterol, sitosteryl-3*β*-acetate and sitosteryl-3*β*-D-glucoside from roots and inner bracts of cardoon, but with no specification regarding to either wild or cultivated variety.

3.5 Phenolic compounds

Phenolic compounds are constituted by one or various aromatic rings, containing one or more hydroxyl substituents. Phenolic compounds can be classified according to basic phenolic skeleton:

(i) simple phenols and benzoquinones (C_6);
(ii) phenolic acids (C_6–C_1);
(iii) phenylacetic acids (C_6–C_2);
(iv) phenylpropenes, cinnamic acid, hydroxycinnamic acids, coumarins and chromones (C_6–C_3);
(v) naphtoquinones (C_6–C_4);
(vi) xanthones (C_6–C_1–C_6);
(vii) stilbenes and anthraquinones (C_6–C_2–C_6);
(viii) flavonoids (C_6–C_3–C_6);
(ix) lignans ((C_6–C_3)$_2$);
(x) biflavonoids ((C_6–C_3–C_6)$_2$);
(xi) tannins (polymers).

Several subgroups of phenolic compounds have been elucidated in the three *C. cardunculus* L. varieties, as shown in this chapter.

3.5.1 Phenolic acids

Phenolic acids are phenolic compounds characterized by having one carboxylic group. Two phenolic acids, namely protocatechuic and gallic acids (Figure 17), were identified in artichoke industrial wastes (bracts, receptacles and floral stems).[84] Vanillic and syringic acids (Figure 17) were also found in wild cardoon leaves.[85]

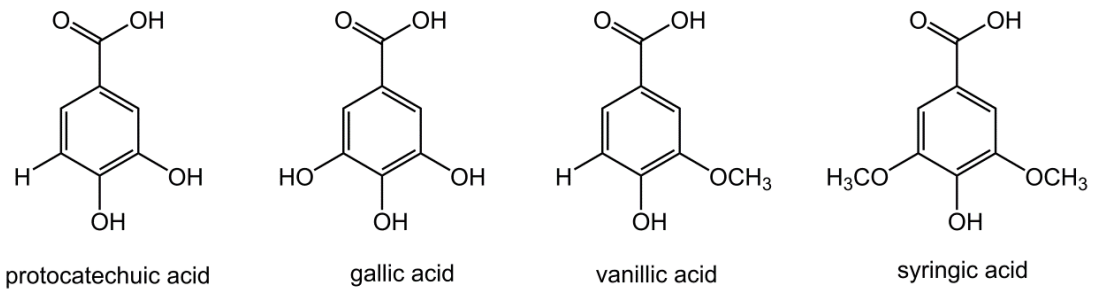


Figure 17: Structures of phenolic acids identified in artichoke by-products and wild cardoon.

3.5.2 Cinnamic and hydroxycinnamic acids, and coumarins

E-cinnamic acid was detected by Falleh, *et al.*[85] in wild cardoon leaves. Hydroxycinnamic acids are hydroxyl derivatives of cinnamic acid. These compounds were commonly found in *C. cardunculus* L. Two hydroxycinnamic acids, known as *p*-coumaric and ferulic acids (Figure 18 and Table 9), were identified in wild cardoon leaves,[85] as well as in capitula of artichoke[86] and cultivated cardoon.[87] Another hydroxycinnamic acid, caffeic acid (Figure 18 and Table 9), was commonly identified in leaves[88–90] and outer bracts of capitula[88] of artichoke. Industrial by-products of artichoke also contained caffeic acid.[84,91]

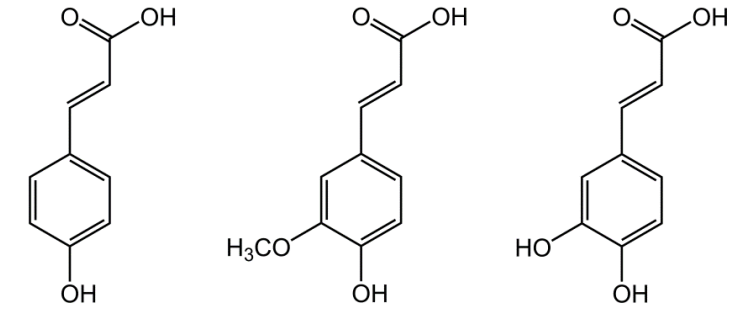


Figure 18: Structures of hydroxycinnamic acids identified in the *C. cardunculus* L. varieties.

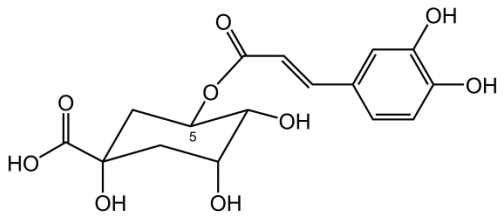
Table 9: Hydroxycinnamic acids identified in several morphological parts of *C. cardunculus* L. varieties.

Hydroxycinnamic acids	Wild cardoon	Artichoke	Cultivated cardoon
Caffeic acid		c,[88] l,[88–90] bp[84,91]	
Ferulic acid	l[85]	r, b[87]	r, b[87]
Ferulic acid glucoside isomer		bp[84]	
Feruloylquinic acid isomer		l, bp[37]	
3-*O*-Feruloylquinic acid		l[58]	
p-Coumaric acid	l[85]	c,[86] r, b[87]	r, b[87]
p-Coumaric acid glucoside isomer		c,[86] bp[84]	
Sinapic acid	l[85]		
Monocaffeoylquinic acids			
1-*O*-Caffeoylquinic acid (pseudochlorogenic acid)	l[9]	fs,[92,93] c,[38,94,95] r,[93,95–97] b,[93,95,97] l,[58,89,92,98] ds, bp[38]	l[9]
3-*O*-Caffeoylquinic acid (neochlorogenic acid)	fs, l[92,99]	fs,[92,93] c,[38,94,95] r,[93,95,96] b,[93,95] l,[37,58,89,90,92] ds,[36–38] bp[38]	fs, l[92]
4-*O*-Caffeoylquinic acid (cryptochlorogenic acid)	l[99]	fs,[93] c,[38,94,95] r, b,[93,95] l,[58,89,90] ds,[36,38] bp[91]	
5-*O*-Caffeoylquinic acid (chlorogenic acid)	fs,[92] r, b,[87] l[9,92,99]	fs,[92,93] c,[38,94,95,98] r,[87,95–97] b,[87,93,95,97] l,[37,58,89,90,92,98,100] ds,[36–38] bp[38,84,91]	fs,[92] l[9,92]
Caffeoylquinic acid diglycoside isomer		l[37]	
Dicaffeoylquinic acids			
1,3-Di-*O*-caffeoylquinic acid (cynarin)	l[99]	fs,[93] c,[38,94,95,98,101] r, b,[87,93,95] l,[37,58,98,100] j,[36–38] bp[38]	r, b[87]
1,4-Di-*O*-caffeoylquinic acid		bp[91]	
1,5-Di-*O*-caffeoylquinic acid	fs,[92] c,[102] l[92,99]	fs,[92,93] c,[38,94,95,102] r, b,[93,95,97] l,[37,58,90,92] ds,[37] bp,[38,91] ds[38]	fs[92]
3,4-Di-*O*-caffeoylquinic acid		fs,[93] c,[38,94] r, b,[93] l,[37,90] ds, bp[38]	
3,5-Di-*O*-caffeoylquinic acid		fs,[92,93] c,[38,94,101] r, b,[93] l,[37,90,100] ds,[37,38] bp[38,91]	
4,5-Di-*O*-caffeoylquinic acid		fs,[93] c,[38,94,101] r,[93,97] b,[97] l,[37,90,100] ds,[38] bp[38,91]	
1,3-Di-*O*-caffeoylquinic acid hexoside isomer		l[37]	
Dicaffeoylsuccinoylquinic acids			
Dicaffeoylsuccinoylquinic acid isomers	fs,[92] l[9]	fs[92]	fs,[92] l[9]
Dicaffeoyldisuccinoylquinic acid isomer	l[9]		

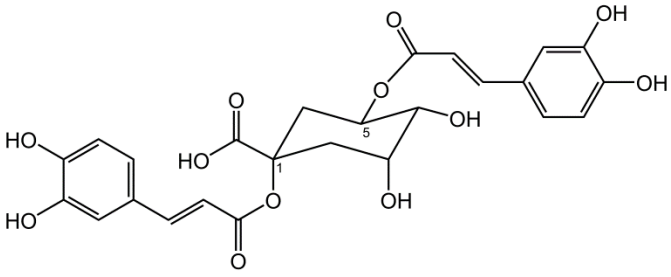
Abbreviations: b, bracts; bp, by-products; c, capitula; ds, dietary supplements; fs, floral stems; j, juice; l, leaves; r, receptacles.

3.5.2.1 Caffeoylquinic acids

Caffeic acid can be found esterified with quinic acid, originating caffeoylquinic acids (Table 9 and Figure 19).



5-*O*-caffeoylquinic acid



1,5-di-*O*-caffeoylquinic acid

Figure 19: Structures of the main caffeoylquinic acids identified in *C. cardunculus* L.

Monocaffeoylquinic acids, namely 1-, 3-, 4- and 5-*O*-caffeoylquinic acids (Table 9), are commonly present in leaves,[37,89,90,92,98,100] floral stems[92,93] and capitula of artichoke,[38,94–96,98,102] more specifically in receptacles, external, intermediate and internal bracts.[87,93,95–97] These compounds were also detected in commercially available dietary supplements (e.g. juices from capitula and capsules containing leaves extract),[36–38] as well as in industrial by-products of artichoke.[38,84,91] According to several studies on the quantitative monocaffeoylquinic acid composition of artichoke,[38,92,94–96,102] 5-*O*-caffeoylquinic acid (Table 9) was the most common compound of this subclass, accounting for 13.3 mg/g dw in the floral stems.[92] Regarding the two varieties of cardoon, 5-*O*-caffeoylquinic acid also given as the most abundant monocaffeoylquinic acid in floral stems[92] and leaves,[9,92] reaching 21.91 and 26.11 g/kg dw in leaves of respectively wild and cultivated types.[9]

Six dicaffeoylquinic acids, namely 1,3-; 1,4-; 1,5-; 3,4-; 3,5- and 4,5-di-*O*-caffeoylquinic acids (Table 9), were identified in floral stems,[92,93] leaves,[37,90,92,98,100] and capitula[38,94,95,98,101,102] of artichoke. These compounds were also detected in the constitutive parts of capitula, such as receptacles, outer, intermediate and inner bracts.[87,93,95,97] Furthermore, dicaffeoylquinic acids were also detected in commercial dietary supplements,[36–38] as well as in artichoke industrial residues.[38,91] The 1,5-di-*O*-caffeoylquinic acid (Table 9) was

the most abundant compound of this subclass in the studied morphological parts of artichoke,[37,92,93,95,97] achieving about 28.8 g/kg dw in capitula.[95] In what concerns to cardoon varieties, 1,5-di-*O*-caffeoylquinic acid was found in floral stems of cultivated cardoon,[92] as well as in floral stems,[92] capitula[102] and leaves[92,99] of wild cardoon. This compound accounted for 13.3 g/kg dw and 6.2 g/kg dw in floral stems of wild cardoon and cultivated cardoon, respectively.[92]

Gouveia, *et al.*[37] also discovered glucosylated derivatives of caffeoylquinic acids in artichoke leaves, namely 1,3-di-*O*-caffeoylquinic acid hexoside and a caffeoylquinic acid diglycoside isomer (Table 9).

Dicaffeoylsuccinoylquinic acids are dicaffeoylquinic acids that present one or more succinoyl moieties esterified with quinic acid. An isomer (Table 9) was identified in the floral stems of artichoke.[92] Furthermore, two dicaffeoylsuccinoylquinic acid isomers were also detected in floral stems[92] and leaves[9] of both cardoon types. Only one dicaffeoyldisuccinoylquinic acid isomer was detected in wild cardoon leaves.[9]

3.5.2.2 Coumarins

Coumarins are hydroxycinnamic acid lactone derivatives, constituted by fused benzene and *α*-pyrone rings.[46,103] Sánchez-Rabaneda, *et al.*[84] reported esculin (Figure 20) in artichoke by-products.[84] Moreover, Grancai, *et al.*[104] isolated scopoletin and its glucosylated form, i.e. scopolin (Figure 20), from capitula involucral bracts of cardoon, albeit they did not refer the variety.

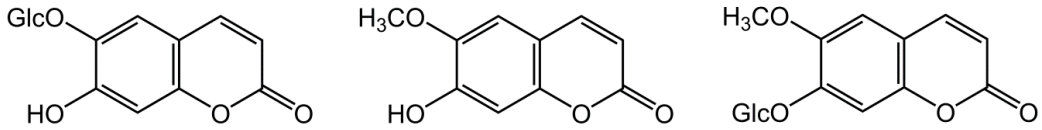


Figure 20: Structures of coumarins identified in *C. cardunculus* L. plants. Abbreviation: Glc, glucosyl.

3.5.3 Flavonoids

Flavonoids derive from a cinnamoyl-CoA starting unit and a chain extension with three molecules of malonyl-CoA.[46] These compounds are constituted by two aromatic rings joined through a three carbon chain (C_6-C_3-C_6). The central propane chain may form a closed pyran ring with one of the two aromatic rings. These compounds can be organized in several subclasses, based on the oxidation state of the central pyran ring, as follows (Figure 21): chalcones, aurones, flavanones, flavanonols, flavones, flavonols, isoflavones, flavan-3-ols and anthocyanidins.[105] Mono-, di-, tri- and tetrasaccharide units are frequently attached to the

flavonoid nucleus. Aromatic and aliphatic acids, prenyl, methylenedioxyl, isoprenyl, methyl, methoxyl, hydroxyl or sulfate groups are often part of flavonoid chemical structure.[105]

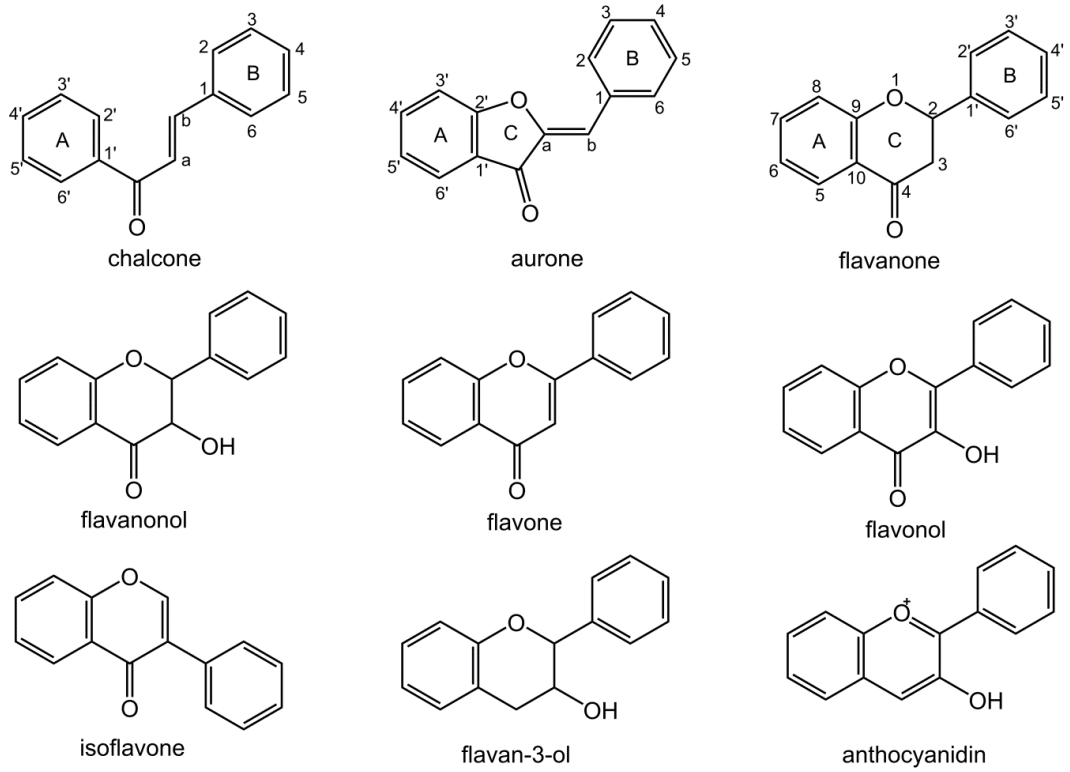


Figure 21: Backbone structures of the flavonoid subclasses commonly found in the plant kingdom.

3.5.3.1 Flavanones

Several studies[38,84,86,93,94,98] investigated the presence of flavanones in capitula, commercial products and industrial residues of artichoke, as depicted in Table 10.

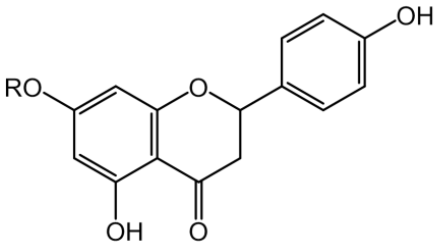
Table 10: Flavanones identified in artichoke morphological parts.

Flavanones	Artichoke
Naringenin	fs,[93] c,[94] r, b,[93] bp[84]
Naringenin 7-*O*-glucoside (prunin)	fs,[93] c,[38,94] r, b,[93] j,[38] bp[38,84]
Naringenin 7-*O*-neohesperidoside (naringin)	bp[84]
Naringenin 7-*O*-rutinoside (narirutin)	c,[38,86,94,98] j, bp[38]
Eriodictyol glucuronide isomer	bp[84]

Abbreviations: b, bracts; bp, by-products; c, capitula; fs, floral stems; j, juice; r, roots.

Naringenin and two glycosylated forms, namely naringenin 7-*O*-glucoside and 7-*O*-rutinoside (Figure 22), were found in capitula[38,86,94,98] and their constitutive parts, such as

receptacles, outer and inner bracts[93] (Table 10). Naringenin 7-*O*-glucoside was the most abundant naringenin derivative, accounting for 106.8 mg/kg dw in artichoke receptacles.[93] Naringenin 7-*O*-neohesperidose (Figure 22) was only detected in the artichoke by-products.[84]



R=H	naringenin
R=Glc	naringenin 7-*O*-glucoside
R=Glc-Rha (rhamnosyl(*α*1→2)-glucoside)	naringenin 7-*O*-neohesperidoside
R=Glc-Rha (rhamnosyl(*α*1→6)-glucoside)	naringenin 7-*O*-rutinoside

Figure 22: Structures of naringenin and derivatives identified in artichoke. Abbreviations: Glc, glucosyl; Rha, rhamnosyl.

An eriodictyol glucuronide isomer was discovered in artichoke by-products (Table 10 and Figure 23).[84]

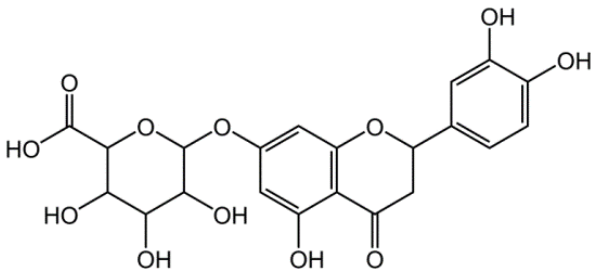


Figure 23: Structure of eriodictyol 7-*O*-glucuronide.

3.5.3.2 Flavones

Flavones, such as apigenin, chrysoeriol and luteolin and their derivatives, were identified in wild cardoon, artichoke and cultivated cardoon, as explained in Table 11.

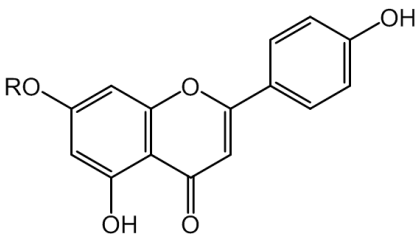
Table 11: Flavones identified in several morphological parts of *C. cardunculus* L. plants.

Flavones	WC	Artichoke	CC
Apigenin derivatives			
Apigenin	fs,[92] c,[102,106] l[9,85,92]	fs,[92,97] c,[102] r, b,[87,95–97] l,[58,92] bp[84]	fs,[92] c,[102] l[9,92]
Apigenin 7-*O*-acetylglucoside		c[86]	
Apigenin 7-*O*-glucoside	l[92,99]	fs,[97] c,[38,94,95] r, b,[93,95–97] l,[58,92,100] d[37]	l[92]
Apigenin 7-*O*-glucuronide	l[9,92]	fs,[92,97] c,[38,94,95,102] r, b,[93,95–97] l,[107] ds[37] bp[84]	c,[102] l[9,92]
Apigenin 7-*O*-malonylglucoside isomer	fs, l[92]	fs,[92,97] c,[102] r, b,[96,97] l[92]	fs,[92] c,[102] l[92]
Apigenin 7-*O*-rutinoside (isohoifolin)	c,[102,106] l[9,92]	c,[38,94,95,98] r,[93] b,[93,95–97] l,[92,100] bp[84]	c,[102] l[9,92]
Chrysoeriol		bp[84]	
Luteolin derivatives			
Luteolin	c,[102,106] l[9,92]	fs,[92,97] c,[95,102,107] r, b,[87,95–97] l,[90,92,107] bp[84]	fs,[92] r, b,[87] l[9,92]
Luteolin 7-*O*-acetylglucoside		ds[37]	
Luteolin 7-*O*-galactoside		bp[84]	
Luteolin 7-*O*-glucuronide	l[9,92]	c,[38,94,95,102] r, b,[93,95–97] l,[37,92,107] ds,[37] bp [84]	fs,[92] l[9,92]
Luteolin 7-*O*-glucoside (cynaroside)	fs,[92] l[9,92,99]	fs,[92,97] c,[38,94,95,102] r, b,[93,95–97] l,[58,90,92,98,100,107] ds[36] bp[84]	fs,[92] l[9,92]
Luteolin 7-*O*-malonylglucoside	l[9]	fs,[97] l[107]	l[9]
Luteolin 7-*O*-malonylglucoside isomer	fs[92]	l[92]	
Luteolin 7-*O*-neohesperidoside		bp[84]	
Luteolin 7-*O*-rhamnoside		bp[84]	
Luteolin 7-*O*-rutinoside (scolymoside)	fs,[92] l[9,92]	fs,[92,97] c,[94,95,102] r, b,[93,95–97] l,[58,92,98,100,107] bp[84]	l[9]

Abbreviations: b, bracts; bp, by-products; c, capitula; CC, cultivated cardoon; ds, dietary supplements; fs, floral stems; l, leaves; r, receptacles; WC, wild cardoon.

Apigenin and several glycosylated derivatives, namely apigenin 7-*O*-glucoside, 7-*O*-glucuronide and 7-*O*-rutinoside (Figure 24), and an apigenin malonylglucoside isomer, were identified in the floral stems,[92,97] capitula,[38,94,95,98,102] receptacles and bracts,[87,93,95–97] and leaves[92,100,107] of artichoke (Table 11). With the exception of apigenin malonylglucoside isomer, all of these apigenin derivatives were also found in dietary supplements,[37] as well as in industrial by-products of artichoke.[84] These compounds were mainly concentrated in capitula of artichoke,[38,93,94,96,97,102,107] with apigenin glucuronide as the most abundant one,

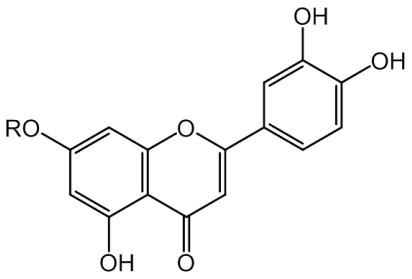
achieving ≈6.2 g/kg dw in receptacles.[93] Regarding to cardoon, apigenin and its derivatives were also present in floral stem, capitula and leaves of wild[9,85,92,99,102,106] and cultivated types[9,92,102] (Table 11). Furthermore, Mucaji, *et al.*[108] isolated apigenin 7-*O*-methylglucuronide (Figure 24) from cardoon capitula, without citing which was the variety. Apigenin 7-*O*-glucuronide was shown as the most common apigenin derivative in both cardoon varieties,[9,92,102] reaching about 10.59 g/kg dw in leaves of wild cardoon[9] and ≈7.0 g/kg dw in capitula of cultivated cardoon.[102]



R=H	apigenin
R=GlcAc	apigenin 7-*O*-acetylglucoside
R=Glc	apigenin 7-*O*-glucoside
R=GlcUA	apigenin 7-*O*-glucuronide
R=GlcMetUA	apigenin 7-*O*-methylglucuronide
R=Glc-Rha	apigenin 7-*O*-rutinoside

Figure 24: Structures of apigenin and derivatives identified in *C. cardunculus* L. varieties. Abbreviations: GlcAc, acetylglucosyl; Glc, glucosyl; GlcMetUA, methylglucuronyl; GlcUA, glucuronyl; Rha, rhamnosyl.

Luteolin 7-*O*-glucoside, 7-*O*-glucuronide, 7-*O*-rutinoside, 7-*O*-malonylglucoside and luteolin (Figure 25) were identified in the floral stems,[92,97] capitula[38,86,90,92–98,100,102,107] and leaves[37,90,92,93,95–98,100,107] of artichoke (Table 11). Luteolin 7-*O*-glucoside and 7-*O*-glucuronide were also found in dietary supplements of artichoke.[36,37] Furthermore, luteolin 7-*O*-galactoside, 7-*O*-rhamnoside and 7-*O*-neohesperidoside (Figure 25) were detected in industrial artichoke by-products.[84] Luteolin 7-*O*-glucuronide displayed the highest abundance among luteolin derivatives of artichoke,[38,92–97,102,107] achieving about ~1.6 g/kg dw in receptacles.[93] In what concerns to cardoon, luteolin 7-*O*-glucoside, 7-*O*-glucuronide, 7-*O*-rutinoside and 7-*O*-malonylglucoside, and luteolin were detected in the floral stems,[92] capitula[87,102] and leaves[9,92] of wild[9,92,99,102,106] and cultivated cardoon[9,87,92] (Table 11). According to quantitative analysis,[9,92,102] luteolin 7-*O*-malonylglucoside was the most common luteolin derivative in leaves of cultivated cardoon and wild cardoon, approximately at 22.98 and 8.05 g/kg dw, respectively.[9]



R=H	luteolin
R=Gal	luteolin 7-*O*-galactoside
R=Glc	luteolin 7-*O*-glucoside
R=GlcUA	luteolin 7-*O*-glucuronide
R=MalGlc	luteolin 7-*O*-malonylglucoside
R=Glc-Rha (rhamnosyl(*α*1→2)-glucoside)	luteolin 7-*O*-neohesperidoside
R=Rha	luteolin 7-*O*-rhamnoside
R=Glc-Rha (rhamnosyl(*α*1→6)-glucoside)	luteolin 7-*O*-rutinoside

Figure 25: Structures of luteolin and its glycosyl derivatives identified in the three varieties of *C. cardunculus* L. Abbreviations: Gal, galactosyl; Glc, glucosyl; GlcUA, glucuronyl; MalGlc, malonylglucosyl; Rha, rhamnosyl.

Chrysoeriol (Table 11 and Figure 26) is another flavone identified in artichoke by-products.[84]

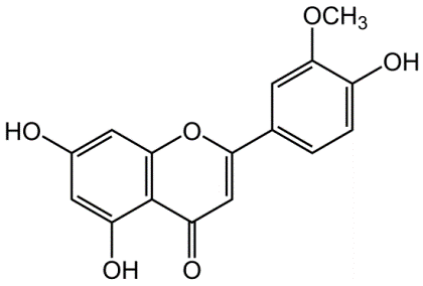


Figure 26: Structure of chrysoeriol identified in artichoke industrial residues.

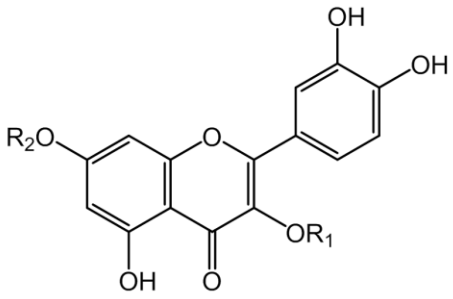
3.5.3.3 Flavonols

Table 12 highlights the flavonols identified in artichoke, namely quercetin and its derivatives. Quercetin and several glycosylated forms, namely quercetin 3-*O*-arabinoside, 3-*O*-galactoside, 3-*O*-glucoside, 3-*O*-rhamnoside, 3-*O*-rutinoside and 7-*O*-neohesperidoside (Figure 27), were detected in artichoke capitula[86] and dietary supplements,[37] as well as in industrial by-products.[84]

Table 12: Flavonols identified in artichoke.

Flavonoids	Artichoke
Quercetin	bp[84]
Quercetin 3-*O*-arabinoside (avicularin)	bp[84]
Quercetin 3-*O*-galactoside (hyperoside)	bp[84]
Quercetin-3-*O*-glucoside (isoquercitrin)	bp[84]
Quercetin 3-*O*-rhamnoside (quercitrin)	bp[84]
Quercetin 3-*O*-rutinoside (rutin)	c,[86] j,[37] bp[84]
Quercetin 7-*O*-neohesperidoside	ds[37]

Abbreviations: bp, by-products; c, capitula; ds, dietary supplements; j, juice.

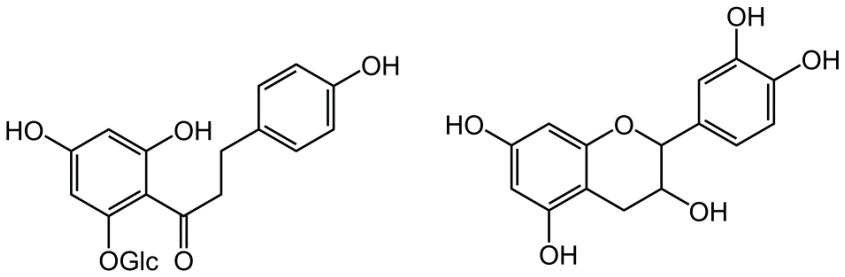


R_1	R_2	
H	H	quercetin
Ara	H	quercetin 3-*O*-arabinoside
Gal	H	quercetin 3-*O*-galactoside
Glc	H	quercetin 3-*O*-glucoside
Rha	H	quercetin 3-*O*-rhamnoside
Glc-Rha (rhamnosyl(α1→6)-glucoside)	H	quercetin 3-*O*-rutinoside
H	Glc-Rha (rhamnosyl(α1→2)-glucoside)	quercetin 7-*O*-neohesperidoside

Figure 27: Structures of quercetin and derivatives identified in artichoke. Abbreviations: Ara, arabinosyl; Gal, galactosyl; Glc, glucosyl; Rha, rhamnosyl.

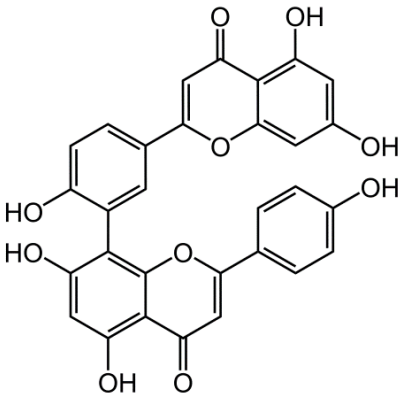
3.5.3.4 Other flavonoid subclasses

Phloretin 2-*O*-glucoside and epicatechin are, so far, the unique chalcone and flavan-3-ol, respectively, identified in *C. cardunculus* L. (Figure 28). Epicatechin was found in wild cardoon leaves,[85] whereas phloretin 2-*O*-glucoside was detected in artichoke by-products.[84] A biflavonoid, named as amentoflavone (Figure 28), was also present in wild cardoon leaves.[85]



phloretin-2-*O*-glucoside

epicatechin



amentoflavone

Figure 28: Other subclasses of flavonoids identified in *C. cardunculus* L. Abbreviation: Glc, glucosyl.

4. Extraction and chemical analysis of plants extractives

4.1 Extraction methods

The extraction process is an important step for the analysis of plants extractable compounds. The solubility of a compound in the extraction solvent is one of the key parameters of extraction process, which may be predicted with the knowledge of polarity.[109] Moreover, the transport of a compound from the plant matrix to the solvent is another important extraction parameter. The mass transfer implies solvent penetration into the matrix and removal of target compounds from the adsorbed sites, which is controlled by partition coefficient, particle size and matrix structure.[110] Another important aspect is related with the fact that, in several cases, target compounds are not freely available, and interacts with other matrix components, namely proteins, carbohydrates and lipids. Therefore, it will be necessary to provide enough energy to break the intermolecular interactions between target compounds and matrix components for a successful extraction.[109]

The extraction method should be selected regarding the plant matrix to be processed and the target compounds to be analyzed. Exhaustive extraction methods are preferentially chosen for the analytical assessment of plants phytochemical profiles, since they virtually enable the complete removal of target compounds from the matrix. Soxhlet extraction and solid-liquid extraction are regarded as exhaustive extraction methods. The exhaustive extraction of plants secondary metabolites can comprise multiple consecutive steps, involving different extractions methods and/or solvents.[109] Our group have demonstrated the usefulness of sequential extraction for analyzing different families of extractable compounds in plant matrices, namely lipophilic and phenolic compounds.[111,112]

4.1.1 Soxhlet extraction

The Soxhlet extraction is a general and well-established technique, which has been used for the extraction of plants secondary metabolites,[109] for instance fatty acids, sterols and pentacyclic triterpenes.[113–115]

The Soxhlet extractor system (Figure 29) is constituted by a condenser, a thimble holder (Soxhlet) and a round-bottomed flask. The ground material plant is placed in a porous cellulose filter thimble. The thimble is located in a glass thimble holder (Soxhlet), which is above the round-bottomed flask and below the condenser. The solvent is placed in the round-bottom flask and heated to its boiling point. The solvent vapor goes through the side tube of Soxhlet, reaching the condenser where it condenses and drips into the thimble holder. When the condensed solvent fills the thimble holder and reaches the top of the siphon of the return tube, it is drained back into the flask through the siphon, carrying the extractable compounds into the solvent reservoir bellow. At this point the extraction thimble does not contain any

solvent. The first cycle of the extraction was thus performed. This cycle repeats many times for a predetermined time period. In the solvent flask, the extractable compounds are separated from extraction solvent by distillation, since they have lower volatility than the solvent. In this manner, the plant sample is always extracted with fresh solvent in each cycle.[109,110]

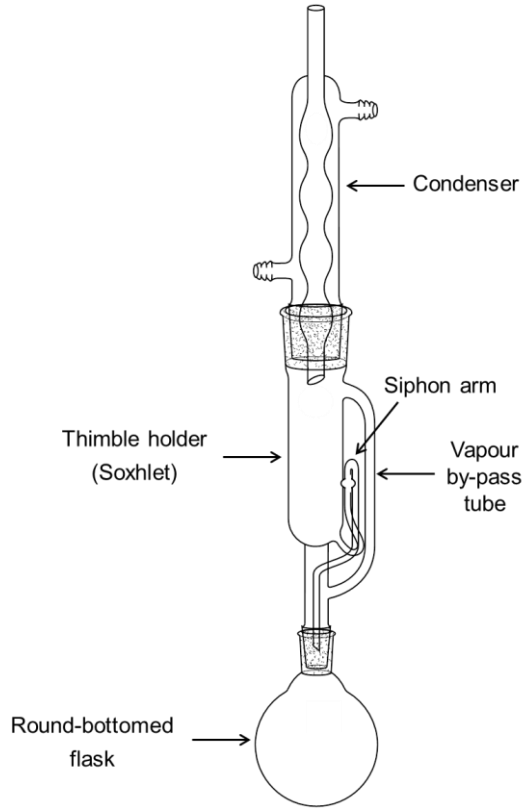


Figure 29: Soxhlet apparatus (adapted from [110]).

Despite its simplicity and the inexpensive extraction apparatus, some disadvantages have been pointed out, namely the long extraction time (6-48 h), and the possibility of degradation of thermolabile compounds, due to the extraction temperature corresponds to the extraction solvent boiling point, for a long time.[109,110]

The Soxhlet method was used to estimate the seed oil percentage of *C. cardunculus* L. var. *altilis* (DC), using ethyl ether as extraction solvent.[19]

4.1.2 Solid-liquid extraction

In the solid-liquid extraction, powdered plant material is placed in contact with the solvent, for many hours or even days, allowing transfer of target compounds from the plant matrix to the solvent. Mechanical stirring and centrifugation are generally included in this method, in order to increase mass transfer. This process is commonly performed under room

temperature, but high temperatures can also be applied to promote the extraction efficiency. Nonetheless, the use of high temperatures should be avoided in what regards to the extraction of thermolabile compounds. This technique has been commonly used for the extraction of plants phenolic compounds.[116,117]

Several studies have used the solid-liquid extraction for the removal of phenolic compounds from *C. cardunculus* L., with organic solvents or mixtures (e.g. methanol and acetone/ethanol/methanol (70:15:15)); hydroalcoholic solutions (e.g., 70% ethanol, adjusted to pH 3.2 and 60-70% methanol); and water. Mechanic stirring, followed by centrifugation was also applied, under low (4 ºC), room or boiling temperatures.[9,38,85,92,99]

4.2 Analytical techniques

Components of complex plant extracts have been mainly analyzed by gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography-mass spectrometry (HPLC-MS). After compounds separation by chromatographic techniques, like GC or HPLC, mass spectrometry provides relevant compound information, namely molecular weight and fragmentation patterns fundamental to establish the chemical structure. Chemical structure of each compound can be established either by comparing mass spectrum with those of pure compounds and library data, or by interpreting the fragmentation pathways.[118]

When the mass spectroscopic data are not enough to unambiguously identify a compound, it is necessary to isolate it through preparative chromatographic techniques, such as adsorption chromatography or thin layer chromatography (TLC), for identification by nuclear magnetic resonance (NMR) spectroscopy, or other spectroscopic techniques (e.g. X-ray).

Some sesquiterpene lactones,[68] pentacyclic triterpenes[83] and phenolic compounds[67,68] were isolated from *C. cardunculus* L. varieties and identified by various NMR spectroscopic techniques, namely ^{1}H and ^{13}C NMR, Distortionless Enhancement Polarization Transfer (DEPT) and two-dimension NMR, such as COrrelated SpectroscopY (COSY), Heteronuclear Multiple Quantum Coherence (HMQC), Heteronuclear Multiple Bond Coherence (HMBC) and Nuclear Overhauser Effect SpectroscopY (NOESY).

In GC-MS and HPLC-MS systems (Figure 30), compounds are separated in the chromatographic columns and transferred to mass spectrometers. Each eluted compound is ionized to gas-phase ionic species, in the ionization source. The excess energy transferred during ionization causes fragmentation of those ionic species to product ions. All ions are thereafter separated by their mass-to-charge ratio (*m/z*) values in the mass analyzer. The type of mass analyzer will decide the type and quality of experimental data obtained regarding to mass accuracy, mass resolution and sensitivity.[119] Finally, the ion current is measured and

amplified in electron multiplier or array detectors. The generated quantity of electrical current will depend on the abundance of ions hitting the detector.[119]

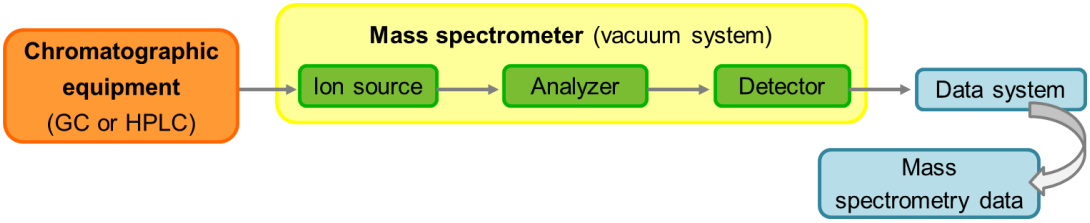


Figure 30: Basic components of chromatographic equipment coupled to mass spectrometer (adapted from [120]).

Table 13 lists the most used devices of GC-MS and HPLC-MS systems in the chemical analysis of plant volatile hydrocarbons and phenolic compounds, respectively.

Table 13: GC-MS and HPLC-MS systems most used in analysis of volatile lipophilic and phenolic compounds, respectively.[118,121,122]

		GC-MS	HPLC-MS
Chromatographic column		Capillary fused-silica	Reversed phase octadecyl silica (C-18)
Interface		Direct coupling interface	Fast atom bombardment
Mass spectrometer	Ionization source	Electron ionization Chemical ionization	Electrospray ionization Atmospheric pressure chemical ionization
	Analyzer	Quadrupole Ion trap Time of flight	Triple quadrupole Ion trap Time of flight Triple quadrupole-ion trap Triple quadrupole-time of flight Ion trap-time of flight

4.2.1 Gas chromatography-mass spectrometry

GC application for the separation of extractives is restricted to volatile and thermally stable compounds.[123] Preliminary derivatization reactions are often needed to transform an analyte in a volatile derivative to be eluted from the GC column. Derivatization will render highly polar compounds to be volatile, in a way they can be eluted without thermal decomposition, at temperatures that often can reach up to 300 ºC. Compounds, exhibiting thiol (-SH), hydroxyl (-OH), amine (-NH) and carboxylic (-COOH) groups, have a tendency to form hydrogen bonds which may affect their volatility, interaction with column packing materials and

thermal stability. Silylation is one of the most prevalent derivatization methods. This reaction relies on the substitution of active hydrogens in those functional groups by trimethylsilyl substituents, lowering the analyte polarity and hydrogen bonding. *N*,*O*-bis(trimethylsilyl)trifluoroacetamide is mainly used in the silylation reaction. The addition of trimethylchlorosilane catalyzes reactions of hindered functional groups, in secondary alcohols and amines. The silylated derivatives are more volatile and more stable, providing narrow and symmetrical chromatographic peaks.[124]

The stationary phase is a film chemically bonded to the inner wall of a capillary fused-silica column. GC columns with 0.25-0.33 μm film thickness are suitable for the separation of higher-boiling and heat-sensitive compounds, whereas thicker stationary phases (1 μm film thickness) for low-boiling compounds. The nonpolar GC stationary phases, like those constituted by dimethylpolysiloxane (DB-1 or equivalent) and 5% phenyl and 95% dimethylpolysiloxane (DB-5 or equivalent), are the most commonly recommended in the separation of lipophilic compounds, providing adequate peak resolution. GC capillary columns with 30-60 m of length are typically used. Shorter columns (5-15 m length) are advantageous for thermally labile and higher-boiling compounds. The internal diameter of the most used GC capillary columns varies between 0.25 and 0.53 mm.

The sample is transferred from a heated injector, where it rapidly volatilizes, through the heated column by the carrier gas (helium in the case of GC-MS system). Compounds are selectively retarded by the stationary phase, being eluted according to their boiling temperature and polarity.[123] Then, at the end of the column, compounds reach the mass spectrometer.

The major problem of GC-MS, when it was developed, consisted of the incompatibility of GC carrier gas high pressure, caused by the high flow rate (e.g. 25 mL/min or more), with the high vacuum required in the mass spectrometer system (10^{-5}-10^{-8} Torr in MS analyzer).[125] This issue was solved with the development of suitable interfaces (e.g., split system, jet concentrator, helium diffuser)[125] which are able to diminish the flow rate by removing the carrier gas before it reaches the ionization source.[120] With the emergence of capillary GC columns, the interface does not exist in the GC-MS, since the mass spectrometer pumping system can handle the low flow rate from the column (1-2 mL/min).[120,123] In this way, the column exit is directly connected to the mass ionization source via a vacuum-tight flange.[120,123,125] This device must be heated above the maximum temperature of the column for the sample, in a way that any compound does not condense before entering into ionization source.[123]

Electron ionization (EI) (Figure 31) is the ionization technique most commonly used in the identification of volatile extractives by GC-MS. For instance, it was applied in the GC-MS analysis of sesquiterpenes present in artichoke volatile oil.[62,63]

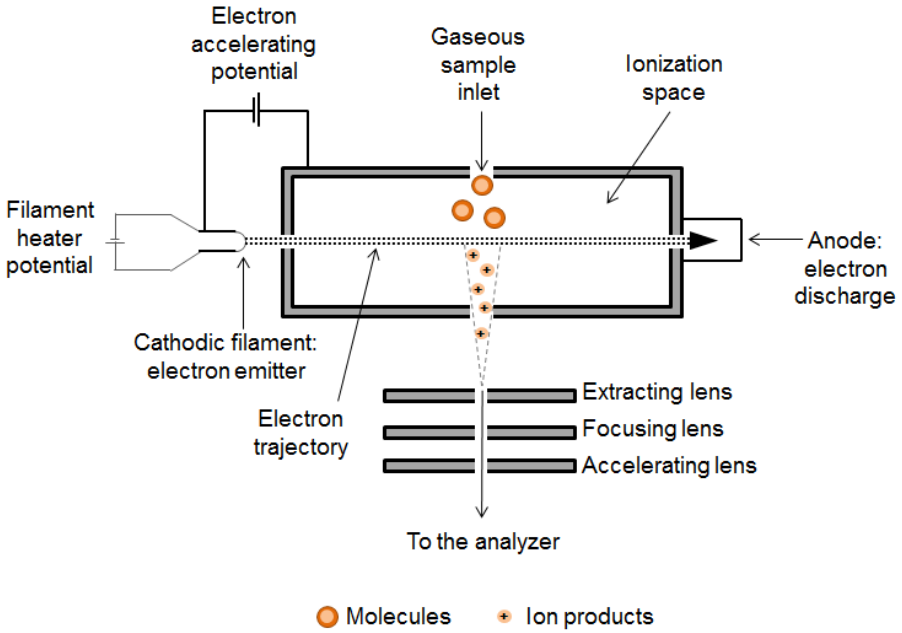


Figure 31: Scheme of EI source (adapted from [126]).

In EI, electrons are emitted from a heated metal filament (cathode, e.g. tungsten) and accelerated by 70 eV towards the anode.[120] Those electrons collide with the gaseous molecules of the analyzed sample, and they may transfer enough energy to remove outer shell electrons, giving rise to an additional free electron and a positive radical molecular ion ($[M]^{+\cdot}$) (Equation (1)). Since the electron beam energy is high, molecular ions often fragment into product ions and neutral fragments (Equation (2)).[123,126] EI mass spectra thus display many product ions, and molecular ion is often absent.[127]

$$M + e^- \longrightarrow [M]^{+\cdot} + 2e^- \quad (1)$$

$$[M]^{+\cdot} \longrightarrow [A]^+ + B^\bullet \quad (2)$$

Quadrupole (Figure 32) is the mass analyzer more frequently installed in the mass spectrometer coupled to GC. It is constituted by four parallel cylindrical metal rods, arranged specifically in a square. A positive direct current (dc) voltage is applied to one pair of opposing rods, whereas the other pair receives a negative dc voltage. Superimposed radiofrequency (rf) voltages are also applied to each pair of rods.[128] The consequent dynamic electric field acts as a filter in that it only allows ions of a given *m/z* ("resonant ions") to pass down the gap between

the four rods, for a certain rf/dc ratio. Ions of different *m/z* values (non resonance ions) have unstable trajectories, and will collide with the rods and become neutralized. Mass-to-charge scanning is accomplished by varying the rf or dc voltages, while keeping rf/dc ratio constant the rf/dc ratio, to obtain the mass spectrum over the required *m/z* range.[119,128]

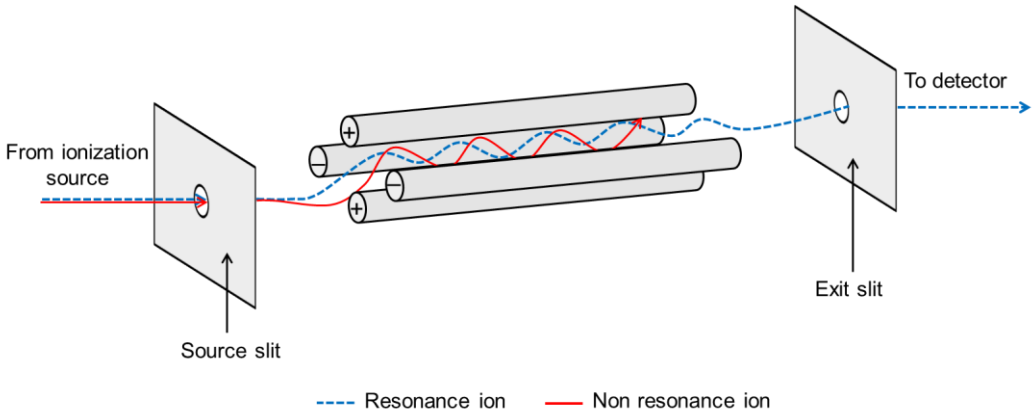


Figure 32: Scheme of a quadrupole (adapted from [119]).

4.2.2 High-performance liquid chromatography-mass spectrometry

In high-performance liquid chromatography (HPLC), the stationary phase is a liquid film covalently bound to silica particles within a stainless steel column, and the mobile phase is a liquid (e.g. organic solvents or water/organic solvent mixtures). Compounds are separated according to their affinity to the stationary or mobile phases. In reversed-phase chromatography, the stationary phase is nonpolar and the mobile phase is polar. In this manner, the more polar compounds are the first to be eluted from the column, while the less polar compounds are retained longer in the column. At the column end, compounds reach the detector, being ultraviolet-visible spectrophotometers and diode array detectors (DAD) the most common. DAD has been important for the identification of analyte phenolic subclass, since the UV-Vis spectrum is specific for each other.[129] HPLC-UV was applied in the quantification of hydroxycinnamic acids and flavonoids detected in *C. cardunculus* L.[9,38,85,87,89,92,97,99,102]

HPLC-UV-MS has revealed to be a powerful in the identification of phenolic compounds. Phenolic compounds of wild cardoon,[9,92,102] artichoke,[38,84,88,89,94,96,102,107] and cultivated cardoon[9,92,102] have been analyzed by HPLC-UV-MS. The separation of phenolic compounds, by HPLC, is generally performed in an octadecyl (C18) bonded silica column (100-250 mm in length and 2.1-4.6 mm in internal diameter). Growing attention has been given to the sub-2 μm particles HPLC columns, known as ultra-HPLC (UHPLC) columns, since these increase the optimum mobile phase velocity, supporting higher flow rates, maintaining high chromatographic efficiency, to decrease analysis time.[130] Farag, *et al.*[58] used UHPLC-UV-

MS to detect 50 secondary metabolites of artichoke leaves within 13 minutes, compared to the 50-90 minutes earlier required.[38,87,93] The UHPLC columns have been operated at temperatures higher than room temperature (50-70 ºC), in the analysis of plant phenolic compounds.[131,132] The use of HPLC columns at higher temperatures decreases the mobile phase viscosity and increases the solute diffusivity, which allows the use of higher mobile linear velocity, leading thereby to faster analyses.[133]

The mobile phase used for the phenolic compounds analysis, through reversed-phase HPLC, is generally constituted by water and less polar organic solvents (e.g. acetonitrile or methanol). Acetic, formic or phosphoric acids are often added to the mobile phase.[121,134]

Coupling HPLC to MS initially was not straightforward, because the normal operating conditions of mass spectrometer are diametrically opposed to those used in HPLC, such as high vacuum, high temperature, gas-phase operation and low flow rates.[135] The currently used ionization sources, like thermospray and electrospray, have solved this issue, working as introduction systems to the mass spectrometer.[125]

Electrospray ionization (ESI) (Figure 33) has been the most utilized ionization technique in the identification of phenolic compounds in *C. cardunculus* L. varieties, by HPLC-MS.[9,38,84,88,89,92,94,96,102,107]

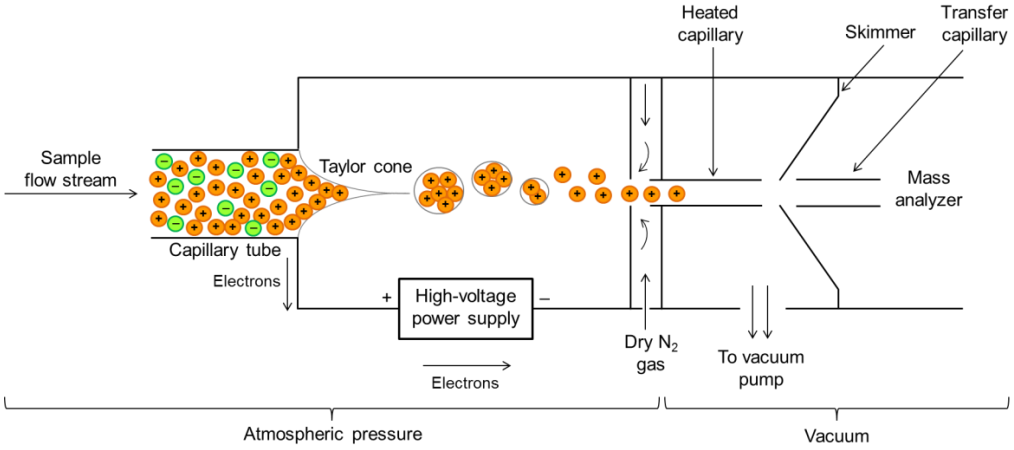


Figure 33: Scheme of electrospray ionization process (positive ion mode) (adapted from [136]).

Within an ESI source, a continuous stream of sample solution is passed through a stainless steel or quartz silica capillary tube. A strong electric field is obtained by applying a potential difference of 3-6 kV between the capillary and the counter-electrode.[126] The counter electrode may be a heated capillary (Figure 33) which drives ions to the mass spectrometer.[136] This field causes a charge accumulation at the liquid surface, at the end of the capillary. which will break to form highly charged droplets.[126] When the pressure of the accumulated charges is higher than the surface tension, the shape of the droplet assumes a conical shape, known as Taylor cone. The Taylor cone originates a fine jet, which is divided in tiny, highly charged

droplets. As the solvent evaporation occurs, the droplet size continuously diminishes, until it reaches the point that the surface tension can no longer sustain the charge, known as the Rayleigh limit. At this point, a Coulombic explosion occurs and the droplet is ripped apart, producing smaller droplets. These small, highly charged droplets will continue to lose solvent, and when the electric field on their surface becomes large enough, desorption of ions from the surface occurs.[119,126] Since ions may be clustered with solvent molecules and other additives, they are subjected to a thermal declustering or "clean-up" stage at a heated capillary. Often, a countercurrent flow of an inert gas (e.g. nitrogen) is used to minimize the entry of solvent vapor into the vacuum region (Figure 33). An electric potential difference is applied between the heated capillary exit and the skimmer originates a second clean-up of ions, through collisional activation.[136]

The dominating signals result from the so-called "quasi-molecular" ions, such as: (i) protonated or sodiated ions, i.e. $[M+H]^+$ or $[M+Na]^+$; (ii) clusters, e.g. $[2M+H]^+$, $[3M+Na]^+$ or $[M+H+solvent]^+$, (iii) multiple charged molecules as $[M+2H]^{2+}$ or (iv) deprotonated or adduct ions, i.e. $[M-H]^-$, $[M+Cl]^-$ and $[M+HCOO]^-$. This is a very soft method of ionization as very little residual energy is retained by the analyte upon ionization, resulting in low fragmentation.[119,137] For the detection of positive ions, electrons have to be provided from the capillary to the circuit, which will occur through oxidation of molecules in the solution at the capillary tip. For negative ions, electrons have to be consumed through reduction.

Triple quadrupole (QqQ) (Figure 34) has been the commonest MS analyzer used to identify phenolic compounds in *C. cardunculus* L. plants, by HPLC-MS.[9,38,84,88,92,96,102,107] Successive ion fragmentations (MS/MS) can be produced in QqQ analyzer. The first quadrupole (Q1) separates and selects the precursor ion of a specific *m/z*. Then, the ion is transferred to a collision cell (q), namely a quadrupole, hexapole or octapole, which operates in rf-only mode.[126,138] At the collision cell, the ion suffers a collision induced dissociation (CID), a process by which the translational energy of the ion accelerated towards a neutral target species (or "collision gas", such as argon, helium or nitrogen) is partitioned in internal energy, with the consequent decomposition in product ions.[139] Finally, in the last quadrupole (Q2), the product ions coming from CID are separated and directed to the detector.[138]

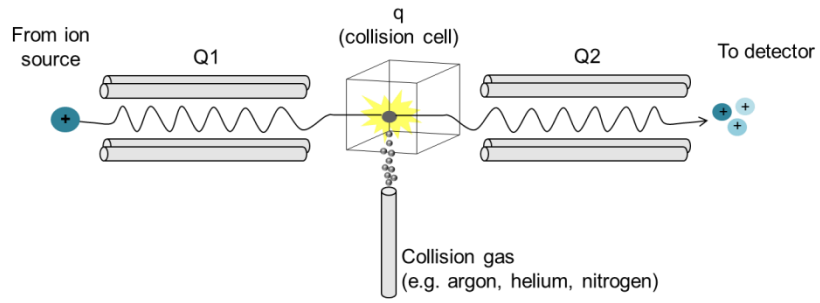


Figure 34: Scheme of triple quadrupole (adapted from [121]).

Ion trap (Figure 35) is a three-dimensional analogue of the quadrupole mass analyzer, containing a central ring electrode between two identical end-capped electrodes, with a hyperbolic geometry. One of the end-cap electrodes has a small aperture which allows ion injection into the analyzer from an external source. The other end-cap electrode has several perforations for the ejection of ions towards the detector.[120] This analyzer is named as trap, because it enables ions to be trapped in an electric field by applying an rf voltage to the ring electrode.[119] The mass separation is achieved by storing the ions in the trapping space (1-30 ms), and by manipulating their motion in time, under an oscillating electric field.[119,120] Mass spectrum of trapped ions is obtained by the mass-selective axial ejection. The increase of rf voltage destabilizes the trajectories of ions, and ejects them sequentially from the ion-trap region, through the end cap electrode.[119] Ion trap is particularly adequate for multi-step mass spectrometry without additional mass analyzers, because ions are stored and fragmented by CID in the same device. CID is accomplished by applying an excitation waveform which accelerates and then decelerates the ions, in the presence of a collision gas (e.g. helium). Slow and multiple collisions of ions lead to highly efficient and low energy CID.[122] CID is performed after ejecting all ions from the trap except the selected precursor, which is energized to originate product ions (Figure 35). Then, a specific product ion can be selected, trapped and collided with the helium gas to give rise product ions for another mass spectrum.[138] Fragmentation of ions can be repeated in a sequential manner, allowing to obtain valuable structural information. This type of analyzer was used in the identification of phenolic compounds in artichoke.[93,94]

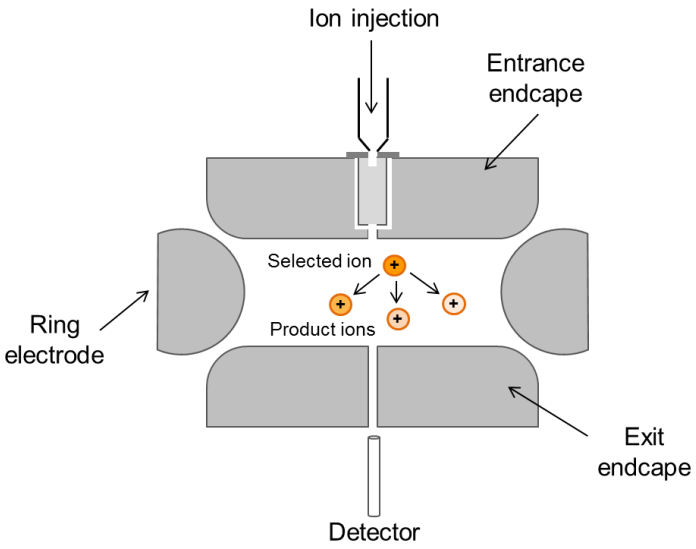


Figure 35: Scheme of ion trap (adapted from [119]).

5. Phytotherapy

The use of herbal teas, poultices and powders for relieving illness is as old as the humanity's history. The phytotherapy term was firstly used by Henri Leclerc (1870-1955) for the study of herbal drugs in the treatment of human diseases, regarding their effectiveness and limitation.[140] The scientific knowledge on phytotherapy is currently one of the fastest growing areas of biomedical research, given the molecular biology advancements in the last years. Actually, the number of publications related to the biological activity assessment of medicinal plants increased more than four times in the last decade (2004-2014) (Figure 36).

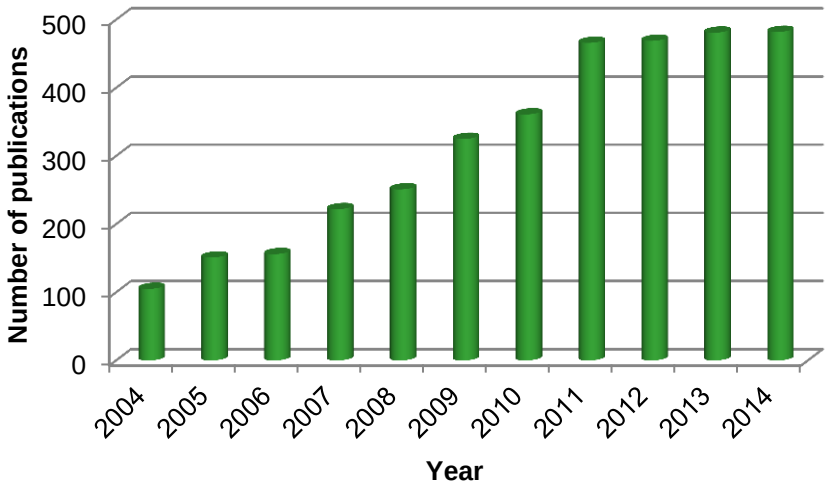


Figure 36: Number of publications in the last decade (2004-2014) with the keywords in topics search "medicinal plant" and "activity" of Web of Science (2015 May).

The wide use of medicinal plants in developing countries, namely in African countries, is frequently associated with its accessibility and affordability, specially in rural areas where people have hardly access to allopathic health care.[141] On the other hand, the consumption of herbal products is becoming more common in developed countries, for several reasons: (i) the preference for natural therapies towards chemical products; (ii) the concern relatively to the undesirable side effects of synthetic medicines; (iii) the preference for preventive medicine, as a consequence of population ageing, and (iv) the scientific evidence supporting efficacy and safety of herbal products.[142]

Phytotherapeutic agents can be seen as an alternative or complementary treatment to conventional medicines against various chronic diseases, such as cardiovascular diseases, cancer, diabetes and mental disorders. More than 100 million of Europeans are nowadays users of traditional and complementary medicine (TCM), with 20% preferring health care including medicinal plant products.[143] In addition to European countries, there is a great TCM

consumption in Asiatic and North American countries.[142,143] In fact, relevant policy issues have been implemented for the appropriate usage of TCM.[143]

After a stagnation in the development of new molecular entities (NMEs) during the last years, the pharmaceutical industry is more and more dedicated in the search for new NMEs, among the secondary metabolites.[144] These compounds display "privileged" chemical structures, since they bind naturally to biological macromolecules and exhibit pleiotropic mechanisms of action.[144–148] The drug discovery process has been estimated to take around 10 years.[144] Figure 37 shows the main steps for the development of NMEs based on plant secondary compounds.

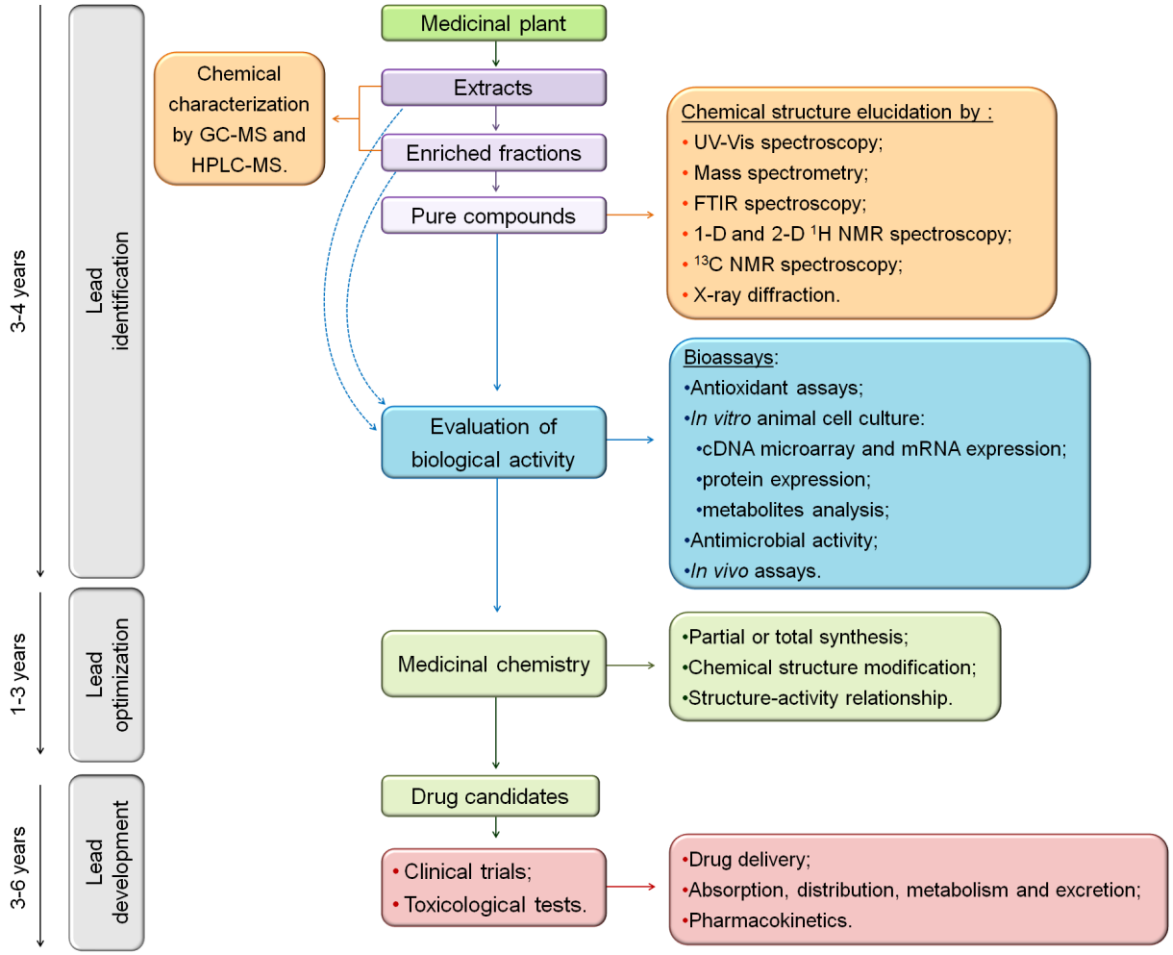


Figure 37: Drug development process, derived from plant secondary metabolites (adapted from [149–151]). Abbreviations: cDNA, complementary DNA; FTIR, Fourier transformed infrared; mRNA, messenger RNA; UV-Vis, ultraviolet-visible spectroscopy.

After the botanical identification of the medicinal plant, the drug development process (Figure 37) is initiated with the extracts preparation and their chemical characterization through GC-MS and HPLC-MS analysis.[152] These plant extracts are then submitted to the

evaluation of a diversity of biological activities, including: (i) antioxidant activity; (ii) *in vitro* animal cell cultures assays, through genomics, transcriptomics and metabolomics-based approaches (iii) antimicrobial action; and (iv) *in vivo* assays (e.g. rodent and non-rodent mammals).[147,150]

The biologically active plant extracts are sequentially fractionated, through chromatographic techniques, namely thin layer chromatography, solid-phase extraction and advanced high-performance liquid chromatographic technique.[153] Each fraction is also subjected to biological activity tests, in order to isolate the most promising bioactive compound. This process is known as bioactivity-guided fractionation.[150] The chemical structure of the lead compound is elucidated through spectroscopic methods, such as: (i) ultraviolet-violet spectroscopy; (ii) mass spectrometry; (iii) Fourier transformed infrared spectroscopy; (iv) one and two dimensional proton (^{1}H) and carbon (^{13}C) NMR spectroscopic techniques.[153]

The following step of the drug development consists of tentatively synthesizing the lead compound. The lead optimization also comprehends the synthesis of lead derivatives, lying on combinatorial chemistry, in order to understand the structure-activity relationship.[149] Either the large-scale isolation, or the synthesis of drug candidates is needed for the clinical and toxicological trials, centered in the future therapeutic application.[150]

6. Biological activity of *C. cardunculus* L. extracts

C. cardunculus L. leaves infusions are quite utilized in the traditional medicine of Europe and North Africa, due to their hepatoprotective and anticholestatic effects.[30,154] These biological effects have been related with the phenolic composition, namely hydroxycinnamic acids and flavonoids.[26,29] Adzet, *et al.*[26] observed that cynarin and luteolin 7-*O*-glucoside prevented, in a dose-dependent manner, the carbon tetrachloride-induced leakage of both aspartic and alanine transaminases. Moreover, Saénz Rodriguez, *et al.*[29] also demonstrated that a commercial artichoke leaves aqueous extract, containing 1.5% caffeoylquinic acids and 0.5% flavonoids, increased the bile flow and bile acids excretion in Wistar rats (100-400 mg/kg).

Table 14 lists some of the studied biological activities of artichoke and wild cardoon extracts, and their relationship with the secondary metabolites composition.

Table 14: Some of *C. cardunculus* L. extracts biological activities, and relationship with the chemical composition.

***C. cardunculus* L. extracts**	**Biological activity**	**Main responsible secondary metabolites**	**Source**
Artichoke leaves	Inhibition of olive oil-induced triglycerides elevation in rat serum	Cynaropicrin, aguerin B, grosheimin, luteolin 7-*O*-glucoside and 7-*O*-rutinoside	[68]
	Cholesterol biosynthesis inhibition	Luteolin and luteolin 7-*O*-glucoside	[155]
	Nitric oxide increased production and eNOS activity up-regulation		[156]
Wild cardoon	eNOS activity induction	Luteolin 7-*O*-glucoside	[157]
	Vasomotion recuperation in rats	Luteolin and apigenin	[158]

Abbreviations: eNOS, endothelial nitric oxide synthase.

Shimoda, *et al.*[68] noted that artichoke leaves methanol extract inhibited the serum triglycerides elevation in olive oil-loaded mice (Table 14), with cynaropicrin as the most effective agent, followed by grosheimin and aguerin B. This research group proposed that the oxygen functional group, as well as the *exo*-methylene moiety in the *α*-methylene-*γ*-butyrolactone ring were essential for the anti-hyperlipidemic activity of guaianolides.[68] Moreover, Gebhardt[155] showed that a commercial artichoke leaves aqueous extract blocked the cholesterol biosynthesis in primary cultured rat hepatocytes, probably due to luteolin and luteolin 7-*O*-glucoside (Table 14).

Three studies indicated the cardiovascular properties of artichoke and wild cardoon extracts[156–158] (Table 14). Li, *et al.*[156] found that a commercial artichoke leaves aqueous extract (10.10% of caffeoylquinic acids and 2.17% of flavonoids), and its ethyl acetate/*n*-butanol (2:1) fraction (26.30% of caffeoylquinic acids and 13.29% of flavonoids), induced endothelial nitric-oxide synthase (eNOS) protein expression. They also proved that luteolin 7-*O*-glucoside and luteolin, known to be present in commercial artichoke leaves extract, increased the eNOS mRNA expression.[156] A wild cardoon 90% ethanol extract (1 µM gallic acid equivalents (GAE)) was also demonstrated to induce the eNOS activity, in primary porcine aortic endothelial cells, after 16 h.[157] Moreover, Rossoni, *et al.*[158] demonstrated that a water-soluble extract of wild cardoon, containing luteolin and apigenin, could restore proper vasomotion, in aged rats, to a similar degree of young animals.

Artichoke capitula are worldwide consumed as a vegetable, specially in the Mediterranean countries. Several epidemiological studies have correlated the Mediterranean diet with a lower incidence of coronary heart diseases and cancer.[159–161] These health benefits can be associated with the high intake of fish, olive oil, fruit and vegetables, which have revealed to be sources of ω-3 fatty acids, phytosterols and phenolic compounds.

Phenolic compounds are well-known by their antioxidant activity. Several studies have tried to understand the mechanism of action of phenolic compounds on tumor cell models, through their effects on the antioxidant-prooxidant balance.[162,163] Plant lipophilic components have also shown antiproliferative effects on human tumor cell lines, for instance sesquiterpene lactones[50] and pentacyclic triterpenes.[79]

The antioxidant and the antitumoral effects of *C. cardunculus* L. extracts have been vastly assayed in the last years. Seeking for natural antibiotics, the antimicrobial action of *C. cardunculus* L. extracts have also been approached. These three important biological activities of *C. cardunculus* L. extracts and their relationship with the secondary metabolites composition will be exposed, as it follows.

6.1 Antioxidant activity

Oxidative stress is caused by disruption in the balance between production of free radicals and antioxidant defense mechanisms. Several reactive oxygen species (ROS) and reactive nitrogen species (Figure 38) can cause oxidative damage to lipids, proteins and nucleic acids, promoting membrane destruction, enzyme inactivation and DNA breakage.[41,164] These oxidative damages are allied to the initiation and progression of degenerative diseases, namely inflammation, cancer, ageing and cardiovascular diseases.[165]

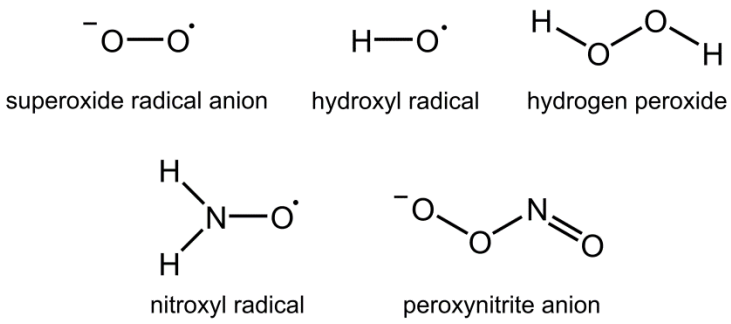


Figure 38: Structures of common reactive oxygen species and reactive nitrogen species.

Making part of medicinal plants, spices, fruit and vegetables, antioxidants may aid to protect the human body against the ROS injuries. The antioxidant concept was defined as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate". Antioxidants may act either by inhibiting free radicals formation, or by directly scavenging free radicals. Moreover, antioxidants might raise the levels of endogenous antioxidant defenses, for instance by upregulating expression of the genes encoding superoxide dismutase, catalase or glutathione peroxidase.[166]

Several studies have devoted to the antioxidant potential of *C. cardunculus* L. phenolic-containing extracts and their components, by using *in vitro* and *in vivo* assays.

6.1.1 *In vitro* assays

Several *in vitro* methods have been utilized to investigate the antioxidant activity of *C. cardunculus* L. extracts and their phenolic constituents, based on hydrogen atom transfer (HAT) and single electron transfer (SET) assays, as well as on the ROS scavenging effect.

6.1.1.1 Hydrogen atom transfer assays

HAT-based methods determine the ability of an antioxidant to quench free radicals, by giving one hydrogen atom, based on the Equation (3):

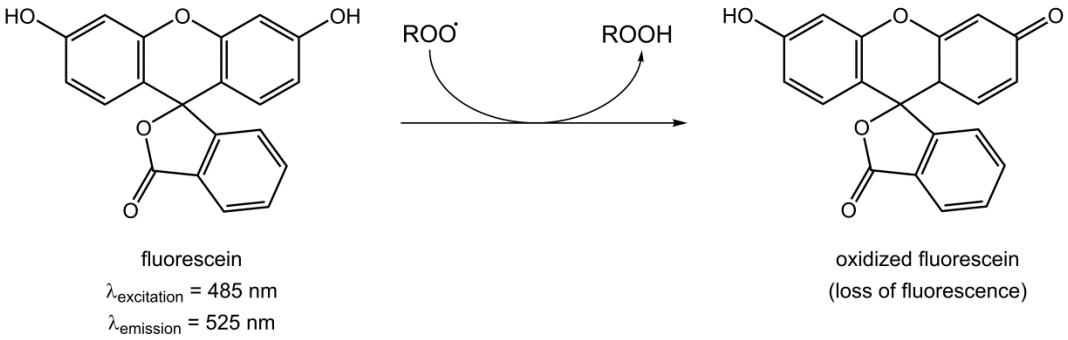
$$X^{\cdot} + AH \longrightarrow XH + A^{\cdot} \quad (3)$$

Oxidation of low-density lipoprotein (LDL) is a HAT-based method that can be applied for evaluating the antioxidant effect in a more physiologically relevant system. This antioxidant test involves the LDL oxidation induced by Cu(II) ions or 2,2´-azobis(2-amidinopropane)dihydrochloride, giving rise to lipid peroxides. Lipid peroxides can be monitored by detecting conjugated dienes (λ_{max} = 234 nm),[167] or by quantifying their decomposition products, like malonaldehyde (MDA), through the thiobarbituric acid method.[88]

Brown, *et al.*[168] demonstrated that a commercial artichoke leaves aqueous extract was able to delay Cu^{2+}-induced LDL oxidation, in a concentration-dependent way (1-20 µg/mL). The major phenolic compound of this extract, luteolin 7-*O*-glucoside, also prevented the LDL oxidation, probably by chelating Cu^{2+} ions.[168] Coinu, *et al.*[88] noted that ethanol and ethyl acetate extracts, derived from artichoke outer bracts and leaves, strongly decreased the production of LDL diene conjugates, though no correlation with the total contents of phenolic compounds, caffeoylquinic acids or flavonoids was pointed out. With the exception of apigenin 7-*O*-glucoside, the main identified phenolic compounds in artichoke extracts inhibited Cu^{2+}-induced LDL peroxidation, as follows: caffeic acid > 5-*O*-caffeoylquinic acid > luteolin 7-*O*-glucoside > 1,5-di-*O*-caffeoylquinic acid > luteolin.[88]

Pistón, *et al.*[164] approached the scavenging effect of artichoke leaves extracts against peroxyl ($ROO^{\cdot}$) radicals, by using the oxygen radical absorbance capacity (ORAC) assay. This assay evaluates whether a test compound is able to transfer a hydrogen atom to $ROO^{\cdot}$ radicals, which reflects a classical radical chain breaking antioxidant effect.[167] The $ROO^{\cdot}$ radicals may be prepared by thermodecomposition of *α*,*α´*-azodiisobutyramidine.[164] Then, the antioxidant potential is evaluated by monitoring the fluorescence decay resulting from the

ROO$^{\cdot}$-induced oxidation of fluorescein (3´,6´-dihydroxyspiroisobenzofuran-13*H*,9´,9*H*-xanthen-3-one) (Equation (4), Figure 39).[164,167] In the presence of an antioxidant, the fluorescein decay is retarded (Equation (5)). The reaction is monitored at 485 nm (excitation)/525 nm (emission) for fluorescence variations, during reaction time.[169] The antioxidant effect is determined through the integrated areas under the fluorescence decay curves (AUC) [AUC_{sample} – $AUC_{no\ sample}$].[167]



(4)

$$ROO^{\cdot} + AH \longrightarrow ROOH + A^{\cdot} \quad (5)$$

Figure 39: Peroxyl radical scavenging effect of a test antioxidant, by using the oxygen radical absorbance capacity (adapted from [167]).

Using the ORAC method, Pistón, *et al.*[164] observed that an artichoke leaves infusion, containing 108 mg/g extract of identified phenolic compounds, was more active in inhibiting the fluorescein oxidation by ROO$^{\cdot}$ radicals, comparatively to the aqueous (decoction) and ethanol/water (70:30) extracts (63 and 73 mg/g extract of identified phenolic compounds, respectively).

6.1.1.2 Single electron transfer assays

SET-based methods are related with the ability of an antioxidant (*A*) to transfer one electron to reduce metals (Equation (6)) and radicals (Equations (7)-(9)):

$$M(III) + AH \longrightarrow AH^{+} + M(II) \quad (6)$$

$$X^{\cdot} + AH \longrightarrow X^{-} + AH^{\cdot +} \quad (7)$$

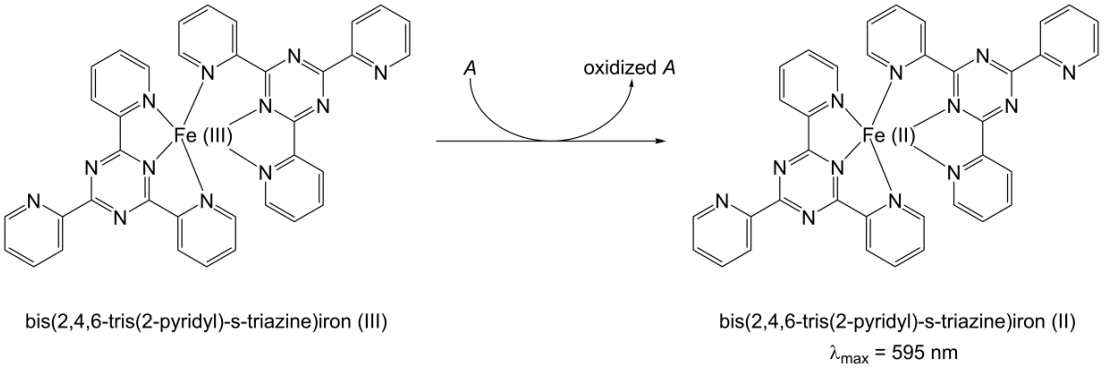
$$AH^{\cdot +} \overset{H_2O}{\rightleftharpoons} A^{\cdot} + H_3O^{+} \quad (8)$$

$$X^{-} + H_3O^{+} \longrightarrow XH + H_2O \quad (9)$$

Phenolic compounds have shown reducing ability, which can depend on the hydroxylation degree and the extent of double bond conjugation.[170] Two SET-based assays

were applied in the assessment of *C. cardunculus* L. extracts reducing power, namely Ferric Reducing Antioxidant Power (FRAP)[92] and reducing power.[171]

FRAP consists of the donation of an electron by the antioxidant to reduce bis(2,4,6-tris(2-pyridyl)-*s*-triazine)iron (III) to bis(2,4,6-tris(2-pyridyl)-*s*-triazine)iron (II) (Equation (10)) which is monitored at 595 nm (Figure 40).[167] This assay was primarily created to measure the reducing power of antioxidants in plasma, but currently it is also used to study the antioxidant effect of plant extracts.[170] This method may predict the free radical scavenging action of phenolic compounds.[167] In this manner, Pandino, *et al.*[92] observed that the FRAP of 70% methanol extracts, derived from *C. cardunculus* L. floral stem and leaves, was linearly and positively correlated with hydroxycinnamic acids (r = 0.95) and luteolin derivatives contents (r = 0.97), respectively.



(10)

Figure 40: Redox reactions involved in the ferric reducing antioxidant power assay (adapted from [167]).

The reducing power assay is based on the capacity of an antioxidant to reduce the ferricyanide ion ($[Fe(CN)_6]^{3-}$) to $[Fe(CN_6)]^{4-}$ ion (Equation (11)). Then, the $[Fe(CN_6)]^{4-}$ ion reacts with ferric ion (Fe(III)), generating $Fe[Fe(CN)_6]^-$ (Equation (12)). In this method, the $Fe[Fe(CN)_6]^-$ is monitored at 700 nm, by measuring the absorbance of Prussian blue salt ($KFe[Fe(CN)_6]$).[172]

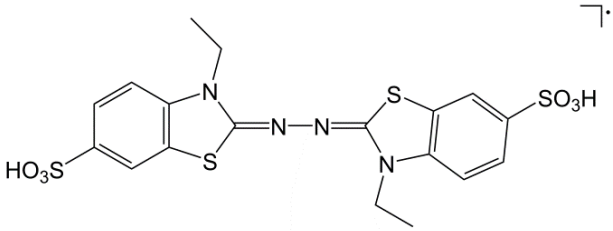
$$[Fe(CN)_6]^{3-} + A \rightleftharpoons [Fe(CN)_6]^{4-} + \text{oxidized } A \quad (11)$$

$$[Fe(CN)_6]^{4-} + Fe^{3+} \rightleftharpoons Fe[Fe(CN)_6]^- \quad (12)$$

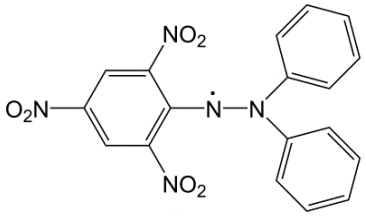
Using this method, our group[171] tested the reducing power of methanol aqueos extracts, derived from stalks, capitula and leaves of wild and cultivated cardoon. Nonetheless, this research group observed a weak linear correlation between the reducing power and the total phenolic contents (r = -0.553).[171]

6.1.1.3 Hydrogen atom transfer and single electron transfer assays

The scavenging effect assays of 2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) (Figure 41) are usually classified as SET reactions. However, both synthetic organic radicals may be neutralized either by direct reduction via electron transfer (SET), or by radical quenching via hydrogen atom transfer (HAT).[167]



2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$)



2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$)

Figure 41: Structures of 2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$), and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) free radicals.

The $ABTS^{\cdot+}$ scavenging effect is monitored through the decrease of $ABTS^{\cdot+}$ radical absorbance at 415 or 734 nm, in the presence of the tested compound. The $ABTS^{\cdot+}$ radical is previously formed through the ABTS oxidation induced by $ROO^{\cdot}$ radicals or other oxidants. A compound can reduce $ABTS^{\cdot+}$ radical if it has a redox potential lower than that of $ABTS^{\cdot+}$/ABTS ($\mathcal{E}^{o}$ = 0.68 V). The inhibitory concentration at 50% (IC_{50}) of the tested compound, regarding to $ABTS^{\cdot+}$ scavenging effect, is usually expressed as Trolox equivalent antioxidant capacity (TEAC).[167] Llorach, *et al.*[91] described the scavenging effect of methanol and aqueous extracts, derived from artichoke by-products towards $ABTS^{\cdot+}$ radicals, observing a weak linear correlation between TEAC and total phenolic contents (r = 0.78). Betancor-Fernández, *et al.*[173] approached the $ABTS^{\cdot+}$ scavenging effect of four fractions of different polarities (acetone/water (75:25), methanol/water (70:30), ethanol and water), derived from commercial artichoke leaves extract. This group noted that TEAC of artichoke fractions was linearly correlated with the total phenolic contents (r = 0.973).[173]

The DPPH assay consists of monitoring the decay of DPPH radicals absorbance (λ_{max} = 515-517 nm), while the antioxidant neutralizes the DPPH free radicals to DPPH-H. This is one of the most used tests to evaluate the antioxidant capacity of plant phenolic-containing extracts.[167] Phenolic compounds can react with DPPH radicals through mechanisms of HAT (Equation (13))[174] or SET (Equations (14)-(16)).[175] According to Foti, *et al.*,[174] the transference of an electron from the phenoxide anion (ArO^{-}) to $DPPH^{\cdot}$ free radicals is prevalent in polar solvents, like methanol and ethanol, and HAT path is a marginal reaction.

HAT $DPPH^{\cdot} + AH \longrightarrow DPPH\text{-}H + A^{\cdot}$ (13)

SET $ArOH \rightleftharpoons ArO^{-} + H^{+}$ (14)

$ArO^{-} + DPPH^{\cdot} \longrightarrow ArO^{\cdot} + DPPH^{-}$ (15)

$DPPH^{-} + H^{+} \longrightarrow DPPH\text{-}H$ (16)

The steric accessibility is also important in the DPPH scavenging effect, since small molecules that have better access to the radical site have higher apparent antioxidant effect in this test. Moreover, DPPH is a stable nitrogen radical that is not similar to the highly reactive $ROO^{\cdot}$ radicals involved in lipid peroxidation. Many antioxidants that react quickly with $ROO^{\cdot}$ radicals may react slowly or even do not react with DPPH due to steric inaccessibility.[167]

Wang, *et al.*[98] assayed the DPPH scavenging effect of seven phenolic compounds, isolated from artichoke capitula and leaves, reporting the following antioxidant capacity order: 1,3-di-*O*-caffeoylquinic acid > luteolin 7-*O*-rutinoside ≈ luteolin 7-*O*-glucoside > 1-*O*-caffeoylquinic acid ≈ 5-*O*-caffeoylquinic acid > naringenin 7-*O*-rutinoside > apigenin 7-*O*-rutinoside. The two caffeoyl moieties may actually contribute for the antioxidant ability of 1,3-di-*O*-caffeoylquinic acid. It was proposed that caffeic acid neutralizes DPPH free radicals, through the formation of *o*- or *p*-quinones,[175] with resonance stabilization across the aromatic ring connected with the highly conjugated side chain CH=CH-COOH (Figure 42).[176,177]

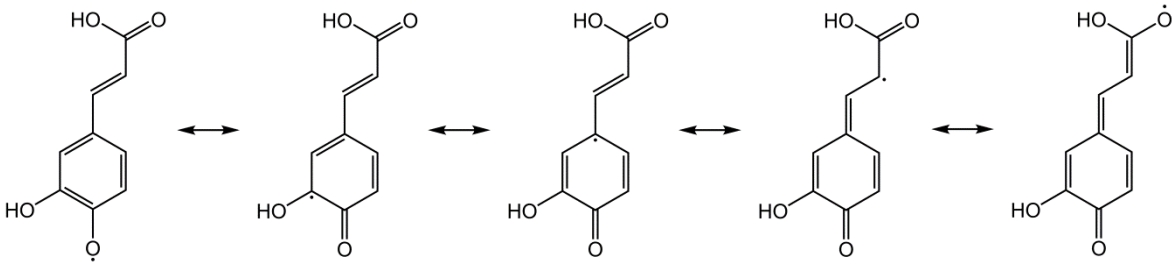


Figure 42: Resonance structures of caffeic acid radical.

Wang, *et al.*[98] also noticed that luteolin 7-*O*-rutinoside and 7-*O*-glucoside represented the more active flavonoids, isolated from artichoke capitula and leaves, in neutralizing the DPPH free radicals. This can be related with the fact that the luteolin aglycone has two of the three essential structural characteristics for the maximum scavenging effect: (i) the presence of 3´,4´-dihydroxystructure in the B-ring and (ii) the presence of a 2,3-double bound in conjugation with the 4-oxo group in the heterocycle, allowing for conjugation between A- and B-rings. The third structural condition is associated with the presence of 3- and 5-hydroxyl groups in the A ring, together with a 4-oxo function in A and C rings.[170]

Table 15 summarizes the antioxidant action of *C. cardunculus* L. extracts assessed through the DPPH radicals scavenging assay.

Table 15: DPPH scavenging effect of *C. cardunculus* L. extracts, and its correlation with the phenolic composition.

***C. cardunculus* L. extracts**		**Correlation with phenolic compounds**	**Source**
Artichoke	By-products	Linear correlation with total phenolic content (r = -0.98)	[91]
	Capitula, leaves	Linear correlation with the total phenolic content (r = -0.96)	[98]
Artichoke and wild cardoon	Stalks, seeds	Linear correlation with total phenolic content (r = -0.832) and condensed tannins content (r = -0.915).	[178]
Wild cardoon	Capitula, leaves, seeds	Linear correlation with total phenolic content (r = -0.93), total flavonoid content (r = -0.98) and condensed tannins content (r = -0.96)	[85]
Wild and cultivated cardoon	Stalks, capitula, leaves	Weak linear correlation with total phenolic contents (r = -0.716)	[171]

Both reports of Llorach, *et al.*[91] and Wang, *et al.*[98] (Table 15) indicated that the antioxidant activities of methanol extracts,[91,98] derived from industrial wastes (floral stems and capitula),[91] capitula and leaves of artichoke,[98] was linearly correlated with the total phenolic contents, by employing the DPPH assay. Furthermore, Falleh, *et al.*[85] and Soumaya, *et al.*[178] (Table 15) referenced that the DPPH scavenging effects of methanol extracts from wild cardoon and artichoke were linearly correlated with the total phenolic, total flavonoid and condensed tannins contents.[85,178] Kukic, *et al.*[10] studied the DPPH scavenging effect of a 96% ethanol extract of capitula wild cardoon, as well as four derived-fractions, namely chloroform, ethyl acetate, butanol and water. They observed that the DPPH scavenging effect of artichoke fractions was dependent on the total phenolic contents, being the ethyl acetate the strongest (0.203 mg GAE/mg extract). Our research group[171] published data regarding to the DPPH scavenging effect of methanol aqueous extracts from stalks, capitula and leaves of wild and cultivated cardoon, collected at the flowering and senescence stages. The antioxidant power of these extracts were weakly linearly correlated with the total phenolic contents.[171]

6.1.1.4 Scavenging effect of reactive oxygen species

Some works have examined the scavenging effect of *C. cardunculus* L. extracts on several ROS, namely superoxide radical anion ($O_2^{\cdot-}$), hydroxyl radical ($HO^{\cdot}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2).[165,179]

Non-cellular systems-based assays

Three reports[85,99,164] showed the scavenging effect of *C. cardunculus* L. extracts upon several ROS, such as $O_2^{\cdot-}$, $HO^{\cdot}$ and $^1O_2$. Table 16 depicts several assays used to evaluate ROS scavenging effect of artichoke and wild cardoon extracts.

Table 16: ROS scavenging assays used for assessing the *C. cardunculus* L. extracts antioxidant effect.

ROS	*C. cardunculus* L. extracts		Assay	Source
$O_2^{\cdot-}$	Artichoke	Leaves infusion	PMS/NADH/O_2	[164]
	Wild cardoon	Leaves infusion	PMS/NADH/O_2 and X/XO	[99]
		Leaves methanol extract	PMS/NADH/O_2	[85]
$HO^{\cdot}$	Wild cardoon	Leaves infusion	Fe(III)-EDTA/ascorbic acid	[99]
$^1O_2$	Artichoke	Leaves infusion, decoction and ethanol/water (70:30) extract	Dihydrorhodamine 123 oxidation	[164]

Abbreviations: NADH, nicotinamide adenine dinucleotide in the reduced form; PMS, phenazine methosulfate; X/XO, xanthine/xanthine oxidase system.

The superoxide anion radical is the first ROS generated from the reduction of oxygen, during mitochondrial electron transport. Due to its solubility properties, $O_2^{\cdot-}$ causes considerable damages to membrane phospholipid components.[41] Pistón, *et al.*[164] evaluated the $O_2^{\cdot-}$ scavenging effect of artichoke leaves extracts, applying a non-enzymatic system constituted by a mixture of phenazine methosulfate (PMS) and nicotinamide adenine dinucleotide (NADH). This mixture, in the presence of oxygen, generates $O_2^{\cdot-}$ through the univalent oxidation of PMS. Thereafter, $O_2^{\cdot-}$ is monitored through reduction of nitroblue tetrazolium to a formazan product which is quantified by UV-Vis spectroscopy (λ_{max} = 560 nm).[180] Pistón, *et al.*[164] observed that artichoke leaves infusion was the most effective to scavenge $O_2^{\cdot-}$ radical anions, which was associated with higher concentrations of phenolic compounds and chlorogenic acid. Falleh, *et al.*[85] observed weak linear correlations between the $O_2^{\cdot-}$ scavenging effect of wild cardoon methanol extracts, and the contents of phenolic compounds (r = -0.81), condensed tannins (r = -0.75) and flavonoids (r = -0.68).[85] Besides the PMS/NADH/O_2 assay, Valentão, *et al.*[99] also examined the $O_2^{\cdot-}$ scavenging effect of wild cardoon leaves infusion, through the xanthine/xanthine oxidase (X/XO) system. Different IC_{50} values were obtained with PMS/NADH/O_2 (145.2 µg/mL) and X/XO systems (35.0 µg/mL), being justified with the higher $O_2^{\cdot-}$ level, generated by the former system.[99]

The $HO^{\cdot}$ radical is one of the strongest oxidizing agents, which reacts with polyunsaturated fatty acids, giving rise to $ROO^{\cdot}$ radicals and, consequently, initiating lipid peroxidation.[164] Valentão, *et al.*[99] observed that wild cardoon leaves infusion exhibited $HO^{\cdot}$ scavenging ability, in a dose-dependent way (3.1-800 µg/mL). The $HO^{\cdot}$ radical was generated

by H_2O_2 reaction with Fe (II) (Fenton reaction), in the presence of EDTA and ascorbic acid.[99] EDTA was added to the mixture, in order to prevent Fe(II) chelation by the test antioxidant, while ascorbic acid was added to act as pro-oxidant through reduction of Fe(III) to Fe(II), and therefore to generate a constant flux of $HO^{\cdot}$ radicals, in the presence of excess H_2O_2.[181] These radicals were reacted with 2-deoxy-D-ribose, forming MDA which was further quantified spectrophotometrically after its reaction with thiobarbituric acid (λ_{max} = 532 nm).[99]

Singlet oxygen is a highly excited state, resulting from sufficient energy, absorbed by dioxygen (O_2), to shift an unpaired electron to a higher orbital. It is more reactive than $HO^{\cdot}$ radicals.[41] Pistón, *et al.*[164] observed the scavenging effect of three artichoke leaves extracts against $^1O_2$, through a fluorescence method based on the $^1O_2$ induced-oxidation of dihydrorhodamine 123 to the fluorescent rhodamine 123 ($\lambda_{excitation}$ = 485 nm and $\lambda_{emission}$ = 528 nm[182]). Among artichoke leaves extracts, Pistón, *et al.*[164] referred that ethanol/water (70:30) extract (73 mg/g of identified phenolic compounds) was the most active extract in preventing the oxidation of dihydrorhodamine 123 induced by $^1O_2$.

Cellular systems-based assays

Some researchers have studied the *in vitro* inhibition action of artichoke extracts, upon ROS production in animal cell cultures assays. Pérez-García, *et al.*[183] noticed that a commercial artichoke leaves aqueous extract (1.5% caffeoylquinic acids and 0.7% flavonoids) reduced, in a dose-dependent way (0.001-100 µg/mL), the ROS production in human leukocytes, stimulated by H_2O_2 and *N*-formylmethyl-leucyl-phenylalanine. It was indicated that the antioxidant power of artichoke leaves extract could be related with the phenolic composition.[183] Moreover, Gebhardt[184] also described that a commercial artichoke leaves aqueous extract (5 and 500 µg/mL), as well as chlorogenic acid and cynarin, inhibited the MDA retention originated by the *tert*-butylhydroperoxide (*t*-BHP) stimulated lipid peroxidation, in rat hepatocytes. In fact, the antioxidant effect of this artichoke extract was higher than that of chlorogenic acid plus cynarin mixture (2-fold).[184] Gebhardt[184] further observed that artichoke leaves extract (0.5-1000 µg/mL) inhibited the GSH loss in *t*-BHP stimulated rat hepatocytes, principally when the cells were pretreated with diethylmaleate, a GSH depleting compound. Similarly, Miccadei, *et al.*[185] demonstrated that artichoke capitula extract (1 mM chlorogenic acid equivalents (CAE)) treatment prevented the GSH loss, as well as the MDA accumulation in H_2O_2-stimulated rat hepatocytes.

Our group[171] reported the protective effect of methanol aqueous extracts from stalks, capitula and leaves of cultivated cardoon on the H_2O_2 injury on the *Saccharomyces cerevisiae* proliferation. It was verified that cultivated cardoon extracts could prevent the H_2O_2 cytotoxicity on *Saccharomyces cerevisiae*, at 25 µg/mL GAE, being comparable to the synthetic antioxidant 2-*tert*-butyl-4-methoxyphenol (25 µg/mL).[171]

6.1.2 *In vivo* assays

Living organisms developed antioxidant defense mechanisms to protect themselves towards the oxidative stress, namely enzymatic systems, such as superoxide dismutase, catalase and glutathione peroxidase.[166] At the current knowledge, only one report[186] examined the physiological effect of artichoke capitula methanol/water (50:50) extract on the rat antioxidant status, after 3 weeks of intake. Jiménez-Escrib, *et al.*[186] explained that the phenolic-containing extract of artichoke capitula did not significantly change the radical scavenging effect in the plasma, but it increased the GSH peroxidase activity in erythrocytes. Jiménez-Escrib, *et al.*[186] also suggested that either flavones or hydroxicinnamic acids probably contributed for the antioxidant effect of artichoke capitula extract.

6.2 Antitumoral activity

The development of cancer is incited by the increased proliferation of tumor cells, which reflects a breakdown in the balance between *de novo* cell replication and the programmed cell death.[187,188] Several environmental factors have been implicated in the etiology of this disease, as follows: (i) chemical carcinogens, as those found in tobacco smoke (e.g. benzo(*a*)pyrene, dimethylnitrosamine, and nickel compounds); (ii) physical carcinogens, such as UV radiation and asbestos; and (iii) infection by pathogenic bacteria and viruses, namely *Helicobacter pylori*, human papilloma virus and human hepatitis B and C virus.[189] They alter the function of key cellular regulatory proteins in two ways, namely through: (i) the formation of DNA adducts, and subsequent somatic mutations, changing the protein structure and function; (ii) errors that affect DNA methylation pattern in the promoter region of certain genes, affecting mRNA level and protein expression. The cellular transformations, stimulated by environmental factors, also depend on the germline mutations of each individual which determine the relative risk to cancer predisposition.[189]

Cancer is originated by alterations in critical regulatory genes that control cell proliferation, differentiation and survival, like proto-oncogenes, oncogenes and tumor suppressor genes.[187] Proto-oncogenes are important cellular regulatory genes, in many cases encoding proteins implicated in signal transduction pathways, controlling normal cell proliferation (e.g., *src*, *ras*, and *raf*). The oncogenes are abnormally expressed or mutated forms of the respective proto-oncogenes. The progressive acquisition of activating mutations in oncogenes drives to abnormal cell proliferation and tumor development.[187,188] Besides this, the inactivation of tumor suppressor genes is also responsible for tumor development. Tumor suppressor genes usually act to inhibit cell proliferation and tumor development. In many tumors, these genes are lost or inactivated and, consequently, the negative regulators of cell proliferation are downregulated, contributing to the abnormal tumor cells regulation.[187]

Mutations of tumor suppressor genes are generally inherited and contribute to familial cancers.[188]

The carcinogenesis is indeed a complex multistep process, comprehending mutation and selection in favor to the transformed cells, with increasing ability for proliferation, survival, invasion and metastasis.[187] This process comprises three major steps, namely: (i) initiation, (ii) promotion and (iii) progression. Initiation results from genetic alterations, leading to abnormal cell proliferation. During the promotion step, abnormal cells replicate and may generate a focus of preneoplastic cells.[190] Primary tumor is formed as preneoplastic cells grow.[191] In the progression step, additional mutations allow the gradual conversion of premalignant cells to neoplastic ones, with higher invasiveness and metastatic potential.[187] Nutrients and oxygen needs of primary tumor will cause segregation of angiogenic inducers to the extracellular medium, being a stimulus for cell growth and angiogenesis, progressing with the formation of secondary tumors at distant locals, known as metastases.[187,190]

Chemoprevention includes the administration of natural and/or synthetic compounds during the initiation, promotion and progression steps of carcinogenesis.[192] Plant secondary metabolites have shown to be promising chemopreventive agents, due to: (i) reduced toxicity; (ii) desired range of efficacy; and (iii) ability to influence simultaneously multiple signaling pathways.[193]

Cancer chemopreventive compounds can be separated in two main groups: (i) blocking agents, which inhibit the mutagenic initiation, and (ii) suppressing agents, which stops the promotion or progression of lesions that have already been established.[189] At the promotion phase, the suppressing agents can induce cell cycle arrest or apoptosis, since these can inhibit or slow down cell division, restoring the balance between cell proliferation and death. At the progression step, chemopreventive agents should prevent malignant cells tumors to migrate, to invade, to stimulate angiogenesis and to decrease the route of metastasis formation.[190]

Several groups have studied, in the last decade, the antitumoral potential of *C. cardunculus* L. extracts, as well of isolated terpenic and phenolic components, by using *in vitro* and *in vivo* assays, as indicated in this chapter.

6.2.1 *In vitro* assays

Two works revealed the antiangiogenic[171] and antimigratory[194] potential of *C. cardunculus* L. phenolic extracts. Our group[171] reported the antiangiogenic potential of a cultivated cardoon leaves methanol aqueous extract, based in the assessment of intersegmental vessels number, in a transgenic zebrafish model (Tg(fli1:EGFP)), observing the decreasing number of intersegmental vessels as response of the increased cultivated cardoon leaves methanol extract concentration (0.15-30 mg/mL). The antiangiogenic effect of

cultivated cardoon leaves methanol extract showed an IC_{50} of 10.05 mg/mL. Mileo, *et al.*[194] indicated that 24 h and 48 h-treatments with artichoke capitula methanol extract (200 µM CAE) diminished greatly the MDA-MB-231 cell invasion ability, almost 2-fold compared to control cells.

The majority of the studies[171,185,194–197] approached the antiproliferative potential of *C. cardunculus* L. phenolic-containing extracts, against human tumor cell lines of leukemia,[195] hepatocellular carcinoma (HCC)[185,196,197] and triple-negative breast cancer (TNBC).[171,194]

Nadova, *et al.*[195] investigated the antiproliferative effect of an ethyl acetate fraction (21.26% of caffeoylquinic acids and 3.25% of flavonoids), derived from artichoke capitula ethanol extract, on human promyelocytic leukemia HL-60 cell line (Figure 43A). This fraction prevented the HL-60 cell growth, in a dose-dependent manner (500-2500 µg/mL), during 24 h.[195]

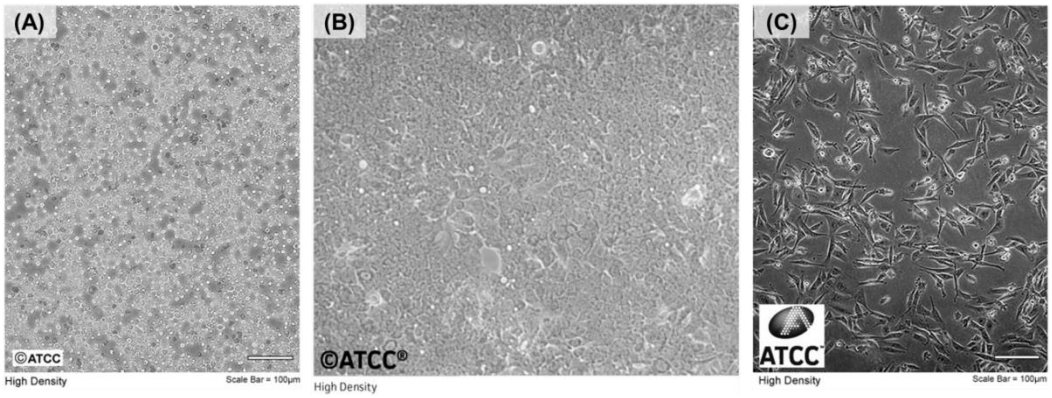


Figure 43: Representative photographs of (A) HL-60, (B) HepG2 and (C) MDA-MB-231 cell lines (Source: American Type Cell Culture[198]).

Miccadei, *et al.*[185] studied the antiproliferative action of an artichoke capitula methanol extract, presenting hydroxycinnamic acids and flavonoids, against the HCC HepG2 cell line (Figure 43B). They reported the ability of this extract to decrease the HepG2 cellular viability, in a dose- (400-1200 µM CAE) and time- (24, 48 and 72 h) dependent ways. Furthermore, Menghini, *et al.*[196] proved that an artichoke leaves phenolic-containing extract (13.3% of caffeoylquinic acids) prevented the HepG2 cell growth, for 48 h, in a dose-dependent manner (75 and 100 µg/mL). The 48 h-treatment of an artichoke leaves infusion (20.82 mg GAE/g) also inhibited the HepG2 cellular viability (IC_{50} = 52.06 µg/mL).[197]

Mileo, *et al.*[194] noted that an artichoke capitula methanol extract, mainly constituted by caffeoylquinic acids, reduced the TNBC MDA-MB-231 cellular viability (Figure 43C), for 24 h, in a dose-dependent manner (200-800 µM CAE). TNBC is defined as absence of estrogen (ER) and progesterone (PR) receptors and lacking overexpression of human epidermal growth

factor receptor type 2 (HER2).[199] Moreover, Mileo, *et al.*[194] observed that increasing concentrations of artichoke phenolic-containing extract decreased the cellular viability of other cell lines of human BC, namely T47D (ER positive), MCF-7 (ER positive) and BT549 (ER negative). At the same concentration range, this artichoke phenolic-containing extract did not have impact on the cellular viability of MCF-10A, an immortalized human breast epithelium cell line.

Our group[171] also studied the inhibitory effects of methanol aqueous extracts, derived from leaves and capitula of wild and cultivated varieties of cardoon on MDA-MB-231 cell growth, during 48 h. Cultivated cardoon leaves methanol extract was the most effective (IC_{50} = 259.4 µg/mL).[171]

Three independent research works[185,194,195] have given insights relatively to the regulating role of artichoke phenolic-containing extracts, upon cell cycle and apoptosis signaling pathways in HL-60,[195] HepG2 cell lines[185] and MDA-MB-231[194] (Table 17).

Table 17: Effects of artichoke capitula phenolic-containing extracts on cell cycle and apoptosis signaling pathways, in human tumor cell lines.

Artichoke extracts	Human tumor cell line	Cell cycle [(I)]	Apoptosis [(I)]	Source
Hydroxycinnamic acids and flavonoids	Promyelocytic leukemia HL-60 cells	G0/G1 cell cycle arrest	↑: cytochrome-c; active caspases-9 and -3; ↓PARP-1	[195]
Caffeoylquinic acids, luteolin and apigenin glycosides	HCC HepG2 cells		↑ cleaved caspase-3	[185]
	TNBC MDA-MB-231 cells	↑p21	↑: caspases-9 and -8; Bax; ↓Bcl-2	[194]

[(I)] The arrow indicates increase (↑) or decrease (↓) in protein expression level, relatively to control. Abbreviations: HCC, hepatocellular carcinoma; PARP, poly(ADP-ribose) polymerase; TNBC, triple-negative breast cancer.

6.2.1.1 Effects on cell cycle

Cell cycle embraces the DNA replication and segregation of replicated chromosomes to two separate cells. This process occurs during two main consecutive steps (Figure 44): (i) interphase, which comprehends G1, S and G2 phases; and (ii) mitosis, which includes prophase, metaphase, anaphase and telophase. DNA replication takes place in the S phase. The S phase is preceded by G1 phase, during which the cell is prepared for DNA synthesis, and followed by the G2 phase, during which the cell growth continues and proteins are synthesized for mitosis. In the presence of extracellular growth factors, animal cell passes through a decision point, in late G1, known as restriction point, and enter into S phase.[187,200] Once it passed through the restriction point, the cell no longer needs mitogenic stimuli to

undergo cell division.[187,201] Accordingly, cells can not pass the restriction point in the absence of growth factors, entering thus into the quiescent step G0 phase. At G0, cells remain metabolically active, although they cease growth and have reduced protein synthesis rates.[187,200] Cells can exit from G0 and reenter into cell cycle, towards growth factor stimulation.[187]

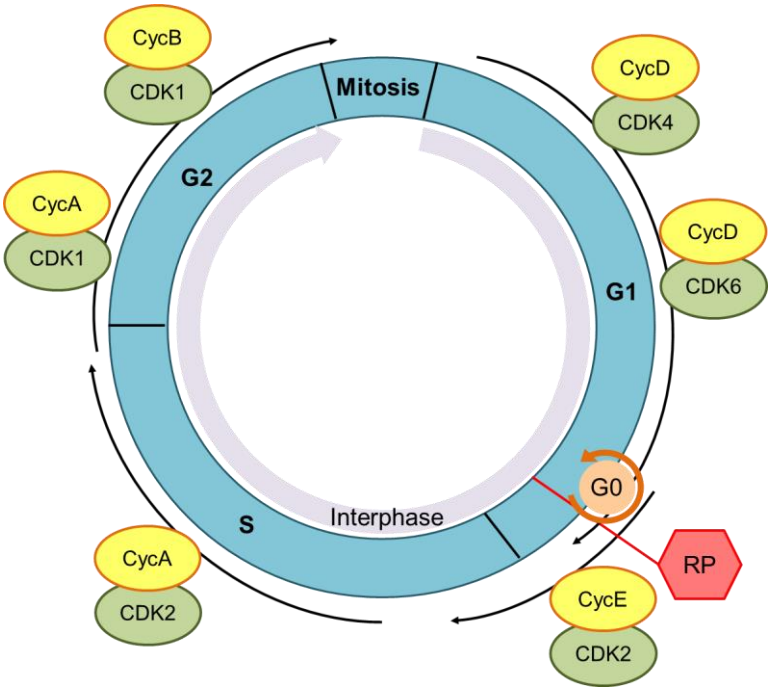


Figure 44: Mammalian cell cycle phases and CDK/cyclin complexes. Abbreviations: CDK, cyclin-dependent kinase; Cyc, cyclin; G1, G1 cell cycle phase; G2, G2 cell cycle phase; RP, restriction point and S, S cell cycle phase (adapted from [187,201]).

Cell cycle regulation is dependent on several cell cycle checkpoints, which prevent the cell entry into the next step, until the events of preceding step have been finished. These checkpoints “recognize” unreplicated or damaged DNA, resulting in cell cycle arrest to activate DNA replication or repair and, in some cases, programmed cell death.[187,200] Cell cycle checkpoints at G1, S and G2 phases are regulated by a family of serine/threonine protein kinases, denominated as cyclin-dependent kinases (CDK) (Figure 44). Four CDKs are active during the cell cycle, specifically: (i) CDK4, CDK6 and CDK2 in G1; (ii) CDK2 in S; (iii) CDK1 in G2 phase and mitosis.[187,200] These are activated by binding with the respective cyclins (Figure 44), as explained herein: (i) cyclin D can bind to CDK4 and to CDK6; CDK4,6/cyclin D complexes are essential in G1 phase for the progression through the restriction point; (ii) cyclin E associates to CDK2, for regulating G1/S transition; (iii) cyclin A connects to CDK2 during progression throughout S phase; iv) cyclin A links to CDK1 for S/G2 transition; v) cyclin B binds to CDK1 for passage from G2 to mitosis.[201]

The CDK kinase activity is regulated by at least four mechanisms:[187] (i) formation of CDK/cyclin complexes, depending on the cyclin synthesis and degradation; (ii) activating phosphorylation of a conserved CDK threonine (Thr) residue around position 160, which is catalyzed by the CDK-activating kinase (CAK) (CDK7/cyclin H complex); (iii) inhibitory phosphorylation of tyrosine (Tyr)-15 and Thr14 residues; iv) binding of CDK inhibitors (CKI).[187]

Two CKI families regulate the different CDK/cyclin complexes (Figure 45), namely Ink4 ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, $p19^{INK4d}$) and Cip/Kip ($p21^{Waf1,\ Cip1}$ (referred hereafter as p21), $p27^{Cip2}$ and $p57^{Kip2}$) families. The Ink4 specifically inactivates G1 phase CDKs (CDK4 and 6), because they generate stable complexes with those CDKs, before cyclin D binding.[200] The Cip/Kip family bind to complexes of CDK2, 4 and 6 with cyclins A, D and E, throughout G1 and S phases.[187,200] Additionally, p21 can inhibit the kinase activity of CDK1/cyclin B complexes.[202]

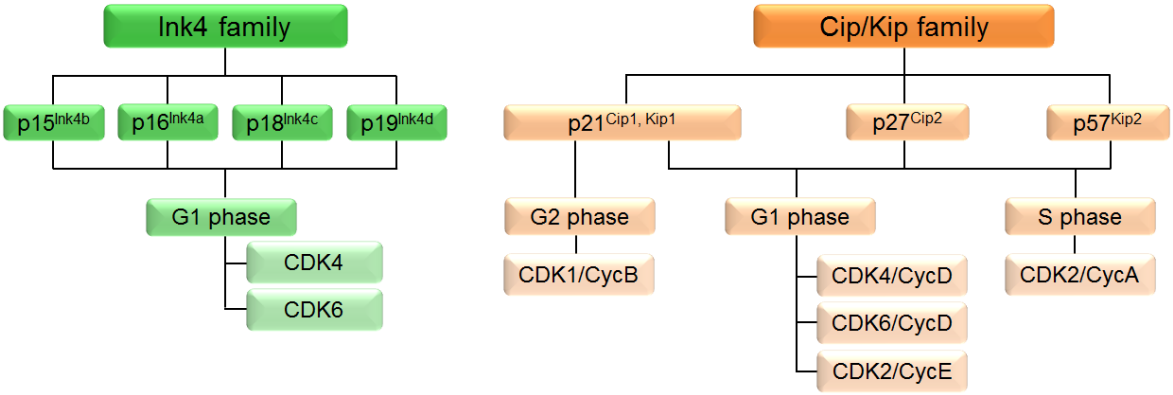


Figure 45: The inhibitor families of G1, S and G2 CDKs.[187,200,202]

When DNA is damaged, two protein kinases are activated, known as ataxia-telangiectasia-mutated (ATM), and ataxia and rad3 related (ATR). Active ATR and ATM then phosphorylate and activate the downstream kinases Chk1 and Chk2, respectively. Chk1 and Chk2, in turn, phosphorylate cell cycle checkpoint proteins, leading to cell cycle arrest at G1, S and G2 phases.[187]

The cell cycle arrest at G1 checkpoint is mediated by the transcription factor p53 (Figure 46). ATM phosphorylates and stabilizes p53, resulting in a rapid protein level increase. p53 upregulates p21 expression which inhibits CDK4,6/cyclin D complexes, leading to cell cycle blockage.[187,200] When p53 loses its function, the G1 arrest is inhibited. Therefore, the damaged DNA is replicated and passed into daughter cells instead of being repaired. This inheritance of damaged DNA results in an increment of mutations frequency and cellular genome instability, which ultimately contributes to carcinogenesis.[187]

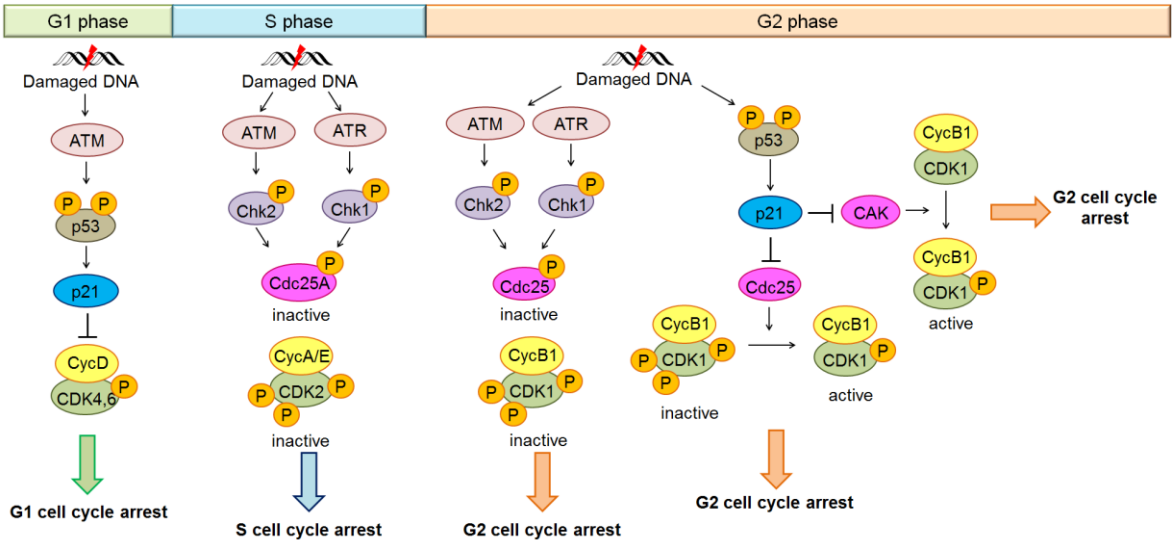


Figure 46: Main mechanisms of cell cycle arrest at G1, S and G2 phases.[187,201–203]

If DNA is damaged in the S phase (Figure 46), active Chk1 and Chk2 protein kinases will phosphorylate Cdc25A. Cdc25A is responsible for the dephosphorylation and activation of CDK2/cyclin E and CDK2/cyclin A complexes. Cdc25A phosphorylation causes its rapid degradation, originating cell cycle arrest at S phase.[187]

The CDK1/cyclin B1 complex, also recognized as mitosis promoting factor (MPF), has a critical function in the cell cycle transition from G2 phase to mitosis. Cyclin B1 protein level increases during S phase, accumulating and forming complexes with CDK1 (MPF) at S and G2 phases.[187] In cycling cells, MPF is kept inactive, during S and G2 phases, by inhibitory phosphorylations of CDK1 at Thr-161, Tyr-15 and Thr-14. The phosphorylations of Tyr-15 and Thr-14 are mediated by the protein kinases Wee1/Mik1 and Myt1, respectively. The prophase onset correlates with the Cdc25 mediated-dephosphorylation of CDK1 at Thr-14 and Tyr-15.[202] Towards damaged DNA, G2 cell cycle arrest can be mediated by Chk1 and Chk2 which phosphorylate Cdc25 members, preventing the MPF activation (Figure 46).[187,202] Other pathway was suggested to support the MPF inactivation, implying p53-dependent p21 upregulation (Figure 46).[202] On one hand, p21 can inhibit the MPF activation, by interfering with CDK1 phosphorylation mediated by CAK;[202,203] on the other hand, p21 can block the CDK1 dephosphorylation mediated by Cdc25 phosphatase.[202]

In what concerns the effect of *C. cardunculus* L. extracts on cell cycle regulation, Nadova, *et al.*[195] observed that the 24 h-treatment of human leukemia HL-60 cells, with an artichoke capitula ethyl acetate fraction, blocked the cell cycle at G0/G1 phases (Table 17, page 71), in a dose-dependent manner (500-2500 µg/mL). Other study reported the action of an artichoke capitula methanol extract (200-800 µM CAE), upon p53 and p21 proteins regulation in MDA-MB-231 cells, for 24 h (Table 17, page 71).[194] Mileo, *et al.*[194] referenced

that this extract upregulated p21 in MDA-MB-231 cells, specially at lower concentrations (200 and 400 µM CAE), being stronger than the known cytostatic paclitaxel (1 µM). Nevertheless, p53 protein levels were practically unaffected at the tested artichoke extract concentrations, relatively to control cells.[194] They suggested that the antiproliferative effect of artichoke phenolic-containing extract, on MDA-MB-231 cells, could be related to cell cycle regulation.[194]

6.2.1.2 Effects on apoptosis

Apoptosis is considered as an important mode of "programmed" cell death, i.e. a genetically cell "discard". This comprises a cascade of molecular events, which occurs as a homeostatic mechanism to maintain cell populations in tissues. There are two main pathways (Figure 47): (i) the extrinsic or death receptor pathway ("type I"), and (ii) the intrinsic or mitochondrial pathway ("type II").[204] These pathways converge to the execution pathway which is started with the caspase-3 cleavage, originating DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and, ultimately, uptake of phagocytic cells.[204]

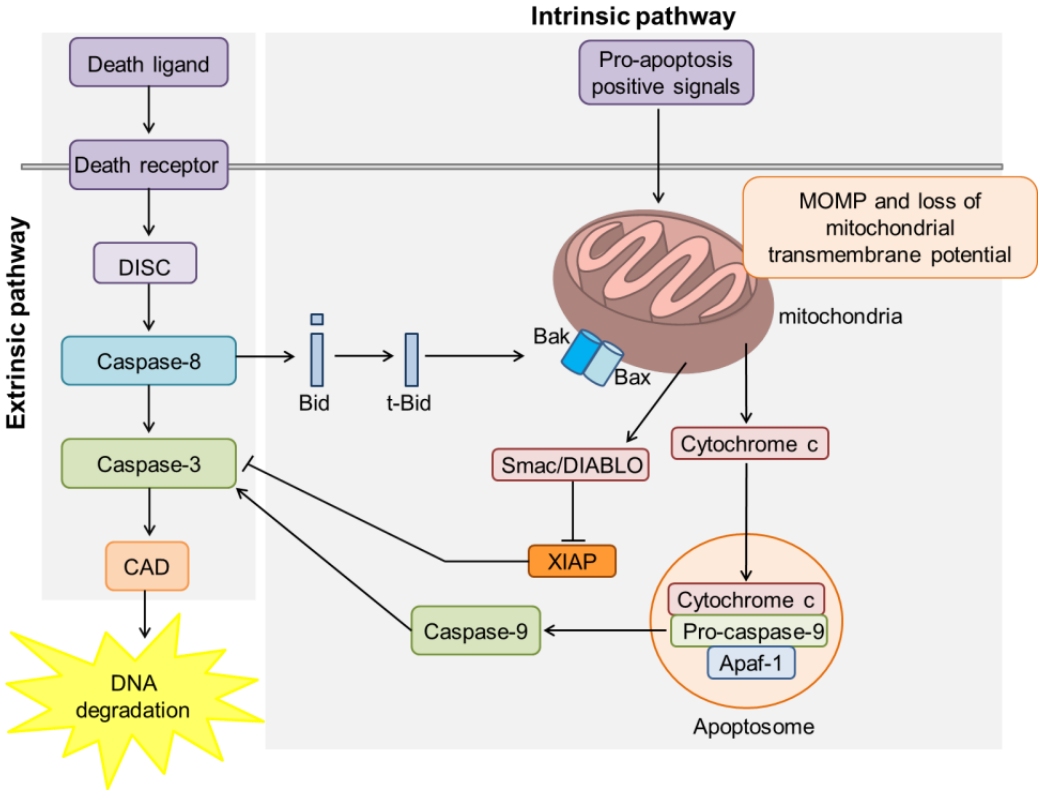


Figure 47: Extrinsic and intrinsic pathways of apoptosis (adapted from [205,206]). Abbreviations: CAD, caspase-activated DNase; DISC, death-inducing signaling complex; MOMP, mitochondrial outer membrane permeabilization.

The extrinsic pathway (Figure 47) involves binding of death ligand to the corresponding death receptors, members of the tumor necrosis factor (TNF) receptor gene superfamily. TNF receptor family members have a cytoplasmic “death domain” critical for transmitting the death signal, from the cell surface to the intracellular signaling pathways. Once death ligand is bonded to the death receptor, the death-inducing signaling complex (DISC) is triggered to be formed, resulting in autocatalytic activation of initiators procaspases-8 and -10.[204] Active caspases-8 and -10 at DISC cleave directly the effector procaspases-3 and -7. Therefore, active caspases-3 and -7 cleave cytokeratins, nuclear lamins and the inhibitor of caspase-activated DNase.[206] Caspase Activated DNAse activation then causes chromosomal DNA degradation and chromatin condensation.[204]

The intrinsic pathway (Figure 47) does not involve receptor-mediated stimuli, but it implicates instead stimuli that promote mitochondrial-mediated intracellular signals. Pro-apoptosis positive signals comprehends several factors, such as radiation, toxins, hypoxia, hyperthermia, viral infections and free radicals.[204] All of these stimuli induce the formation of pores in the mitochondrial membrane, causing mitochondrial outer membrane permeabilization (MOMP), and loss of the mitochondrial transmembrane potential. Bcl-2 protein family members regulate the mitochondrial pore formation, which can be classified in four groups, as follows: (i) “effectors” Bax and Bak, whose oligomerization creates pores; (ii) “inhibitors” of Bax and Bak association, namely Bcl-2, Mcl1 and BclxL; (iii) “activators” of Bax and Bak, e.g. Bid and Bim; and iv) “sensitizers”, such as Bad, Bik, and Noxa which antagonize the antiapoptotic Bcl-2-like proteins. Initiator caspases-8 and -10, activated at the DISC, cleave Bid to tBid, which leads to conformational changes and oligomerization of Bax and Bak. Bax and Bak oligomers thereafter originate mitochondrial outer membrane pores, as well as promote cytoplasmic translocation of critical apoptosis regulators, such as cytochrome c and Smac/DIABLO which are usually in the mitochondrial intermembrane space.[206] Once in the cytosol, cytochrome c binds and activates Apaf-1 and procaspase-9, forming the apoptosome. Caspase-9 activates effector procaspases-3 and -7.[204] However, XIAP associates to the catalytic pocket of active effector caspases-3 and -7, thereby inhibiting their protease activity and promoting their ubiquitin-dependent degradation. Binding of Smac/DIABLO to XIAP relieves this inhibition, allowing full activation of effector caspases and consequently cell death.

Some reports[185,194,195] have examined the apopotic action of artichoke phenolic-containing extracts in three human tumor cell lines. Nadova, *et al.*[195] noticed that the 24 h-treatment of human leukemia HL-60 cells, with the artichoke capitula ethyl acetate fraction, caused apoptosis (Table 17, page 71), probably through the extrinsic pathway. This artichoke extract induced, in a dose-dependent manner (1250-2500 µg/mL), the cytochrome-c release, as well as caspases-9/-3 activation and protein expression decrease of poly(ADP-ribose)

polymerase (PARP), the caspase-3 substrate.[195] Internucleosomal DNA fragmentation was also detected in HL-60 cells, after 24 h-treatment with artichoke capitula ethyl acetate fraction at 2500 µg/mL.[195]

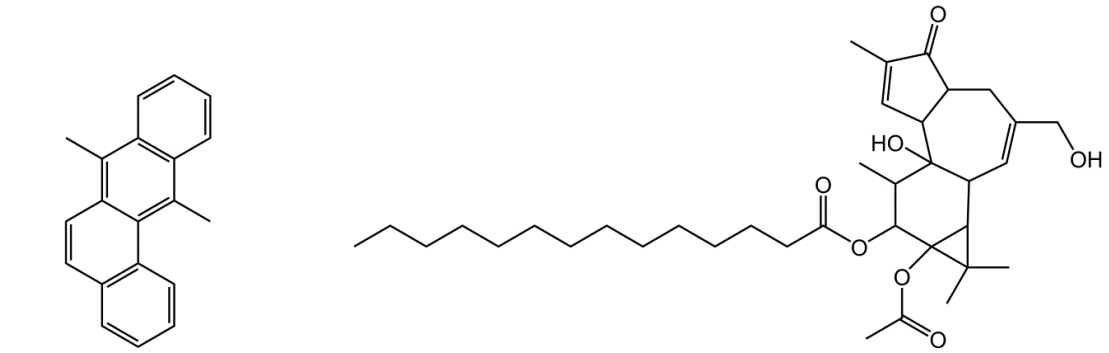
Miccadei, *et al.*[185] observed that 24 h-treatment of HepG2 cells with artichoke capitula methanol extract (400-1200 µM CAE) increased, in a dose-dependent manner, the apoptotic cell population, to a maximum of 64.5%. This extract, at 1200 µM CAE, caused 3.1-fold higher apoptotic cell percentage than 1200 µM 5-*O*-caffeoylquinic acid.[185] Another evidence of apoptotic action was shown through an higher protein expression of cleaved executioner caspase-3 in HepG2 cells (Table 17, page 71), compared to control, after 24 h of exposition with artichoke methanol extract, at 400 and 800 µM CAE.[185]

Mileo, *et al.*[194] analyzed several apoptotic molecular targets in MDA-MB-231 cells, after 24 h-treatment with artichoke capitula methanol extract (200-800 µM CAE). This extract increased the apoptotic cell population percentage, relatively to control cell population, reaching 45% at 400 µM.[194] They proposed that artichoke capitula methanol extract could induce apoptosis, on one hand, through the extrinsic pathway, due to increased protein expression of caspase-8, relatively to control cells. On the other hand, the intrinsic pathway of apoptosis could also be boosted (Table 17, page 71), since the artichoke capitula methanol extract upregulated the apoptosis effector Bax and downregulated the inhibitor Bcl-2, as well as increased the caspase-9 protein level. Additionally, the 24 h-incubation of MDA-MB-231 cells with artichoke capitula methanol extract, at 200 µM CAE, caused a drop in mitochondrial membrane potential.[194]

6.2.2 *In vivo* assays

Yasukawa, *et al.*[80] studied the inhibitory effect of an artichoke capitula methanol extract against the formation of mouse skin tumor. The tumor formation was induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) and promoted by 12-*O*-tetradecanoylphorbol-13-acetate (TPA)[80] (Figure 48). It was noted that the topical application of artichoke capitula extract (1 mg), during 2 weeks, delayed the occurrence of the first skin tumor, as well as decreased the average number of tumors per mice (73%).[80] Yasukawa, *et al.*[80] also referred a 27% reduction in the number of tumor-bearing mice, after 20 weeks of tumor promotion with DMBA plus TPA and artichoke extract treatment. They suggested that the antitumoral potential of artichoke capitula extract could be ascribed to pentacyclic triterpenes, such as taraxasterol, *ψ*-taraxasterol, *α*- and *β*-amyrin and their acetate derivatives.[80] Indeed, Yasukawa, *et al.*[207] had earlier proven that the application of taraxasterol (2 µmol) in mouse skin markedly decreased the tumor-bearing mice number (73%), and the number of mouse skin papillomas (86%), during the 20 weeks of tumor promotion with DMBA plus TPA. By using the same two-step

carcinogenesis mouse skin model, Takasaki, *et al.*[208] noted that the topical application of taraxasteryl acetate (85 nmol) was much weaker than taraxasterol (85 nmol) to inhibit mouse skin tumor formation, after 20 weeks, being comparable to positive control.



7,12-dimethylbenz[*a*]anthracene (DMBA)

12-*O*-tetradecanoylphorbol-13-acetate (TPA)

Figure 48: Structures of 7,12-dimethylbenz[*a*]anthracene (DMBA, inducer) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA, promotor) used in the mouse skin two-stage carcinogenesis assay.

6.3 Antimicrobial activity

The antimicrobial effects of artichoke leaves[100] and wild cardoon capitula extracts[10] were studied, in what regards to antifungal and antibacterial actions.

6.3.1 Antifungal activity

Few groups reported the antifungal action of *C. cardunculus* L. and their main phenolic compounds.[10,100]

Zhu, *et al.*[100] found that 5-*O*-caffeoylquinic acid was the most active phenolic compound, isolated from artichoke leaves, in inhibiting growth of four yeasts, namely *Candida albicans*, *C. lusitaniae*, *Saccharomyces cerevisiae* and *S. carlsbergensis*, within the range 50-200 µg/mL of minimum inhibitory concentration (MIC) values. Moreover, luteolin 7-*O*-glucoside demonstrated the highest inhibitory action on growth of four molds, namely *Aspergillus niger*, *Penicillium oxalicum*, *Mucor mucedo* and *Cladosporium cucumerinum*, with MIC of 50 µg/mL.[100]

Kukic, *et al.*[10] investigated the antifungal effect of wild cardoon capitula 96% ethanol extract, and derived-fractions (chloroform, ethyl acetate, butanol and water) against the growth of eight fungi, namely *Aspergillus niger*, *A. ochraceus*, *A. flavus*, *Penicillium ochrochloron*, *P. funiculosum*, *Trichoderma viride*, *Fusarium tricinctum* and *Alternaria alternate*. Ethyl acetate fraction, containing the highest total phenolic content (0.203 mg GAE/mg extract), evidenced to be the most active in preventing growth of those fungi, within ranges 1000-1500 µg/mL of

MIC and Minimum Fungicidal Concentration (MFC) values. Nonetheless, wild cardoon capitula 96% ethanol extract, and derived-fractions were weaker than miconazole (ranges 0.2-2.0 µg/mL and 1.0-5.0 µg/mL of MICs and MFCs, respectively). It was noted that luteolin presented the highest antifungal action, among the isolated compounds from wild cardoon capitula (ranges 30-100 µg/mL and 50-100 µg/mL of MICs and MFCs, respectively). However, the inhibitory actions of luteolin against the tested fungi were weaker compared to miconazole.[10]

6.3.2 Antibacterial activity

Bacteria are classified in two groups, namely Gram-negative and Gram-positive (Figure 49). Gram-negative bacteria have a complex outer membrane covering the thin peptidoglycan cell wall, while the outer membrane is absent in Gram-positive bacteria.[209]

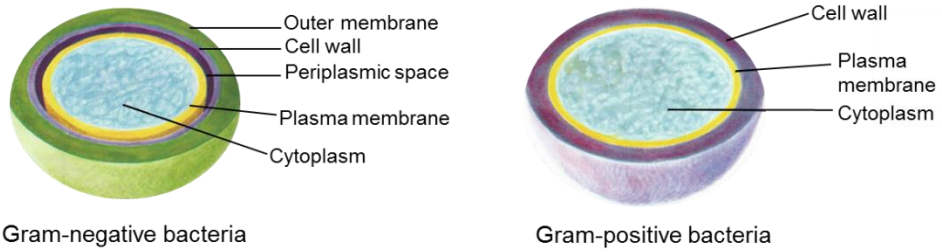


Figure 49: Morphological aspect of Gram-negative and Gram-positive bacteria (adapted from [209]).

Nowadays, pharmaceutical companies are urgently searching for new antibacterial drugs, principally due to occurrence and spread of multidrug-resistant (MDR) bacteria which has resulted by misuse and, consequently, effectiveness loss of antibiotics. Through an initiative of the European Centre for Disease Prevention and Control and the Centers for Disease Control and Prevention, a group of international experts proposed a definition for MDR bacteria, as non-susceptible "to at least one agent in three or more antimicrobial categories".[210] MDR bacteria represent a serious burden to public health worldwide, contributing for an increased risk of morbidity and mortality.[211] It is estimated that MDR bacterial infections can cause 25000 deaths per year in Europe.[212] MDR bacteria are often the main responsible for the health care-associated infections, formerly known as "nosocomial" or "hospital" infections. Examples of MDR bacteria are the Gram-negative *Escherichia coli*, *Klebsiella pneumonia* (Figure 50A), *Acinetobacter* species and *Pseudomonas aeruginosa* (Figure 50B), as well as the Gram-positive *Enterococcus faecalis* (Figure 50C), *E. faecium*, *Streptococcus pneumonia* and *Staphylococcus aureus* (Figure 50D).[212]

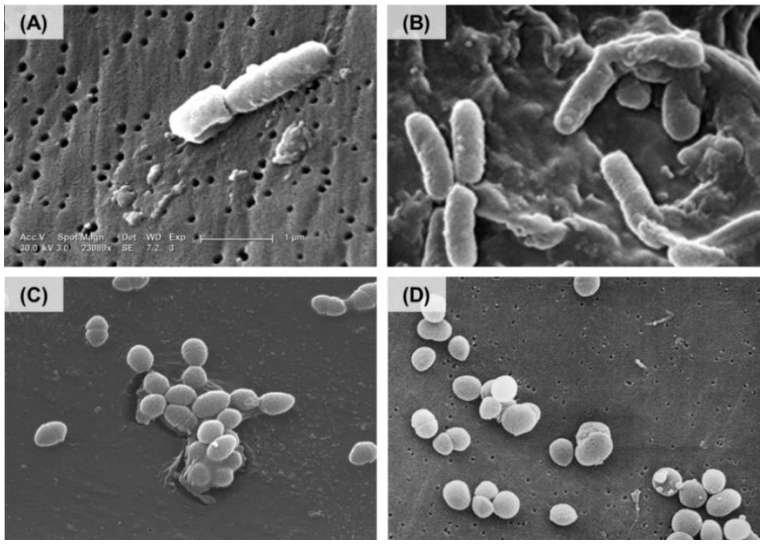


Figure 50: Representative scanning electron microscopy photographs of the Gram-negative (A) *Klebsiella pneumonia*, (B) *Pseudomonas aeruginosa*, and the Gram-positive (C) *Enterococcus faecalis* and (D) *Staphylococcus aureus* (Source: Centers for Disease Control and Prevention[213]).

Zhu, *et al.*[100] assessed the antibacterial activity of phenolic compounds isolated from artichoke leaves, namely caffeoylquinic acids, apigenin and luteolin glycosides. Among these compounds, they noticed that 4,5-di-*O*-caffeoylquinic acid and luteolin 7-*O*-glucoside were the most active phenolic compounds, with Minimum Inhibitory Concentration (MIC) of 50 µg/mL, on growth of *Micrococcus luteus* and *S. aureus*, respectively. Nevertheless, the antibacterial effects of these compounds were less active than ampicillin, streptomycin and kanamycin sulfate (range 5-40 µg/mL of MICs).[100]

Kukić, *et al.*[10] investigated the antibacterial of wild cardoon capitula 96% ethanol extract, and fractions (chloroform, ethyl acetate, *n*-butanol and water), by determining the MIC and the Minimum Bactericidal Concentration (MBC) values (Table 18). The ethyl acetate fraction, presenting 20.3% of GAE, was the most active in preventing growth of the tested bacteria, with MIC values of 1000-1500 µg/mL and MBC values of 1500-2000 µg/mL. Nevertheless, wild cardoon capitula 96% ethanol extract and fractions were less potent than streptomycin (range 0.5-1.0 µg/mL of MICs and MBCs).[10] Moreover, it was shown that luteolin was the most active antibacterial compound isolated from wild cardoon capitula, among sterols, triterpenic saponins and phenolic compounds, with MIC values of 50-100 µg/mL and MBC values of 50-100 µg/mL. However, this compound was weaker than streptomycin (MIC and MBC values of 0.5-1.0 µg/mL).[10]

Table 18: Minimum inhibitory and bactericidal concentrations (MICs and MBCs) of wild cardoon capitula 96% ethanol extract, and fractions (chloroform, ethyl acetate, *n*-butanol and water).[10]

Bacteria	MIC (µg/mL)	MBC (µg/mL)
Gram-negative		
Escherichia coli	1000-1500	1500-2000
Salmonella typhimurium	1500-2000	2000-2500
Gram-positive		
Bacillus subtilis	1000-2000	1000-2500
Staphylococcus aureus	1500	2000
Staphylococcus epidermidis	1500	2000

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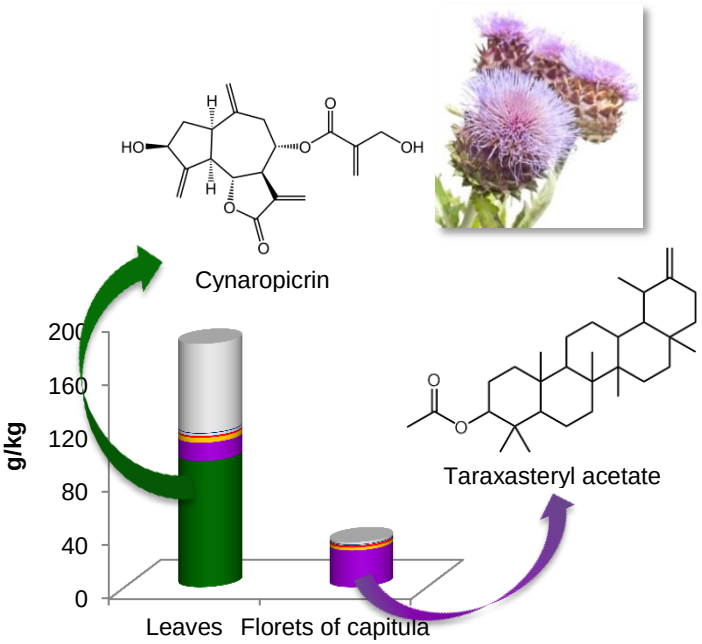
Part B

Chemical characterization of *Cynara cardunculus* L. var. *altilis* (DC)

Chapter III

Chemical characterization of *Cynara cardunculus* L. var. *altilis* (DC) lipophilic fraction



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Abstract

Lipophilic extracts of *Cynara cardunculus* L. var. *altilis* (DC) from the south of Portugal (Baixo Alentejo) were studied by gas chromatography-mass spectrometry which allowed the analysis of 65 components. Thirty three compounds were here identified and quantified, for the first time, as *C. cardunculus* L. components: 6 aromatic compounds, 9 fatty acids, 2 hydroxyfatty acids, 8 long chain aliphatic alcohols, 4 sterols, 1 pentacyclic triterpene and 3 other compounds (inositol, 2,3-dihydroxypropyl hexadecanoate, *E*-squalene). Four triterpenyl fatty acid esters were also identified for the first time. In addition to these compounds, 2 aromatic compounds, 2 fatty acids, 1 sesquiterpene lactone and 3 pentacyclic triterpenes were reported, for the first time, as cultivated cardoon components, namely vanillic and syringic acids, (9Z)-hexadec-9-enoic (15) and tetracosanoic acids, deacylcynaropicrin, and acetates of *β*- and *α*-amyrin and *ψ*-taraxasteryl acetate. Sesquiterpene lactones and pentacyclic triterpenes were the major lipophilic families, accounting for respectively ≈94.5 g/kg dw in leaves and ≈27.5 g/kg dw in florets. Cynaropicrin was the most abundant sesquiterpene lactone, while taraxasteryl acetate was the main pentacyclic triterpene. Fatty acids and sterols, mainly represented by respectively hexadecanoic acid and *β*-sitosterol, were present at lower amounts, accounting for ≈3.7 g/kg dw in leaves and ≈1.4 g/kg dw in florets. Long chain aliphatic alcohols and aromatic compounds were detected at low abundances.

1. Introduction

Cynara cardunculus L. (Asteraceae) is a Mediterranean plant species that includes three varieties, namely var. *sylvestris* (Lamk) Fiori (wild cardoon), var. *scolymus* (L.) Fiori (globe artichoke) and var. *altilis* (DC) (cultivated cardoon).[1] The wild cardoon grows spontaneously in clay soils in the Mediterranean basin and Macaronesia (Madeira and Canary Islands).[2] Several lines of evidence have shown that wild cardoon is the ancestor of both cultivated forms, characterized by different morphological traits, due to agricultural selection.[3] Globe artichoke is cultivated all over the world for its edible immature large capitula, with high economic importance in Italy, Spain, France and Turkey.[2] Cultivated cardoon has been explored for its fleshy stems and leaf petioles. These are generally collected in late autumn-early winter and submitted to a blanching process before cooking. This "delicacy" is much appreciated in regional dishes in Spain, Italy, France.[4] Another traditional application, in the Iberian Peninsula, involves the use of wild cardoon capitula as source of aspartic proteinases (cardosins A and B) for milk clotting during sheep cheeses manufacturing.[5] Furthermore, both artichoke and wild cardoon leaves extracts have been used since ancient times in folk medicine for the hepatobiliary system regulation,[6,7] due to their recognized hepatoprotective,[8] hypocholesterolemic,[9] choleretic and anti-cholestatic[10] actions.

Several industrial applications have been considered for cultivated cardoon biomass, namely for pulp production,[11–13] power generation[11,14] and domestic heating.[15] Moreover, the cultivated cardoon oil seed is suitable for biodiesel production.[11,16,17] Thus, cultivated cardoon has been regarded as a multipurpose perennial crop for a sustainable economic development in south European countries, well adapted to the Mediterranean climatic and soil conditions and with high biomass productivities (in the range 15.2-24.2 t/ha).[11,18]

Besides the lignocellulosic fraction, cardoon plantation can be further valorized through the exploitation of high value extractable compounds, addressing the key goal of the biorefinary concept which is based on the integrated use of all the fractions of any biomass resource.[19] The valorization of any plant biomass requires the detailed knowledge of its chemical composition, the establishment of relationships with traditional applications and the search for new applications. Regarding the dietary and nutraceutical applications, several studies have been focused on the relationship between phenolic composition and biological activity of *C. cardunculus* L. extracts, specifically antioxidant[20] and anti-tumor activities.[21,22] So far, few studies have been devoted to the study of the lipophilic composition of globe artichoke, for example the isolation of sesquiterpene lactones from the leaves[23] and pentacyclic triterpenes from the capitula.[24] However, less attention has been devoted to the lipophilic fraction of *C. cardunculus* L. var. *altilis* (DC), beyond the fatty acids composition of seed oil.[25] Finally, the isolation of pentacyclic triterpenes and sterols from cardoon has already been reported,[26] with no differentiation between wild and cultivated cardoon varieties.

Therefore, considering the growing interest in the exploitation of cultivated cardoon as a biomass resource in the south of Portugal, and the lack of detailed information on the extractives composition, it is given here a detailed chemical characterization of the lipophilic fractions of different morphological parts of *C. cardunculus* L. var. *altilis* (DC) by gas chromatography-mass spectrometry (GC-MS) analysis.

2. Materials and Methods

2.1 Chemicals

Dichloromethane (p.a. ≥99% purity), methanol (p.a., ≥99.8% purity) and light petroleum (p.a., ≥99% purity) were supplied by Fischer Scientific (Pittsburgh, Pennsylvania, USA). Ethyl acetate (p.a., ≥99.8% purity) was purchased from Carlo Erba Reagents (Val de Reuil, France). Hydrochloric acid (HCl) (p.a., ≥37%) was purchased from Fluka Chemie (Madrid, Spain). Potassium hydroxide (KOH) (p.a., ≥88% purity), *N*,*O*-bis(trimethylsilyl)trifluroacetamide (99% purity), trimethylchlorosilane (99% purity), hexadecane (99% purity), hexadecanoic acid (≥99% purity), nonadeca-1-nol (99% purity), vanillin (99% purity), cholesterol (99% purity), lupeol (≥94% purity), deuterated chloroform ($CDCl_3$) (99.8% D), tetramethylsilane (≥ 99.9% purity) and acetyl chloride (p.a. ≥99% purity) were obtained from Sigma Chemicals Co. (Madrid, Spain). Silica gel grade 60 for column chromatography (63-200 μm of particle size) and for thin layer chromatography (5-40 μm of particle size) were purchased from Merck (Darmstadt, Germany). Pyridine (p.a., ≥99.5% purity) was purchased from Panreac (Castellar del Vallès, Spain). Cynaropicrin (≥97.5% purity) and *β*-amyrin (≥98.5% purity) were obtained from Extrasynthese (Genay Cedex, France). Taraxasteryl acetate (≥99.2% purity) was purchased from AvaChem Scientific (San Antonio, Texas, USA).

2.2 Samples preparation

C. cardunculus L. var. *altilis* (DC) was collected during the flowering stage, in June 2010 at the Experimental Center of Agriculture School of the Instituto Politécnico de Beja, southern Portugal. Plants were separated into stalks, capitula and leaves, and preserved at -20 ºC until analysis. Before extraction, samples were freeze-dried. Then the stalks were separated into outer and inner parts and capitula into receptacles, bracts and florets.

2.3 Extraction

All plant fractions were ground to a granulometry of 40-60 mesh prior to extraction. Each sample (6 g of dry weight) was Soxhlet extracted with dichloromethane (150 mL) for 7 h. Dichloromethane was chosen because it is a fairly specific solvent for lipophilic extractives.[27] The solvent was evaporated to dryness at low-pressure. The dried extracts were weighted,

and the results are expressed as percentages of dry biomass material (w/w, %). Two extracts were prepared for each morphological part of cultivated cardoon.

2.4 Alkaline hydrolysis

About 20 mg of each extract was dissolved in 10 mL of 1 M KOH in 10% aqueous methanol. The mixture was heated at 100 ºC, under nitrogen atmosphere, for 1 h. The reaction mixture was cooled, acidified with 1 M aqueous HCl to pH ≈ 2, and then extracted three times with dichloromethane and the solvent was evaporated to dryness.[27] The alkaline hydrolysis reaction was performed to indirectly detect esterified compounds, e.g. triglycerides, steryl esters, among others.

2.5 GC-MS analysis

Before GC-MS analysis, nearly 20 mg of each dried sample was converted into trimethylsilyl derivatives.[27] Each sample was dissolved in 250 µL of pyridine containing 1 mg of hexadecane (internal standard), and compounds with hydroxyl and carboxyl groups were converted into trimethylsilyl (TMS) ethers and esters, respectively, by adding 250 µL of *N,O*-bis(trimethylsilyl)trifluoroacetamide and 50 µL of trimethylchlorosilane. The mixture was maintained at 70 ºC for 30 min.

GC-MS analyses were performed using a Trace gas chromatograph (2000 series) equipped with a Thermo Scientific DSQ II mass spectrometer (Waltham, Massachusetts, USA). Separation of compounds was carried out in a DB-1 J&W capillary column (30 m x 0.32 mm inner diameter, 0.25 µm film thickness) using helium as the carrier gas (35 cm s^{-1}). The chromatographic conditions were as follows: initial temperature, 80 ºC for 5 min; temperature rate of 4 ºC min^{-1} up to 260 ºC; 2 ºC min^{-1} up to 285 ºC which was maintained for 8 min; injector temperature, 250 ºC; transfer-line temperature, 290 ºC; split ratio, 1:33. The mass spectrometer was operated in the electron ionization (EI) mode with the energy of 70 eV and data collected at a rate of 1 scan s^{-1} over a range of *m/z* 33-700. The ionization source was kept at 250 ºC.[28]

To detect the presence of esterified structures, dichloromethane extracts were also analyzed in a short DB-1 J&W capillary column (15 m x 0.32 mm inner diameter, 0.25 µm film thickness); the chromatographic conditions were as follows: initial temperature, 100 ºC for 3 min; temperature gradient, 5 ºC min^{-1}; final temperature, 340 ºC for 12 min; injector temperature, 290 ºC; transfer-line temperature, 290 ºC; and split ratio, 1:33.[27]

Chromatographic peaks were identified by comparing their mass spectra with the equipment mass spectral library (Wiley-NIST Mass Spectral Library), literature data and, when needed, by injection of standards. In some cases, identification was also confirmed based on characteristic retention times (RTs) under the described experimental conditions.[27–29] For

quantitative analysis, GC-MS was calibrated with pure reference compounds, representative of the major lipophilic extractive components (namely hexadecanoic acid, nonadecan-1-ol, vanillin, cynaropicrin, lupeol and cholesterol) relative to hexadecane (the internal standard). The respective response factors were calculated as the average of four GC-MS runs.

For each morphological part of cultivated cardoon, two extracts were analyzed before alkaline hydrolysis, and another two after alkaline hydrolysis. Each extract was injected in duplicate. Results represent the mean value for each morphological part (less than 5% variation between injections of the same extract and between extracts of the same morphological part). The compound contents were expressed as milligram per kilogram of dry weight of plant biomass.

2.6 Nuclear magnetic resonance experiments

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 300 spectrometer (Wissembourg, France) (300.13 and 75.47 MHz, for ^{1}H and ^{13}C, respectively), using chloroform-*d* as the solvent and tetramethylsilane as the internal reference. Unequivocal ^{1}H and ^{13}C assignments were made with the aid of unidimensional distortionless enhancement polarization transfer (DEPT) and bidimensional, namely COSY ($^{1}H/^{1}H$), HSQC ($^{1}H/^{13}C$) and HMBC ($^{1}H/^{13}C$) experiments. The low-pass *J*-filter portion of the HMBC experiment was optimized for an average of one-bond heteronuclear coupling of 145 Hz; the delay for evolution of long-range couplings was optimized for 7 and 2 Hz.

2.7 Isolation and characterization of cynaropicrin and grosheimin from leaves extract

About 50 g of cultivated cardoon leaves was Soxhlet extracted with dichloromethane (750 mL) for 7 h to afford 7.5 g of crude extract. About 1 g of dried extract was dissolved in dichloromethane, subjected to column chromatography on silica gel, and continuously eluted with a gradient of ethyl acetate in light petroleum, collecting 50 mL of each fraction: fractions F1 and F2 were collected with 10% ethyl acetate, fractions F3 and F4 with 20%, fractions F5 and F6 with 40%, fractions F7 and F8 with 50% and fractions F9-F11 with 100%. An aliquot of each fraction was submitted to derivatization and analyzed by GC-MS. Fractions F10 and F11, accounting for 363.7 mg, presented higher relative abundances of cynaropicrin and grosheimin which were separated from each other, as explained thereafter.

2.7.1 Isolation of cynaropicrin

About 312.4 mg of F10 and F11 combined fractions were dissolved in dichloromethane and further fractionated by column chromatography on silica gel eluting with 50% ethyl acetate

in light petroleum to collect five fractions, F1a to F5a (50 mL each). The column was then eluted with 60% ethyl acetate in light petroleum to collect the fractions F6a and F7a (50 mL each) and F8a-F11a (25 mL each). The fraction F11a yielded 10 mg of cynaropicrin (Figure 1) that was identified by comparing its NMR and EI-MS data with literature data.[30]

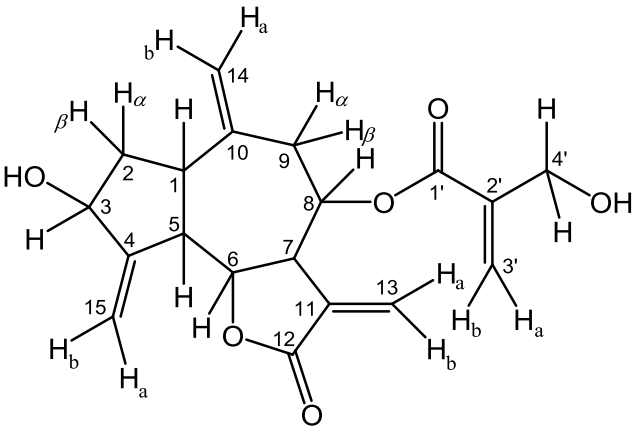


Figure 1: Structure of cynaropicrin.

EI-MS (70 eV) *m/z* (relative intensity, %): 346 $[M]^{+\cdot}$ (2), 262 (18), 245 $[M–C_4H_5O_3]^+$ (7), 244 $[M–C_4H_6O_3]^+$ (48), 226 (27), 216 (21), 198 (31), 148 (33), 91 (47), 85 (100).

^{1}H NMR (300.13 MHz, $CDCl_3$): δ = 1.67 (1H, ddd, *J* = 5.2, 7.9, 15.9 Hz, 2*α*-C$\underline{H}_2$CHOH), 2.18 (1H, dt, *J* = 6.2, 13.2 Hz, 2*β*-C$\underline{H}_2$CHOH), 2.34 (1H, dd, *J* = 3.5, 14.6 Hz, 9*α*-C$\underline{H}_2$COC(=O)), 2.65 (1H, dd, *J* = 3.5, 14.6 Hz, 9*β*-C$\underline{H}_2$COC(=O)), 2.79 (1H, dd, *J* = 8.9, 10.8 Hz, 5-C$\underline{H}$CHOC(=O)), 2.92 (1H, dt, *J* = 5.2, 10.8, Hz, 1-C$\underline{H}$C=CH_2), 3.14 (1H, tt, *J* = 3.2, 10.0 Hz, 7-C$\underline{H}$C=CH_2), 4.20 (1H, dd, *J* = 8.9, 10.0 Hz, 6-C$\underline{H}$OC(=O)), 4.32 (2H, s, 4´-C$\underline{H}_2$OH), 4.50 (1H, tt, *J* = 1.8, 7.9 Hz, 3-C$\underline{H}$OH), 4.89 (1H, d, *J* = 1.4 Hz, 14a-C$\underline{H}_2$=C), 5.08 (2H, m, 8-C$\underline{H}$OC(=O) and 14b-C$\underline{H}_2$=C), 5.31 (1H, t, *J* = 1.8 Hz, 15a-C$\underline{H}_2$=C), 5.44 (1H, t, *J* = 1.8 Hz, 15b-C$\underline{H}_2$=C), 5.57 (1H, d, *J* = 3.2 Hz, 13a-C$\underline{H}_2$=C), 5.90 (1H, q, *J* = 1.1 Hz, 3´a-C$\underline{H}_2$=C), 6.16 (1H, d, *J* = 3.2 Hz, 13b-C$\underline{H}_2$=C), 6.27 ppm (1H, d, *J* = 1.1 Hz, 3´b-C$\underline{H}_2$=C).

^{13}C NMR (75.47 MHz, $CDCl_3$): δ = 37.0 (9-C$\underline{H}_2$COC(=O)), 39.0 (2-C$\underline{H}_2$CHOH), 45.3 (1-C$\underline{H}$C=CH_2), 47.6 (7-C$\underline{H}$COC(=O)), 51.4 (5-C$\underline{H}$COC(=O)), 62.3 (4´-C$\underline{H}_2$OH), 73.7 (3-C$\underline{H}$OH), 74.3 (8-C$\underline{H}$OC(=O)), 78.4 (6-C$\underline{H}$OC(=O)), 113.6 (15-C$\underline{H}_2$=C), 118.2 (14-C$\underline{H}_2$=C), 122.7 (13-C$\underline{H}_2$=C), 127.8 (3´-C$\underline{H}_2$=C), 137.3 (11-C$\underline{=}$CH_2), 139.2 (2´-C$\underline{=}$CH_2), 141.7 (10-C$\underline{=}$CH_2), 152.2 (4-C$\underline{=}$CH_2), 165.3 (1´-C$\underline{=}$(=O)O), 169.0 ppm (12-C$\underline{=}$(=O)O).

2.7.2 Isolation of grosheimin

About 51.3 mg of the F10 and F11 combined fractions were dissolved in dichloromethane and applied to thin layer chromatography plates (TLC), which were eluted

with dichloromethane/methanol (95:5). A main spot was observed at R_f = 0.31 and isolated from TLC plates, yielding 7 mg of pure grosheimin (Figure 2) which was identified by comparing EI-MS data with the equipment mass spectral library.

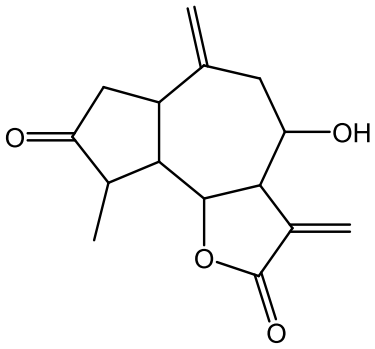


Figure 2: Structure of grosheimin.

EI-MS (70 eV) of grosheimin, *m/z* (relative intensity, %): 262 $[M]^{+\cdot}$ (13), 244 (88), 166 (35), 165 (47), 137 (63), 136 (87), 93 (47), 91 (49), 69 (100), 68 (26), 67 (30), 41 (9).

2.8 Lupenyl acetate preparation

Lupenyl acetate (Figure 3) was prepared by acetylation of lupeol with acetyl chloride in pyridine at room temperature for 12 h.[31] The reaction product was analyzed by GC-MS, and the identification confirmed by comparing its EI-MS data with literature data.[32]

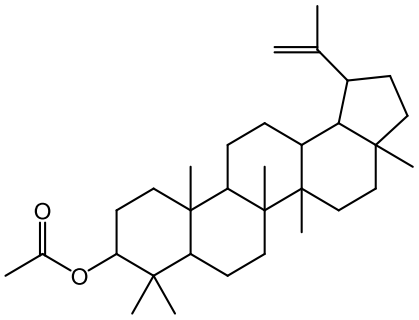


Figure 3: Structure of lupenyl acetate.

EI-MS (70 eV) of lupenyl acetate, *m/z* (relative intensity, %): 468 $[M]^{+\cdot}$ (18), 453 $[M\text{-}CH_3]^+$ (11), 408 $[M\text{-}CH_3COOH]^+$ (6), 249 $[M\text{-}C_{16}H_{27}]^+$ (13), 218 $[M\text{-}C_{16}H_{26}O_2]^+$ (30), 204 $[M\text{-}C_{17}H_{28}O_2]^+$ (32), 203 $[204\text{-}H]^+$ (40), 189 $[M\text{-}C_{18}H_{31}O_2, 249\text{-}CH_3COOH]^+$ (100).

2.9 Statistical analysis

The quantitative analysis results were analyzed using the MIXED procedure option of SAS (SAS Institute Inc., Cary, North Carolina, USA), considering morphological parts of cultivated cardoon and extracts as fixed and random effects, respectively. Where differences existed, the source of the differences at $p < 0.05$ of significance level was identified by all pairwise multiple comparison procedure. The Tukey's test was used for pairwise comparisons.

3. Results and Discussion

3.1 Dichloromethane extractives yield

Dichloromethane extractives yields of *C. cardunculus* L. var. *altilis* (DC) morphological parts (Table 1) varied between 1.0 and 2.0% (w/w) for stalks parts, and 3.3 and 4.5% (w/w) for capitula parts, reaching at maximum 17.3% (w/w) for leaves. The extractives yields of outer and inner parts of stalks are in good agreement with the previously reported results (0.75-0.98%),[12] while the yield shown here for leaves is considerably higher than the previously published values (4% (w/w)).[33]

Table 1: Extractives yields with dichloromethane (%, w/w) for the different morphological parts of *C. cardunculus* L. var. *altilis* (DC).

Morphological parts of *Cynara cardunculus* L. var. *altilis* (DC)		Extractives yield (% w/w)
Stalks	Outer part	1.0 ± 0.1
	Inner part	2.0 ± 0.1
Capitula	Receptacles and bracts	3.3 ± 0.2
	Florets	4.5 ± 0.8
Leaves		17.3 ± 0.5

Results represent the mean estimated from two extracts.

3.2 GC-MS analysis of *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts

GC-MS allowed the identification of 65 compounds in dichloromethane extracts of *C. cardunculus* L. var. *altilis* (DC), before and after alkaline hydrolysis (Figure 4). The identified compounds in cultivated cardoon dichloromethane extracts were grouped into six chemical classes, namely aromatic compounds, fatty acids, long chain aliphatic alcohols, sesquiterpene lactones, pentacyclic triterpenes and sterols.

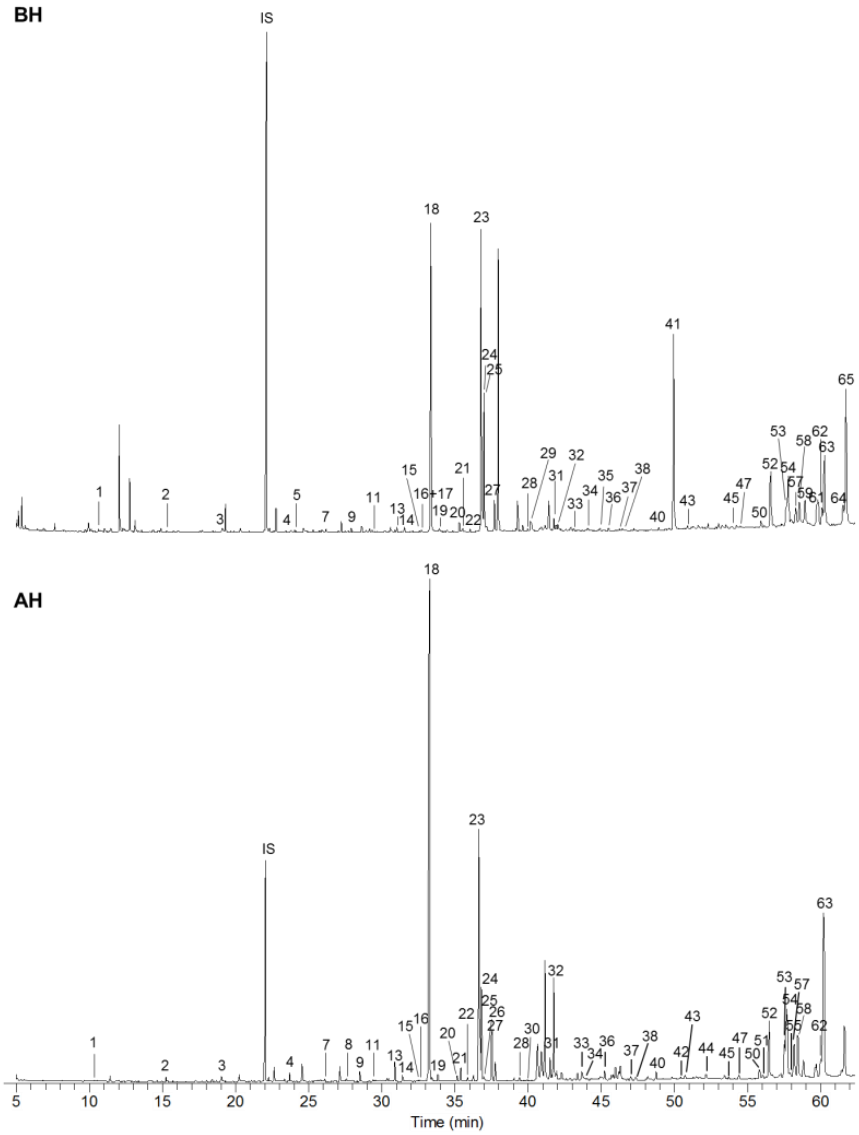


Figure 4: GC-MS chromatograms (DB-1 30 m column) of the trimethylsilyl derivatized dichloromethane extract of stalks outer part from *C. cardunculus* L. var. *altilis* (DC), before (BH) and after (AH) alkaline hydrolysis. Chromatographic peak numbers are those indicated in the **Table 2**. Abbreviation: IS, internal standard.

In Table 2, the retention time of the 65 compounds, identified in the *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts, are indicated. Among these, 33 and 41 were described here for the first time as *C. cardunculus* L. and cultivated cardoon components, respectively. With the exception of sesquiterpene lactones, the compounds detected in the studied extracts were identified by comparing the EI-MS fragmentation of the respective TMS derivatives with literature data and, in some cases, with the injection of standards, as explained along this chapter. Sesquiterpene lactones were identified through the EI-MS data, obtained before the TMS derivatization, since there is no publication regarding the EI-MS data

of the corresponding TMS ethers. In addition to these data, the NMR spectroscopic analysis was also employed to identify cynaropicrin.

Table 2: Retention time (RT) of the compounds identified in the dichloromethane extracts of *C. cardunculus* L. var. *altilis* (DC) by GC-MS analysis.

No.	RT (min)	Compound	Presence in *C. cardunculus* L.
1	10.29	Benzoic acid	RFCC
2	15.27	2-Hydroxyheptanoic acid	RFCC
3	19.07	Vanillin	RFCC
4	23.75	Syringaldehyde	RFCC
5	24.02	2,6-Dimethoxyhydroquinone	RFCC
6	24.29	2-Hydroxyundecanoic acid	RFCC
7	26.14	Vanillic acid	WC[34]
8	27.75	3-Vanillylpropanol	RFCC
9	28.54	Tetradecanoic acid (myristic acid)	WC,[25] CC[17]
10	29.35	Vanillylpropanoic acid	RFCC
11	29.44	Syringic acid	WC[34]
12	29.96	*Z*-Ferulic acid	Art, CC[35]
13	30.94	Pentadecanoic acid	RFCC
14	31.46	Hexadecan-1-ol	RFCC
15	32.50	(9*Z*)-Hexadec-9-enoic acid (palmitoleic acid)	WC[25]
16	32.61	(9*E*)-Hexadec-9-enoic acid	RFCC
17	33.02	Inositol	RFCC
18	33.22	Hexadecanoic acid (palmitic acid)	WC, Art,[25] CC[17,25]
19	33.81	*E*-Ferulic acid	Art, CC[35]
20	35.19	(9*Z*)-Octadec-9-en-1-ol	RFCC
21	35.41	Heptadecanoic acid	CC[17]
22	35.89	Octadecan-1-ol	RFCC
23	36.64	(9*Z*,12*Z*)-Octadeca-9,12-dienoic acid (linoleic acid)	WC, Art,[25] CC[17,25]
24	36.70	(9*Z*,12*Z*,15*Z*)-Octadeca-9,12,15-trienoic acid (linolenic acid)	WC,[25] CC[17]
25	36.84	(9*Z*)-Octadec-9-enoic acid (oleic acid)	WC,[25] Art,[25] CC[17,25]
26	37.00	(9*E*)-Octadec-9-enoic acid	RFCC
27	37.54	Octadecanoic acid (stearic acid)	WC,[25] Art,[25] CC[17,25]
28	39.53	Nonadecanoic acid	RFCC
29	39.91	Grosheimin	Art,[6,23,36–38] C[39]
30	40.03	Eicosan-1-ol	RFCC-AH
31	41.50	Eicosanoic acid (arachidic acid)	WC,[25] CC[17]
32	41.75	Deacylcynaropicrin	Art[40]
33	43.40	Heneicosanoic acid	RFCC

No.	RT (min)	Compound	Presence in *C. cardunculus* L.
34	43.87	Docosan-1-ol	RFCC
35	45.00	2,3-Dihydroxypropyl hexadecanoate	RFCC
36	45.21	Docosanoic acid (behenic acid)	WC,[25] CC[17]
37	46.99	Tricosanoic acid	RFCC
38	47.44	Tetracosan-1-ol	RFCC
39	48.55	*E*-Squalene	RFCC
40	48.69	Tetracosanoic acid (lignoceric acid)	WC[25]
41	49.73	Cynaropicrin	Art,[6,23,36–38,41] C[41]
42	50.50	Pentacosanoic acid	RFCC-AH
43	50.79	Hexacosan-1-ol	RFCC
44	52.19	Hexacosanoic acid	RFCC
45	53.62	Cholesterol	RFCC
46	54.11	*α*-Tocopherol	WC, Art, CC[25]
47	54.45	Octocosan-1-ol	RFCC
48	54.88	Scopolin	C[42]
49	55.45	24-Methylenecholesterol	RFCC
50	55.61	Campesterol	WC, Art, CC[25]
51	56.01	Octacosanoic acid	RFCC
52	56.24	Stigmasterol	WC, Art, CC[25]
53	57.30	*β*-Amyrin	Art,[24] C[43]
54	57.42	*β*-Sitosterol	WC,[25] Art, CC[25]
55	57.62	*β*-Sitostanol	RFCC
56	57.78	Δ^5-Avenasterol	RFCC
57	57.91	*α*-Amyrin	Art,[24] C[43]
58	58.16	Lupeol	Art,[38] C[43]
59	58.55	*β*-Amyrin acetate	Art[24]
60	59.38	*α*-Amyrin acetate	Art[24]
61	59.43	Lupenyl acetate	RFCC
62	59.70	*ψ*-Taraxasterol	Art,[24] C[43]
63	59.88	Taraxasterol	Art,[24] C[26,43]
64	61.12	*ψ*-Taraxasteryl acetate	Art[24]
65	61.31	Taraxasteryl acetate	Art,[24] C[26]

Abbreviations: Art, artichoke; CC, cultivated cardoon; C, cardoon without reference in literature regarding the variety; RFCC, referenced for the first time as *C. cardunculus* L. component; RFCC-AH, referenced for the first time as *C. cardunculus* L. component, after alkaline hydrolysis; WC, wild cardoon.

3.2.1 Aromatic compounds

Eleven aromatic compounds were identified in the cultivated cardoon dichloromethane extracts (Table 2 and Figure 5).

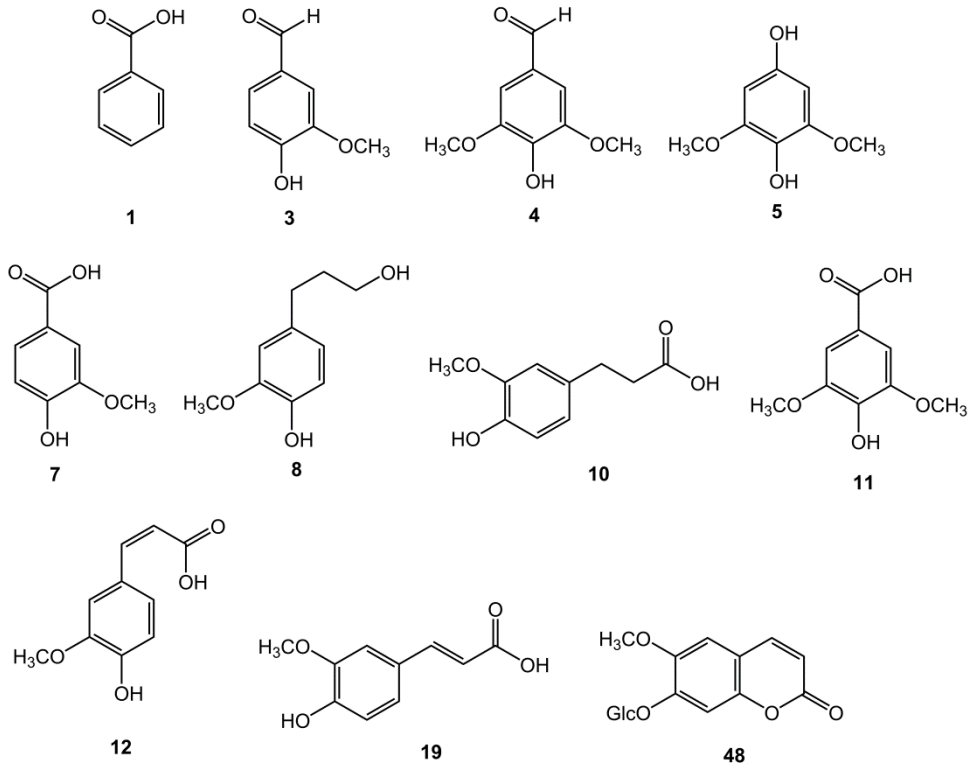


Figure 5: Aromatic compounds identified in the *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts. Abbreviation: Glc, glucosyl.

Six aromatic compounds were referenced for the first time as *C. cardunculus* L. components (Table 2 and Figure 5), namely benzoic acid (**1**), vanillin (**3**), syringaldehyde (**4**), 2,6-dimethoxyhydroquinone (**5**), 3-vanillylpropanol (**8**) and vanillylpropanoic acid (**10**). In addition to these, 2 aromatic compounds were here referred, for the first time, as cultivated cardoon extractives, such as vanillic (**7**) and syringic (**11**) acids. These compounds had previously been identified in the wild cardoon.[34] Scopolin was earlier isolated from cardoon, but without citing the variety.[42]

Aromatic compounds were identified by comparing the EI-MS data of the respective TMS derivatives with those of the equipment mass spectral library and with published data.[27,28,44] The EI mass spectra of the aromatic TMS derivatives were characterized by intense peaks, in what respects to the molecular ion $[M]^{+\cdot}$ as well as to product ions arising from losses of a methyl radical from TMS group (-15 Da), CHO from alkylic aldehydes (-29 Da) and HCHO from methoxyphenyl derivatives (-30 Da).[44] Figure 6 illustrates the EI mass

spectrum of the syringaldehyde TMS ether, with the indication of the molecular ion and the main mass fragmentation product ions.

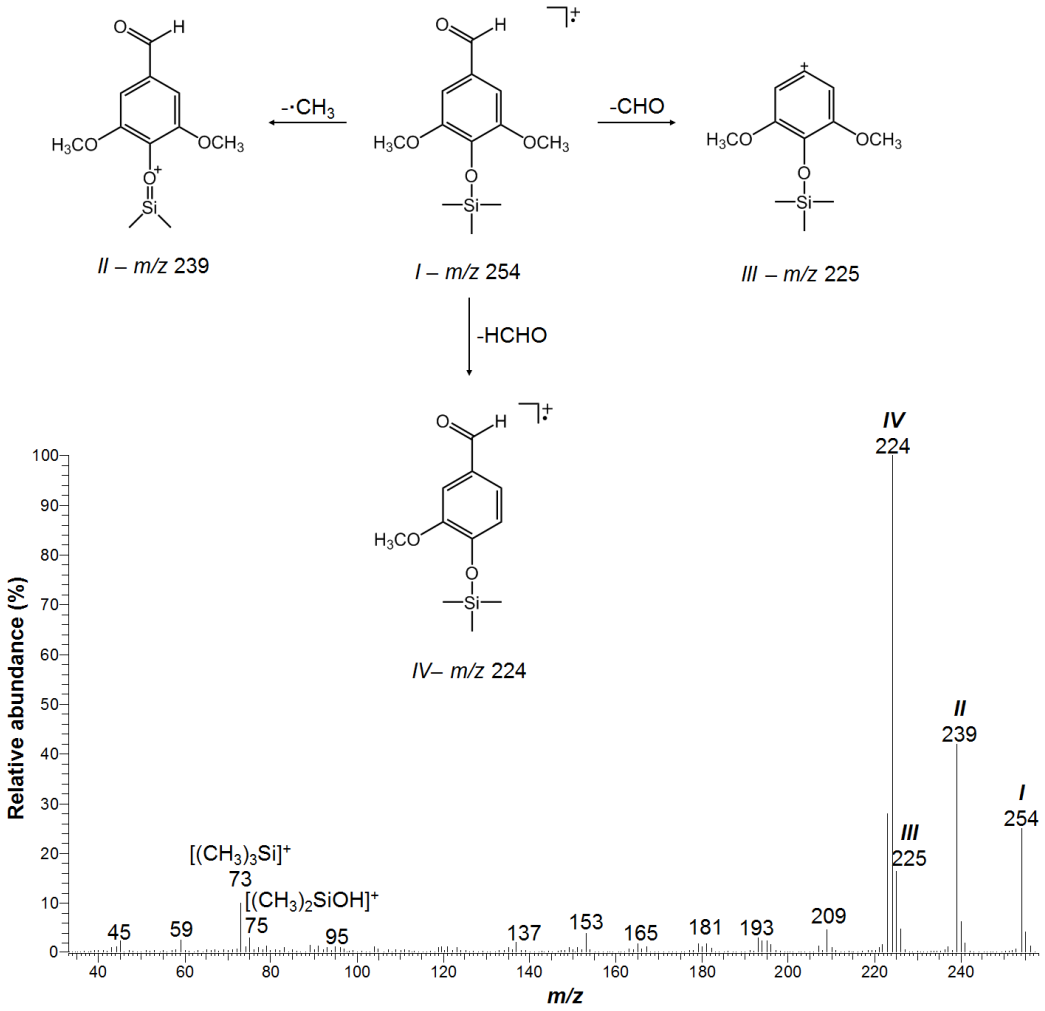


Figure 6: EI mass spectrum and the main fragmentation product ions of the syringaldehyde (**4**) trimethylsilyl ether derivative.

Table 3 summarizes the EI-MS data of the aromatic compounds identified in the cultivated cardoon dichloromethane extracts.

Table 3: EI mass spectroscopic data of the trimethylsilyl (TMS) derivatives of the aromatic compounds identified in *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts.

No.	Aromatic compound TMS derivative	$[M]^{+\cdot}$ (*m/z*)	EI-MS product ions *m/z* (relative intensity %)	Identification
1	Benzoic acid	194	194 $[M]^{+\cdot}$ (4), 179 $[M\text{-}CH_3]^+$ (100), 135 (40), 105 $[M\text{-}(CH_3)_3SiO]^+$ (53), 77 (48), 75 $[(CH_3)_2SiOH]^+$ (26), 73 $[(CH_3)_3Si]^+$ (24)	[28]
3	Vanillin	224	224 $[M]^{+\cdot}$ (28), 209 $[M\text{-}CH_3]^+$ (50), 195 $[M\text{-}CHO]^+$ (17) 194 $[M\text{-}HCHO]^+$ (100), 163 (7), 135 (3) $[M\text{-}(CH_3)_3SiO]^+$, 93 (13), 79 (14), 75 (40), 73 (25)	Co
4	Syringaldehyde	254	254 $[M]^{+\cdot}$ (25), 239 $[M\text{-}CH_3]^+$ (42), 225 $[M\text{-}CHO]^+$ (16), 224 $[M\text{-}HCHO]^+$ (100), 209 (4), 194 $[M\text{-}2HCHO]^+$ (2), 165 $[M\text{-}(CH_3)_3SiO]^+$ (2), 75 (3), 73 (10)	[28,44]
5	2,6-Dimethoxyhydroquinone	314	314 $[M]^{+\cdot}$ (50), 299 $[M\text{-}CH_3]^+$ (10), 284 $[M\text{-}HCHO]^+$ (100), 254 $[M\text{-}2HCHO]^+$ (8), 195 (2), 75 (28), 73 (55)	[27,44]
7	Vanillic acid	312	312 $[M]^{+\cdot}$ (50), 297 $[M\text{-}CH_3]^+$ (100), 282 $[M\text{-}HCHO]^+$ (33), 267 (75), 253 (45), 223 (46), 193 (19), 165 (13), 126 (23), 75 (56), 73 (54)	[28,44]
8	3-Vanillylpropanol	326	326 $[M]^{+\cdot}$ (34), 311 $[M\text{-}CH_3]^+$ (16), 296 $[M\text{-}HCHO]^+$ (3), 236 (16), 221 (15), 206 (100), 179 (26), 148 (10), 75 (19), 73 (33)	[27]
10	Vanillylpropanoic acid	340	340 $[M]^{+\cdot}$ (55), 325 $[M\text{-}CH_3]^+$ (28), 310 $[M\text{-}HCHO]^+$ (25), 222 (21), 209 (100), 192 (44), 179 (30), 149 (9), 89 (6), 75 (81), 73 (62)	Wiley-NIST MS Library
11	Syringic acid	342	342 $[M]^{+\cdot}$ (58), 327 $[M\text{-}CH_3]^+$ (100), 312 $[M\text{-}HCHO]^+$ (66), 297 (67), 283 (23), 253 (38), 223 (15), 149 (17), 141 (23), 75 (25), 73 (41)	[27,28,44]
12	*Z*-Ferulic acid	338	338 $[M]^{+\cdot}$ (100), 323 $[M\text{-}CH_3]^+$ (63), 308 $[M\text{-}HCHO]^+$ (59), 293 (59), 249 (72), 219 (26), 191 (16), 75 (53), 73 (65), 45 (9)	[27,44]
19	*E*-Ferulic acid	338	338 $[M]^{+\cdot}$ (100), 323 $[M\text{-}CH_3]^+$ (64), 308 $[M\text{-}HCHO]^+$ (62), 293 (49), 279 (13), 249 (56), 219 (21), 191 (10), 75 (13), 73 (35)	[27,44]
48	Scopolin	642	450 $[C_6H_5O((CH_3)_3SiO)_4]^+$ (3), 361 (72), 331 (6), 319 (8), 271 (19), 264 (47), 243 (15), 217 (74), 191 $[C_{10}H_7O_4]^+$ (16), 147 (36), 129 (19), 103 $[(CH_3)_3SiOCH_2]^+$ (12), 75 (14), 73 (100)	Wiley-NIST MS Library

Abbreviation: Co, co-injection of authentic standard.

3.2.2 Fatty acids

Twenty fatty acids were detected in the *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts, where 14 were saturated (**9**, **13**, **18**, **21**, **27**, **28**, **31**, **33**, **36**, **37**, **40**, **42**, **44**, **51**) and 6 were unsaturated (**15**, **16**, **23**-**26**). Among these, 9 were identified for the first time in *C. cardunculus* L., namely pentadecanoic acid (**13**), (9*E*)-hexadec-9-enoic acid (**16**), (9*E*)-octadec-9-enoic acid (**26**) (Figure 7), nonadecanoic acid (**28**), heneicosanoic acid (**33**), tricosanoic acid (**37**), hexacosanoic acid (**44**), octacosanoic acid (**51**) and pentacosanoic acid (**42**); the last one after alkaline hydrolysis. In addition to these compounds, (9*Z*)-hexadec-9-enoic (**15**) and tetracosanoic (**40**) acids were identified here for the first time as cultivated cardoon components. Additionally, two 2-hydroxy fatty acids, namely 2-hydroxyheptanoic acid (**2**) and 2-hydroxyundecanoic acid (**6**) (Figure 7), were identified in this study, for the first time, as *C. cardunculus* L. components.

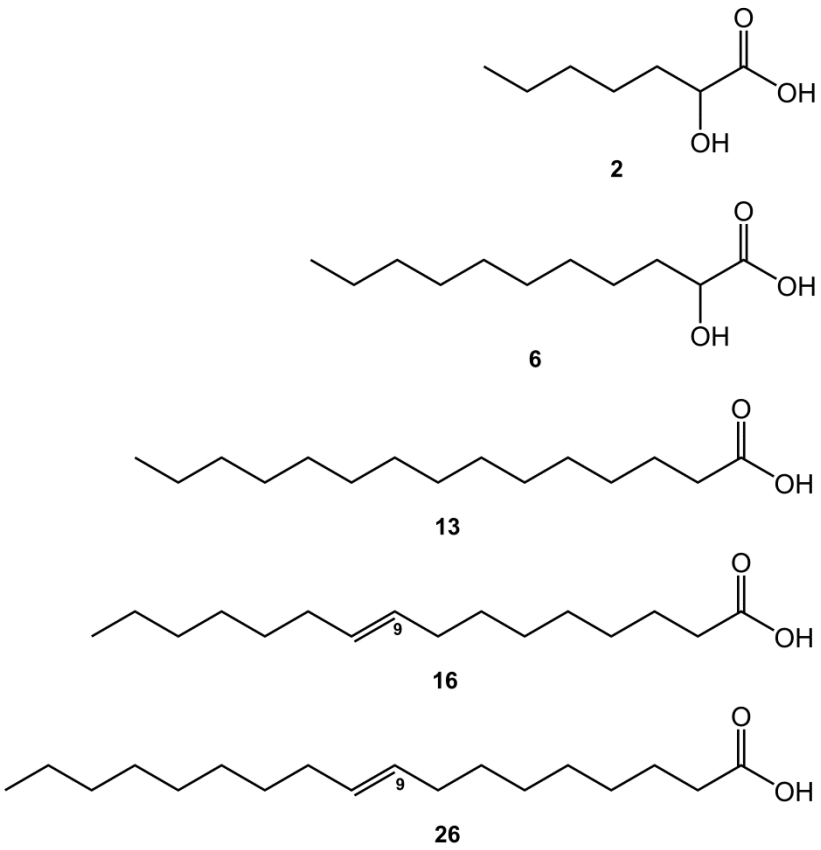


Figure 7: Structures of some of the fatty acids and hydroxyfatty acids identified in the *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts.

Fatty and hydroxyfatty acids were identified based on the EI-MS data of the corresponding TMS esters, as outlined in Table 4.

Table 4: EI mass spectroscopic data of the fatty acid trimethylsilyl (TMS) esters identified in the *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts.

No.	Fatty acid TMS ester	$[M]^{+\cdot}$ (*m/z*)	EI-MS product ions *m/z* (relative intensity %)	Identification
2	2-Hydroxyheptanoic acid	290	275 $[M\text{-}CH_3]^+$ (4), 173 $[M\text{-}COOSi(CH_3)_3]^+$ (100), 147 (66), 103 (17), 93 (40), 75 $[(CH_3)_2SiOH]^+$ (68), 73 $[(CH_3)_3Si]^+$ (81)	Wiley-NIST MS Library
6	2-Hydroxyundecanoic acid	346	229 $[M\text{-}COOSi(CH_3)_3]^+$ (67), 147 (24), 117 (5), 97 (100), 75 (7), 73 (22)	Wiley-NIST MS Library
9	Tetradecanoic acid	300	300 $[M]^{+\cdot}$ (2), 285 $[M\text{-}CH_3]^+$ (83), 145 (16), 132 (25), 129 (41), 117 (84), 75 (87), 73 (100)	[27–29]
13	Pentadecanoic acid	314	314 $[M]^{+\cdot}$ (5), 299 $[M\text{-}CH_3]^+$ (100), 145 (24), 132 (27), 129 (41), 117 (86), 75 (67), 73 (83)	[27,28]
15	(9*Z*)-Hexadec-9-enoic acid	326	326 $[M]^{+\cdot}$ (3), 311 $[M\text{-}CH_3]^+$ (27), 145 (14), 132 (6), 129 (48), 117 (100), 95 (28), 75 (95), 73 (73)	Wiley-NIST MS Library
16	(9*E*)-Hexadec-9-enoic acid	326	326 $[M]^{+\cdot}$ (4), 311 (88), 145 (27), 132 (11), 129 (59), 117 (71), 95 (29), 75 (100), 73 (95)	Wiley-NIST MS Library
18	Hexadecanoic acid	328	328 $[M]^{+\cdot}$ (4), 313 $[M\text{-}CH_3]^+$ (100), 145 (25), 132 (27), 129 (40), 117 (88), 75 (43), 73 (60)	Co
21	Heptadecanoic acid	342	342 $[M]^{+\cdot}$ (5), 327 $[M\text{-}CH_3]^+$ (97), 145 (33), 132 (38), 129 (51), 117 (100), 75 (67), 73 (91)	[27–29]
23	(9*Z*,12*Z*)-Octadeca-9,12-dienoic acid	352	352 $[M]^{+\cdot}$ (3), 337 $[M\text{-}CH_3]^+$ (100), 145 (12), 132 (4), 129 (44), 117 (34), 95 (43), 75 (86), 73 (86)	Wiley-NIST MS Library
24	(9*Z*,12*Z*,15*Z*)-Octadeca-9,12,15-trienoic acid	350	350 $[M]^{+\cdot}$ (4), 335 $[M\text{-}CH_3]^+$ (33), 173 (12), 163 (11), 149 (22), 145 (16), 132 (5), 129 (40), 117 (27), 108 (32), 95 (62), 79 (100), 75 (95), 73 (87)	Wiley-NIST MS Library
25	(9*Z*)-Octadec-9-enoic acid	354	354 $[M]^{+\cdot}$ (4), 339 $[M\text{-}CH_3]^+$ (92), 264 (12), 222 (14), 199 (13), 185 (12), 145 (35), 132 (14), 129 (90), 117 (95), 96 (33), 81 (32), 75 (93), 73 (100)	[29]
26	(9*E*)-Octadec-9-enoic acid	354	354 $[M]^{+\cdot}$ (3), 339 (77), 264 (9), 222 (11), 199 (11), 185 (12), 145 (31), 132 (14), 129 (75), 117 (74), 96 (28), 81 (30), 75 (98), 73 (100)	[29]
27	Octadecanoic acid	356	356 $[M]^{+\cdot}$ (6), 341 $[M\text{-}CH_3]^+$ (86), 145 (39), 132 (38), 129 (58), 117 (100), 75 (77), 73 (94)	[27–29]

No.	Fatty acid TMS ester	$[M]^{+\cdot}$ (*m/z*)	EI-MS product ions *m/z* (relative intensity %)	Identification
28	Nonadecanoic acid	370	370 $[M]^{+\cdot}$ (4), 355 $[M\text{-}CH_3]^+$ (62), 145 (26), 132 (25), 129 (29), 117 (63), 75 (100), 73 (59)	[28]
31	Eicosanoic acid	384	384 $[M]^{+\cdot}$ (9), 369 $[M\text{-}CH_3]^+$ (100), 145 (35), 132 (33), 129 (40), 117 (84), 75 (45), 73 (60)	[27–29]
33	Heneicosanoic acid	398	398 $[M]^{+\cdot}$ (2), 383 $[M\text{-}CH_3]^+$ (100), 145 (41), 132 (39), 129 (44), 117 (88), 75 (69), 73 (80)	[27–29]
36	Docosanoic acid	412	412 $[M]^{+\cdot}$ (12), 397 $[M\text{-}CH_3]^+$ (100), 145 (45), 132 (41), 129 (48), 117 (98), 75 (42), 73 (69)	[27–29]
37	Tricosanoic acid	426	426 $[M]^{+\cdot}$ (3), 411 $[M\text{-}CH_3]^+$ (92), 145 (44), 132 (46), 129 (55), 117 (100), 75 (56), 73 (69)	[27–29]
40	Tetracosanoic acid	440	440 $[M]^{+\cdot}$ (13), 425 $[M\text{-}CH_3]^+$ (89), 145 (50), 132 (47), 129 (53), 117 (100), 75 (49), 73 (71)	[27–29]
42	Pentacosanoic acid	454	454 $[M]^{+\cdot}$ (10), 439 $[M\text{-}CH_3]^+$ (70), 145 (41), 132 (42), 129 (48), 117 (71), 75 (81), 73 (100)	[27–29]
44	Hexacosanoic acid	468	468 $[M]^{+\cdot}$ (2), 453 $[M\text{-}CH_3]^+$ (85), 145 (59), 135 (48), 129 (53), 117 (100), 75 (58), 73 (92)	[27–29]
51	Octacosanoic acid	496	496 $[M]^{+\cdot}$ (15), 481 $[M\text{-}CH_3]^+$ (66), 145 (52), 132 (36), 129 (50), 117 (81), 75 (56), 73 (100)	[28,29]

Abbreviation: Co, Co-injection of authentic standard.

Figure 8 demonstrates the EI mass spectrum of the hexadecanoic acid (**18**) TMS ester. A low relative abundance peak was found at *m/z* 328 (*I*), corresponding to the molecular ion. The base peak of this EI mass spectrum was detected at *m/z* 313 (*II*), due to the loss of a methyl radical (-15 Da) from the TMS group. This product ion is useful in assigning the molecular weight of the TMS fatty acid ester, since the molecular ion peak can be sometimes absent in the EI mass spectra.[45] Four characteristic product ions regarding the EI fragmentation of fatty acid TMS esters were observed at *m/z* 145 (*III*), 132 (*V*), 129 (*IV*) and 117 (*VI*). The product ions at *m/z* 145 (*III*) and 129 (*IV*) resulted from 1,3-hydrogen transfer (*m/z* 145), followed by loss of methane (*m/z* 129).[45] The other two product ions at *m/z* 132 (*V*) and 117 (*VI*) resulted from McLafferty rearrangement (*m/z* 132), and successive loss of a methyl radical (*m/z* 117).[45] Additionally, two high relative abundant peaks, namely at *m/z* 73 $[(CH_3)_3Si]^+$ and *m/z* 75 $[(CH_3)_2SiOH]^+$, were also demonstrated in the EI mass spectrum of hexadecanoic acid (**18**) TMS ester[45] (Figure 8).

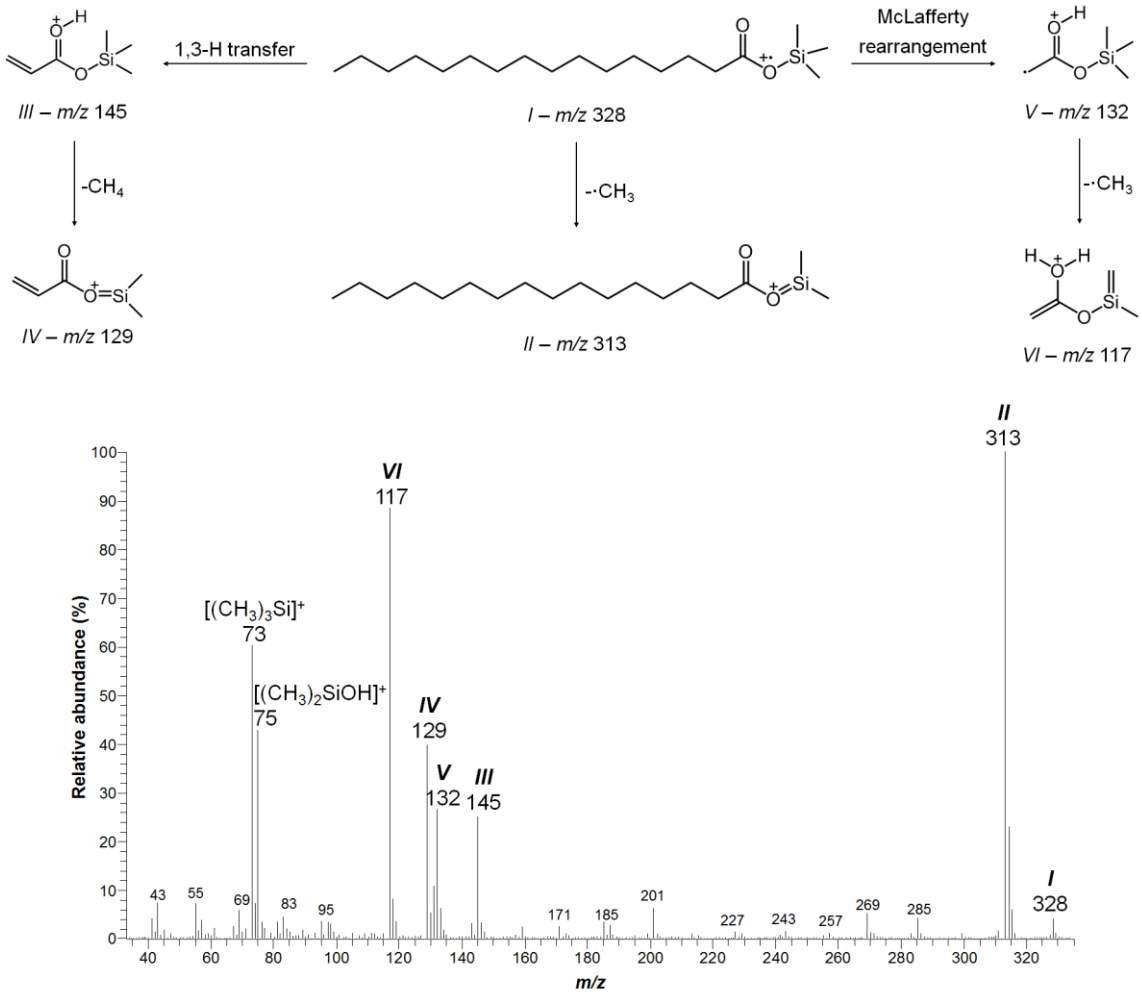


Figure 8: EI mass spectrum and the main fragmentation product ions of hexadecanoic acid (**18**) trimethylsilyl ester.

Two 2-hydroxyfatty acids (**2** and **6**) were identified in the cultivated cardoon dichloromethane extracts (Table 4). In the EI mass spectra of the respective TMS derivatives, the molecular ions were absent. It was common to find a highly abundant product ion originated by the loss of COOTMS group (-117 Da) from the molecular ion.

3.2.3 Long chain aliphatic alcohols

Eight long chain aliphatic alcohols (**14**, **20**, **22**, **30**, **34**, **38**, **43** and **47**) (Figure 9) were identified in dichloromethane extracts of *C. cardunculus* L. var. *altilis* (DC). These compounds were described, for the first time, as *C. cardunculus* L. components, although eicosan-1-ol (**30**) was only noticed after alkaline hydrolysis.

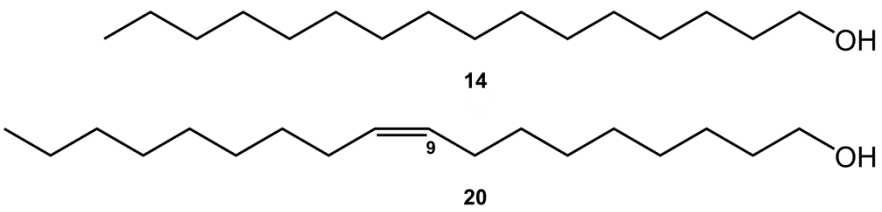


Figure 9: Structures of some of the long chain aliphatic alcohols identified in the *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts.

The EI-MS data was essential to identify the long chain aliphatic alcohols in the cultivated cardoon dichloromethane extracts (Table 5), in addition to the retention time for comparison with the literature data.[27–29]

Table 5: EI mass spectroscopic data of long chain aliphatic alcohol trimethylsilyl (TMS) ethers identified in the *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts.

No.	Long chain aliphatic alcohol TMS ether	$[M]^{+\cdot}$ (*m/z*)	EI-MS product ions *m/z* (relative intensity %)	Identification
14	Hexadecan-1-ol	314	314 (1), 299 $[M\text{-}CH_3]^+$ (100), 103 $[CH_2OSi(CH_3)_3]^+$ (16), 89 $[(CH_3)_3SiO]^+$ (14), 75 (65), 73 (42)	[27,29]
20	(9*Z*)-Octadec-9-en-1-ol	340	340 (5), 325 $[M\text{-}CH_3]^+$ (21), 103 (16), 89 (11), 75 (100), 73 (46)	[27,29]
22	Octadecan-1-ol	342	327 $[M\text{-}CH_3]^+$ (100), 103 (11), 89 (8), 75 (30), 73 (14)	[27,29]
30	Eicosan-1-ol	370	355 $[M\text{-}CH_3]^+$ (100), 103 (14), 89 (9), 75 (88), 73 (84)	Wiley-NIST MS Library
34	Docosan-1-ol	398	383 $[M\text{-}CH_3]^+$ (100), 103 (15), 89 (8), 75 (58), 73 (68)	[27,29]
38	Tetracosan-1-ol	426	411 $[M\text{-}CH_3]^+$ (100), 103 (9), 89 (5), 75 (26), 73 (13)	[29]

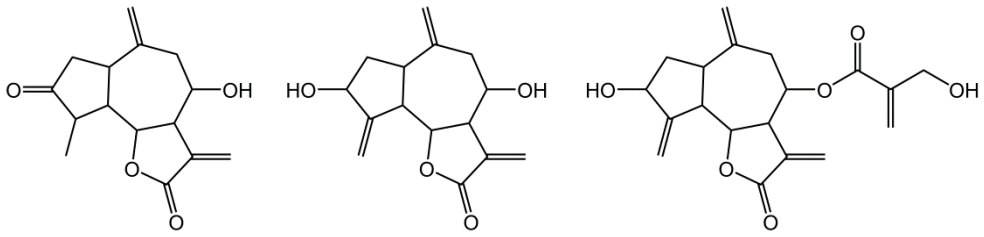
No.	Long chain aliphatic alcohol TMS ether	$[M]^{+\cdot}$ (*m/z*)	EI-MS product ions *m/z* (relative intensity %) [a]	Identification
43	Hexacosan-1-ol	454	439 $[M\text{-}CH_3]^+$ (100), 103 (14), 89 (6), 75 (36), 73 (32)	[28,29]
47	Octocosan-1-ol	482	467 $[M\text{-}CH_3]^+$ (100), 103 (15), 89 (8), 75 (42), 73 (23)	[27–29]

Figure 10 reveals the EI mass spectrum of hexacosan-1-ol (**43**) TMS ether derivative. The molecular of this compound (*m/z* 454) was absent in the EI mass spectrum. The molecular ions of TMS ether derivatives of these compounds are generally absent, or denoted by a low intense peak.[46] The base peak was observed at *m/z* 439 (*I*), due to the loss of a methyl radical from the TMS group. The base peak is very important for determining the molecular weight of the long chain aliphatic alcohol TMS derivative. Two characteristic product ions were registered at *m/z* 103 (*II*) and 89 (*III*).[46] The former was afforded by C1-C2 bond cleavage (*m/z* 103), whereas the latter resulted from the bond cleavage between C1 and oxygen, and transition of a methyl radical from the TMS group to the oxygen (*m/z* 89).[46] The characteristic product ions at *m/z* 75 and *m/z* 73 were also detected with relative high abundance, as earlier explained.[46]

Figure 10: EI mass spectrum and the main fragmentation product ions of hexacosan-1-ol (**43**) trimethylsilyl ether.

3.2.4 Sesquiterpene lactones

Three guaianolide sesquiterpene lactones (Table 2 and Figure 11) were identified in *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts, namely grosheimin (**29**), deacycynaropicrin (**32**) and cynaropicrin (**41**). These compounds were previously isolated from artichoke.[23,36–38,41] Cynaropicrin[41] and grosheimin[39] were also isolated from cardoon, although without referring the wild or cultivated variety. In this manner, deacycynaropicrin (**32**) was identified for the first time as *C. cardunculus* L. var. *altilis* (DC) component.



29 **32** **41**

Figure 11: Structures of guaianolide sesquiterpene lactones identified in the *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts.

Grosheimin (**29**) and deacylcynaropicrin (**32**) were identified by comparing the respective EI-MS data with the equipment mass spectral library and literature data.[30] The identification of cynaropicrin (**41**) was based on the EI-MS and, principally, NMR spectroscopic data, as explained bellow in this chapter. The EI-MS data of the guaianolide TMS ethers, regarding the three guaianolides identified in cultivated cardoon dichloromethane extracts, were reported here for the first time (Table 6).

Table 6: EI mass spectroscopic data of the guaianolide sesquiterpene lactone trimethylsilyl (TMS) derivatives identified in *C. cardunculus* L. var. *altilis* (DC).

No.	Guaianolide sesquiterpene lactone TMS derivative	$[\mathbf{M}]^+$ (*m/z*)	EI-MS product ions *m/z* (relative intensity %)
29	Grosheimin	334	334 $[M]^{+\cdot}$ (3), 319 $[M\text{-}CH_3]^+$ (9), 291 (5), 263 (3), 244 (7), 237 (100), 197 (20), 169 (22), 141 (14), 91 (9), 75 $[(CH_3)_2SiOH]^+$ (17), 73 $[(CH_3)_3Si]^+$ (63)
32	Deacylcynaropicrin	406	406 $[M]^{+\cdot}$ (15), 388 $[M\text{-}CH_3]^+$ (3), 316 (8), 295 (9), 273 (7), 219 (14), 197 (53), 181 (20), 168 (30), 141 (13), 129 (7), 91 (8), 75 $[(CH_3)_2SiOH]^+$ (27), 73 $[(CH_3)_3Si]^+$ (100)
41	Cynaropicrin	490	490 $[M]^{+\cdot}$ (13), 462 (7), 391 (10), 317 (28), 316 $[M–(CH_3)_3SiOCH_2CCH_2CO_2H]^+$ (83), 301 (14), 273 (15), 227 (74), 168 (77), 103 (14), 91 (19), 75 $[(CH_3)_2SiOH]^+$ (37), 73 $[(CH_3)_3Si]^+$ (100)

The EI mass spectrum of cynaropicrin TMS diether (Table 6 and Figure 12) displayed the molecular ion at *m/z* 490, as well as an intense peak at *m/z* 316 ([M-$(CH_3)_3SiOCH_2CCH_2CO_2H]^+$) which was yielded by the loss of the trimethylsilylated ester moiety, through McLafferty rearrangement and inductive cleavage.[6] Additionally, the base peak was observed at *m/z* 73 corresponding to the TMS ion ($[(CH_3)_3Si]^+$), a characteristic EI product ion of trimethylsilylated derivatives.

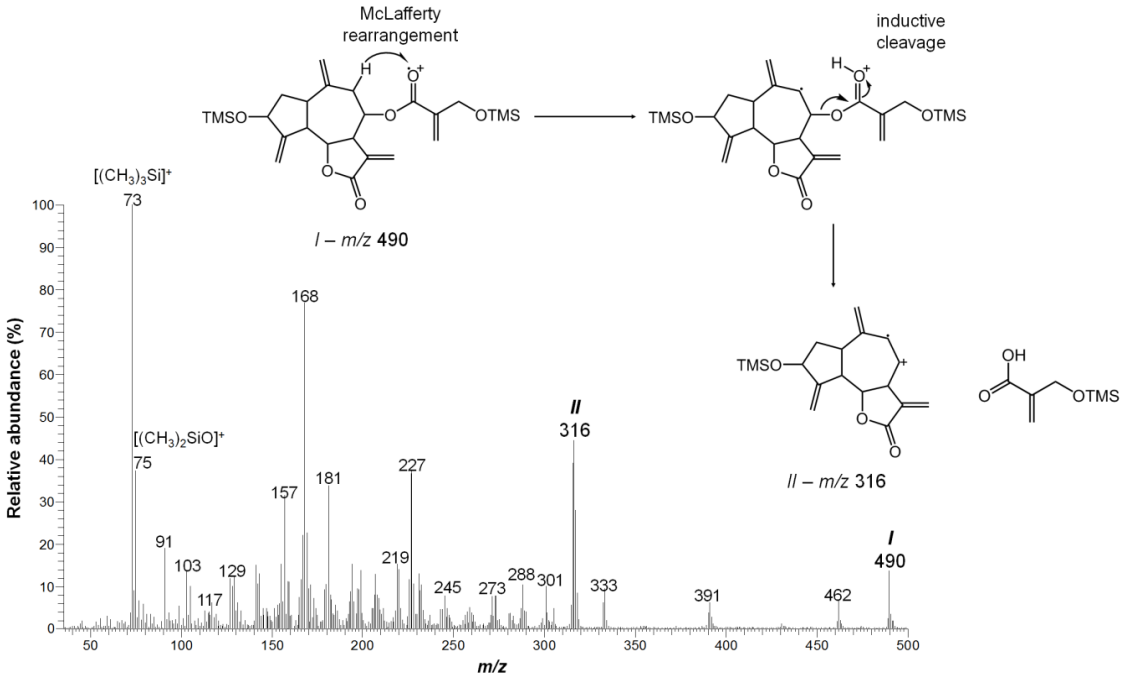


Figure 12: EI mass spectrum and the main fragmentation product ions of the cynaropicrin (**41**) trimethylsilyl (TMS) diether.

The NMR spectroscopic analysis showed to be decisive for the identification of cynaropicrin, because the EI-MS data of its TMS diether derivative was not previously indicated in the literature.

3.2.4.1 Chemical identification of cynaropicrin by NMR spectroscopy

The identification of cynaropicrin was confirmed by NMR spectroscopic analysis. The ^1H and ^{13}C NMR spectra showed respectively 24 and 19 resonances, namely in the saturated (ranges of δ_H 1.67–2.92 ppm and δ_C 37.0-51.4 ppm) and in the alkene regions (ranges of δ_H 5.08–6.27 ppm and δ_C 113.6-152.2 ppm). According to the DEPT analysis, the resonances were ascribed to 7 secondary, 6 tertiary and 6 quaternary carbons.

In the ^1H NMR spectrum, two resonances were assigned to H-4´ (δ 4.32 ppm) and H-3 (δ 4.50 ppm) (Figure 13), corresponding to protons near to alcohol groups, and other two to H-

6 (δ 4.20 ppm) and H-8 plus 14 (δ 5.08 ppm), relatively to protons near to ester groups, but the resonance at H-8 was overlapped with the one at H-14. In the ^{13}C NMR spectrum, four resonances were ascribed to carbons bonded to oxygen atoms (Figure 13), more specifically two carbons bonded to alcohol groups, at C-4´ (δ 62.3 ppm) and C-3 (δ 73.7 ppm), and two carbons bonded to ester groups, at C-8 (δ 74.3 ppm) and C-6 (δ 78.4 ppm). Two resonances detected in the ^{13}C NMR spectrum were assigned to the carbonyls of the ester groups, namely at C-1´ (δ 165.3 ppm) and C-12 (δ 169.0 ppm).

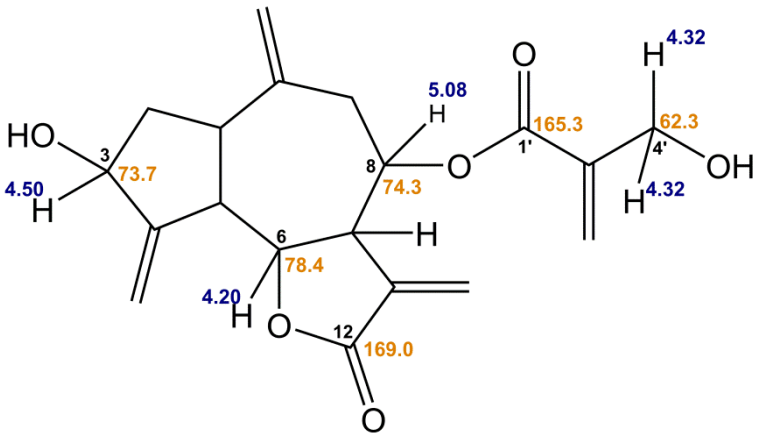


Figure 13: Chemical shifts of relevant ^{1}H (blue) and ^{13}C (orange) NMR data (ppm) of cynaropicrin.

Table 7 indicates the COSY, HSQC and HMBC correlations for cynaropicrin. The two-dimension NMR techniques allowed to identify four methylene groups (C=CH_2), namely at C-13 (*δ* 122.7 ppm) C-14 (*δ* 118.2 ppm), C-15 (*δ* 113.6 ppm) and C-3´ (*δ* 127.8 ppm) resonances.

Table 7: COSY, HSQC and HMBC correlations for cynaropicrin.

Position	COSY	HSQC	HMBC
1	H-2*α*, H-2*β*, H-5	C-1	C-5, C-6, C-10
2*α*	H-1, H-2*β*, H-3	C-2	C-1, C-3, C-10
2*β*	H-1, H-2*α*, H-3	C-2	C-5, C-3, C-4
3	H-2*α*, H-2*β*, H-15a, H-15b	C-3	
5	H-1, H-6, H-15a	C-5	C-4, C-6
6	H-5, H-7	C-6	C-4, C-8
7	H-6, H-8+14b, H-13a, H-13b	C-7	C-6, C-11
8 + 14b	H-9*α*, H-9*β*, H-7, H-14a	C-8, C-14	C-9, C-7, C-1, C-10, C-11
9*α*	H-9*β*, H-8+14b	C-9	C-1, C-7, C-8, C-10, C-14
9*β*	H-9*α*, H-8+14b	C-9	C-1, C-7, C-8, C-14, C-10

Position	COSY	HSQC	HMBC
13a	H-7	C-13	C-7
13b	H-7	C-13	C-7, C-11, C-12
14a		C-14	C-1, C-9
15a	H-3, H-5	C-15	C-3, C-5
15b	H-3	C-15	C-3, C-4, C-5
3´a	H-4´, H-3´b	C-3´	C-1´, C-4´
3´b	H-4´, H-3´a	C-3´	C-1´, C-2´, C-4´
4´	H-3´a, H-3´b	C-4´	C-1´, C-2´, C-3´

Figure 14 indicates the COSY and HMBC correlations that endorsed the identification of the methylene group at C-13 (C=CH_2). According to the HSQC spectrum, two doublets noted at δ 5.57 (H-13a) and 6.16 ppm (H-13b) correlate with the secondary carbon (DEPT) signal at δ 122.7 ppm (C-13). In the COSY spectrum, both H-13a and H-13b correlate with a resonance at δ_H 3.14 ppm. In the HSQC spectrum, the proton resonance at δ 3.14 ppm correlates with the tertiary carbon (DEPT) at δ 47.6 ppm which was assigned to C-7. Both protons at C-13 couple with the proton at C-7 (Figure 14). Moreover, the HMBC spectrum indicated that H-13b correlates with the C-7 and with two quaternary carbons (DEPT) at δ 137.3 and 169.0 ppm, attributed to C-11 and C-12 (HSQC), respectively (Figure 14). The HMBC spectrum also displayed that H-13a correlates with C-7; nevertheless H-13a should also correlates with C-11 and C-12, which it was not noticed in the HMBC spectrum, in the experimental conditions covered.

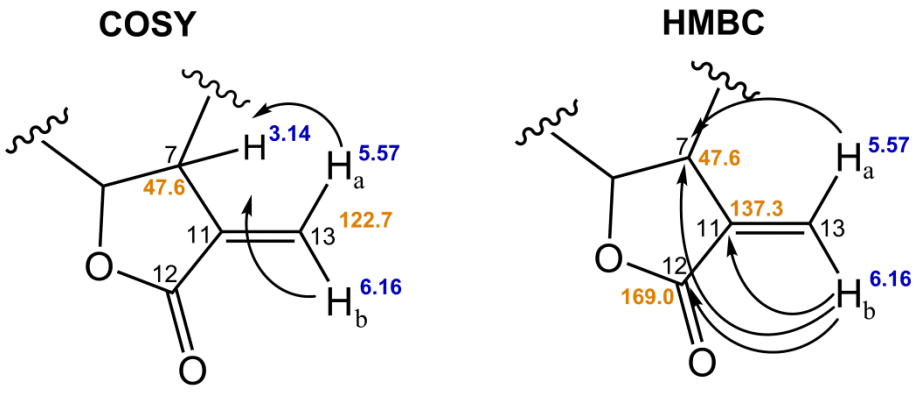


Figure 14: Relevant COSY (left) and HMBC (right) correlations of cynaropicrin, with the chemical shifts of ^{1}H (blue) and ^{13}C (orange) NMR spectroscopic data (ppm).

3.2.5 Sterols

Seven sterols were identified in the *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts (Table 2 and Figure 15): a stanol, named as *β*-sitostanol (**55**), and six Δ^5-stenols, namely cholesterol (**45**), 24-methylenecholesterol (**49**), campesterol (**50**), stigmasterol (**52**), *β*-sitosterol (**54**) and Δ^5-avenasterol (**56**). These sterols can be grouped into four subclasses, namely cholestane (**45**), ergostane (**49**), campestane (**50**) and stigmastane (**52**, **54**-**56**). In the present work, cholesterol (**45**), 24-methylenecholesterol (**49**), *β*-sitostanol (**55**) and Δ^5-avenasterol (**56**) were reported for the first time as *C. cardunculus* L. components. Campesterol (**50**), stigmasterol (**52**) and *β*-sitosterol (**54**) were previously reported in the seed oil composition of the three varieties from *C. cardunculus* L.[25]

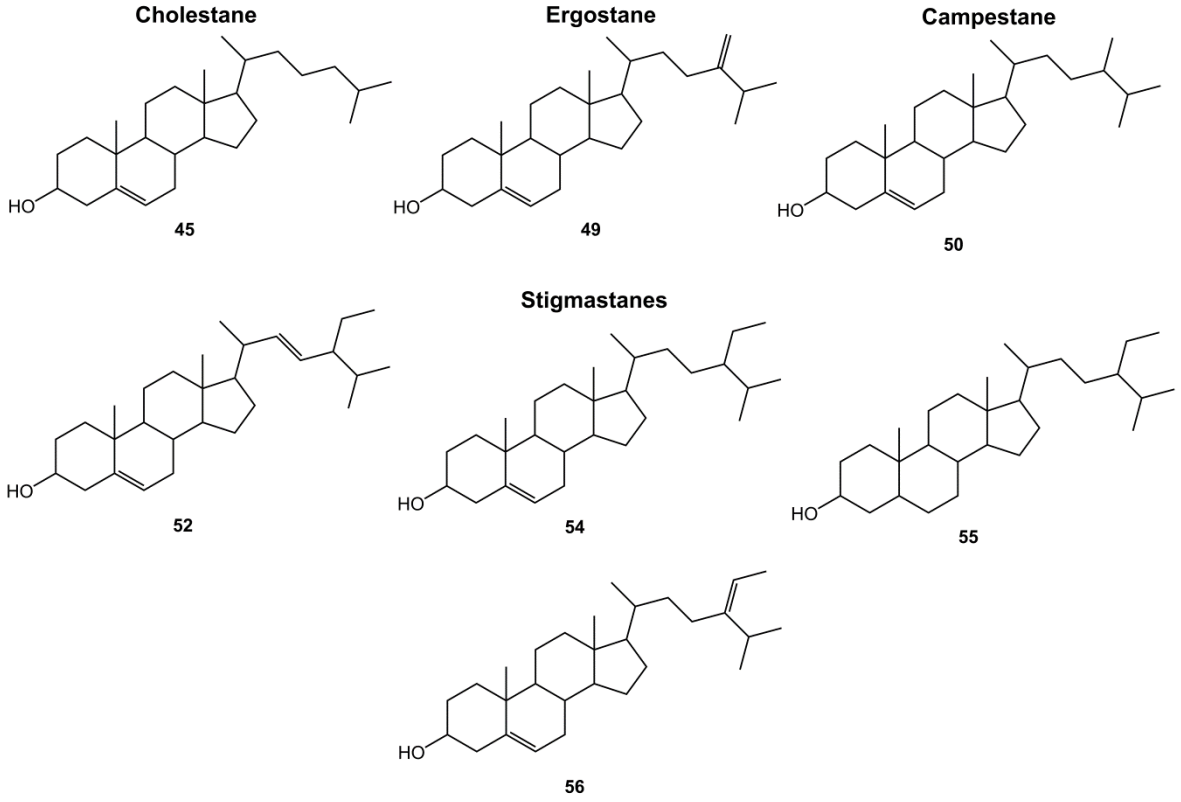


Figure 15: Structures of sterols identified in the *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts.

The identification of sterols in *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts was approached by comparing the EI-MS data, elution order and retention time of the corresponding TMS ethers with the literature data.[27–29,47–50] Table 8 lists the EI-MS data of the TMS ethers of the identified sterols.

Table 8: EI mass spectroscopic data of sterol trimethylsilyl ethers identified in the *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts.

No.	Sterol TMS ether	$[M]^{+\cdot}$ (*m/z*)	EI-MS product ions *m/z* (relative intensity %)	Identification
45	Cholesterol	458	458 $[M]^{+\cdot}$ (23), 443 $[M\text{-}CH_3]^+$ (10), 368 $[M\text{-}TMSOH]^+$ (55), 353 $[M\text{-}TMSOH\text{-}CH_3]^+$ (35), 329 $[M\text{-}129]^+$ (68), 281 (55), 255 $[M\text{-}SC\text{-}TMSOH]^+$ (26), 247 $[M\text{-}TMSOH\text{-}(A+B)]^+$ (190), 213 $[M\text{--}SC\text{-}D\text{-}TMSOH]^+$ (15), 129 $[M\text{-}329]^+$ (89), 75 $[(CH_3)_2SiOH]^+$ (77), 73 (100) $[TMS]^+$	Co
49	24-Methylenecholesterol	470	470 $[M]^{+\cdot}$ (3), 455 $[M\text{-}CH_3]^+$ (9), 386 $[M\text{-}SC_\beta]^+$ (36), 380 $[M\text{-}TMSOH]^+$ (21), 365 $[M\text{-}TMSOH\text{-}CH_3]^+$ (18), 341 $[M\text{-}129]^+$ (38), 296 (25), 259 $[M\text{-}TMSOH\text{-}(A+B)]^+$ (9), 255 $[M\text{-}SC\text{-}TMSOH]^+$ (16), 213 $[M\text{-}SC\text{-}D\text{-}TMSOH]^+$ (17), 129 $[M\text{-}341]^+$ (100), 75 (66), 73 (91)	[47]
50	Campesterol	472	472 $[M]^{+\cdot}$ (32), 457 $[M\text{-}CH_3]^+$ (12), 382 $[M\text{-}TMSOH]^+$ (87), 367 $[M\text{-}TMSOH\text{-}CH_3]^+$ (46), 343 $[M\text{-}129]^+$ (95), 303 $[M\text{-}SC\text{-}D]^+$ (1), 261 $[M\text{-}TMSOH\text{-}(A+B)]^+$ (18), 255 $[M\text{-}TMSOH\text{-}SC]^+$ (25), 213 $[M\text{-}SC\text{-}D\text{-}TMSOH]^+$ (17), 129 $[M\text{-}343]^+$ (100), 75 (40), 73 (55)	[27,28,48,50]
52	Stigmasterol	484	484 $[M]^{+\cdot}$ (23), 469 $[M\text{-}CH_3]^+$ (5), 394 $[M\text{-}TMSOH]^+$ (34), 379 $[M\text{-}TMSOH\text{-}CH_3]^+$ (15), 355 $[M\text{-}129]^+$ (16), 303 $[M\text{-}SC\text{-}D]^+$ (1), 273 $[M\text{-}TMSOH\text{-}(A+B)]^+$ (2), 255 $[M\text{-}TMSOH\text{-}SC]^+$ (53), 213 $[M\text{-}271]^+$ (17), 159 (28), 129 $[M\text{-}355]^+$ (70), 83 (100), 75 (25), 73 (34)	[28,48,50]
54	*β*-Sitosterol	486	486 $[M]^{+\cdot}$ (26), 471 $[M\text{-}CH_3]^+$ (10), 396 $[M\text{-}TMSOH]^+$ (78), 381 $[M\text{-}TMSOH\text{-}CH_3]^+$ (41), 357 $[M\text{-}129]^+$ (86), 303 $[M\text{-}SC\text{-}D]^+$ (6), 275 $[M\text{-}TMSOH\text{-}(A+B)]^+$ (15), 255 $[M\text{-}TMSOH\text{-}SC]^+$ (24), 213 $[M\text{-}SC\text{-}D\text{-}TMSOH]^+$ (16), 129 $[M\text{-}357]^+$ (100), 75 (34), 73 (42)	[28,29,48,50]
55	*β*-Sitostanol	488	488 $[M]^{+\cdot}$ (20), 473 $[M\text{-}CH_3]^+$ (37), 431 (14), 398 $[M\text{-}TMSOH]^+$ (19), 383 $[M\text{-}TMSOH\text{-}CH_3]^+$ (35), 357 (34), 305 $[M\text{-}SC\text{-}D]^+$ (18), 257 $[M\text{-}SC\text{-}TMSOH]^+$ (12), 230 $[M\text{-}SC\text{-}C16\text{-}C17\text{-}TMSOH]^+$ (15), 215 $[M\text{-}SC\text{-}D\text{-}TMSOH]^+$ (99.5), 144 $[M\text{-}344]^+$ (11), 145 $[M\text{-}344\text{-}H]^+$ (36), 147 (46), 129 $[M\text{-}359]^+$ (57), 75 (100), 73 (79)	[48–50]
56	Δ^5-Avenasterol	484	484 $[M]^{+\cdot}$ (8), 469 $[M\text{-}CH_3]^+$ (6), 394 $[M\text{-}TMSOH]^+$ (11), 379 $[M\text{-}TMSOH\text{-}CH_3]^+$ (9), 386 $[M\text{-}SC_\beta]^+$ (100), 355 $[M\text{-}129]^+$ (10), 303 $[M\text{-}SC\text{-}D]^+$ (3), 296 (77), 281 (81), 273 $[M\text{-}TMSOH\text{-}(A+B)]^+$ (5), 257 (39), 255 $[M\text{-}TMSOH\text{-}SC]^+$ (28), 213 $[M\text{-}SC\text{-}D\text{-}TMSOH]^+$ (23), 159 (34), 129 $[M\text{-}355]^+$ (93), 83 (26), 75 (49), 73 (68)	[48,50]

Abbreviations: Co, Co-injection of authentic standard; SC, loss of side chain; SC_β, loss of side chain in the *β*-position of the double bond; A, loss of A-ring; B, loss of B-ring; D, loss of D-ring; TMSOH, trimethylsilanol.

3.2.5.1 Stanols

Figure 16 illustrates the EI mass spectrum and the main fragmentation product ions of the *β*-sitostanol (**55**) TMS ether derivative.

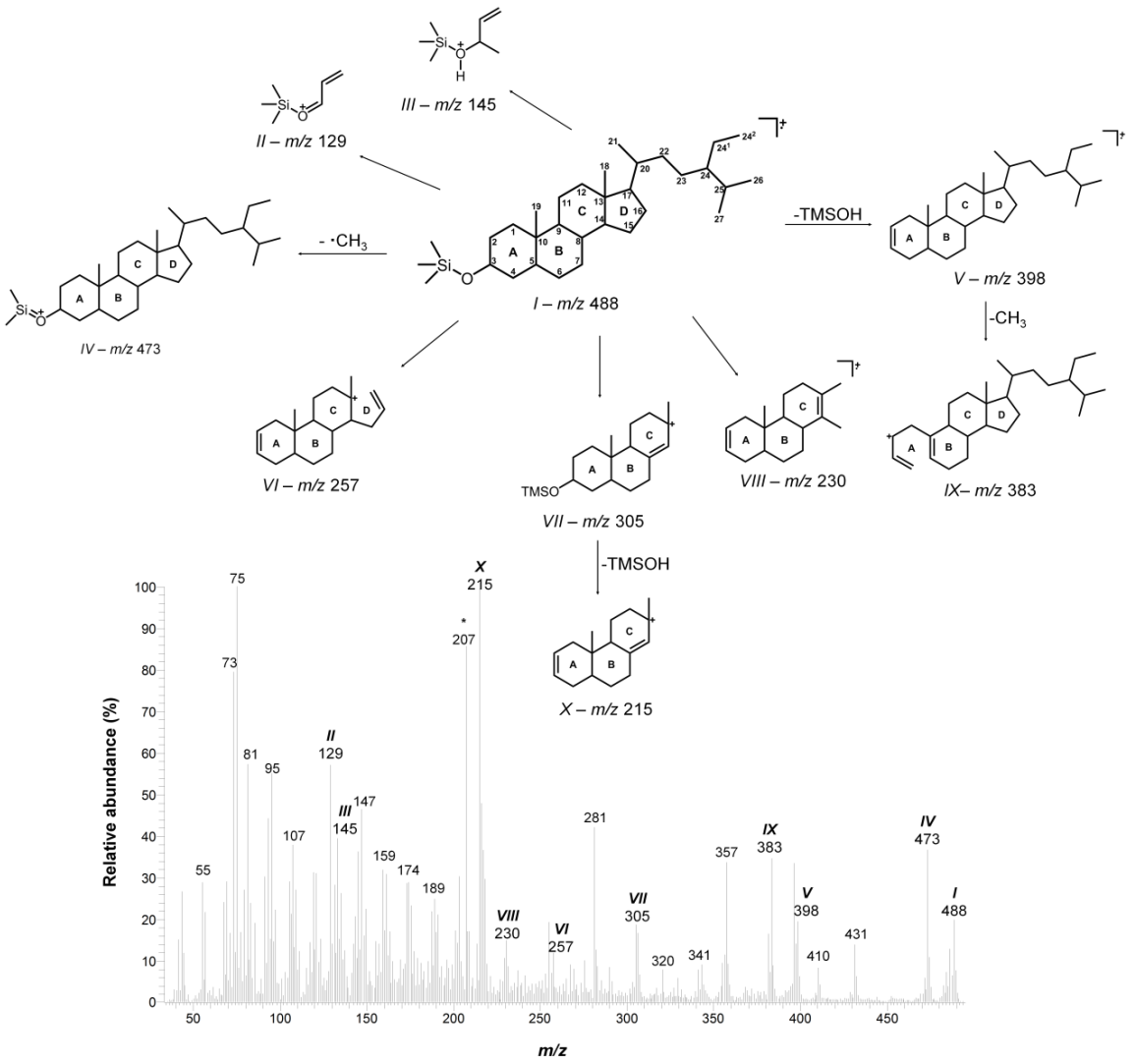


Figure 16: EI mass spectrum and the main fragmentation product ions of *β*-sitostanol (**55**) trimethylsilyl (TMS) ether. *Contamination.

The EI mass spectrum of *β*-sitostanol TMS ether (Figure 16) exhibited an intense peak at *m/z* 488 (*I*), corresponding to the molecular ion. The base peak was detected at *m/z* 215 (*X*), due to the loss of trimethylsilanol (-90 Da) from the ion at *m/z* 305 (*VII*). Three product ions at *m/z* 305 (*VII*), 257 (*VI*) and 230 (*VIII*) were yielded by the loss of the side chain, together with: (i) the loss of C15-C17, for *m/z* 305; (ii) the trimethylsilanol loss, for *m/z* 257 and (iii) the C16-C17 loss, followed by the trimethylsilanol loss for *m/z* 230. The C1-C10 and C3-C4 bonds cleavages originated the product ion at *m/z* 129 (*II*), whereas the C1-C10 and C4-

C5 bonds cleavages gave rise to the product ion at *m/z* 145 (III) (with hydrogen atom transfer).[49]

3.2.5.2 Δ^5-Sterols

Figure 17 indicates the EI mass spectrum of the *β*-sitosterol (**54**) TMS ether derivative, as well as the main fragmentation product ions.

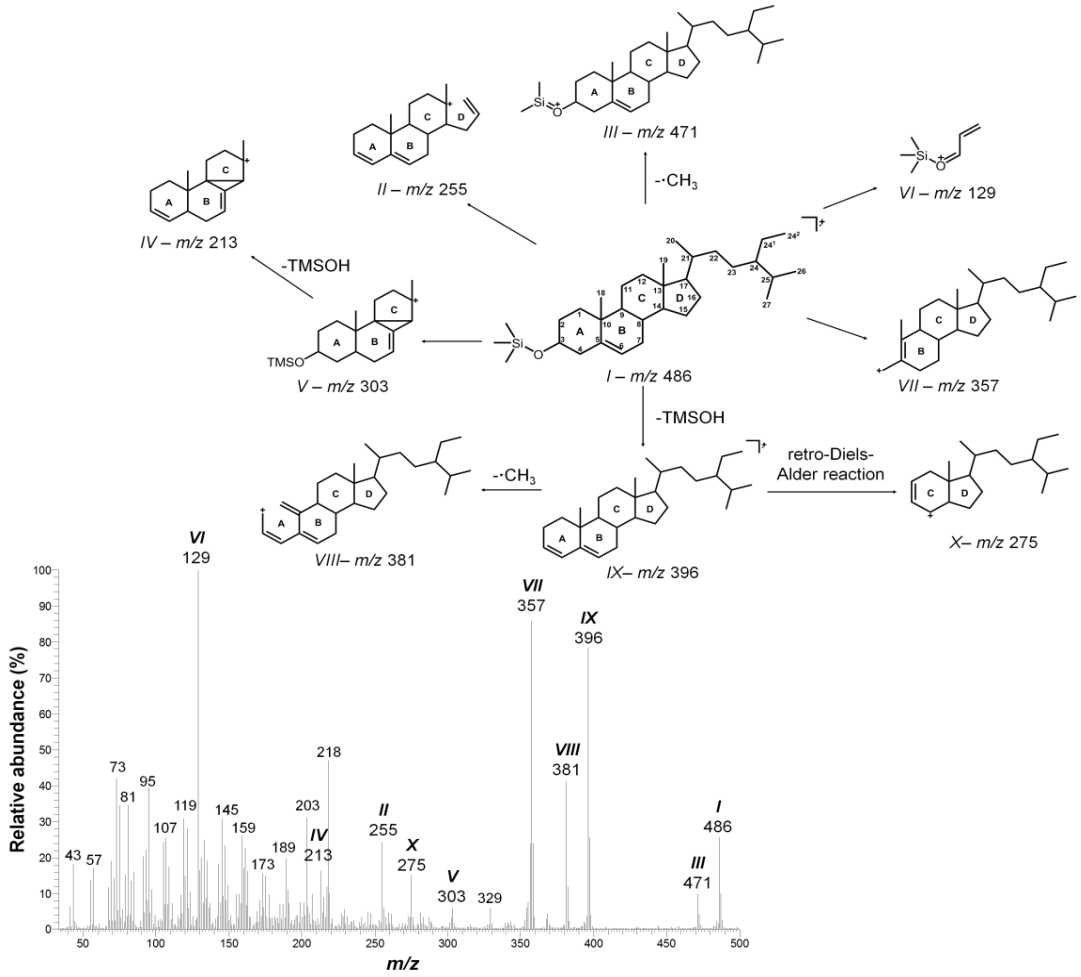


Figure 17: EI mass spectrum and the main fragmentation product ions of *β*-sitosterol (**54**) trimethylsilyl (TMS) ether derivative.

The molecular ion of the *β*-sitosterol (**54**) TMS ether was observed in the EI mass spectrum (Figure 17) like an intense peak at *m/z* 486 (*I*). The loss of a methyl radical (-15 Da) from the TMS substituent of the molecular ion afforded the product ion at m/z 471 (*III*).[51] The pair of product ions observed by *m/z* 129 (*VI*, base peak) and *m/z* 357 (*VII*) ([M-129]$^+$) derived from the C1-C10 and C3-C4 bonds cleavages, being characteristic of the EI fragmentation of TMS derivatives of Δ^5-sterols.[49,50] Other intense peak was noticed at *m/z* 396 (*IX*), which was yielded by the loss of the trimethylsilanol from the molecular ion.[50–52] Moreover, the product

ion at *m/z* 275 (*X*) resulted from the loss of the A and B-rings, through the retro-Diels-Alder reaction on the B ring of the ion at *m/z* 396 (*IX*).[50] A product ion at *m/z* 213 (*IV*) was formed by the loss of the side chain and the D-ring (C15-C17) (*m/z* 303), followed by the loss of the trimethylsilanol (-90 Da).[52] This product ion is usually found in the EI mass spectra of sterol monoenes.[52] An intense peak was also observed at *m/z* 255 (*II*), due to the loss of side chain, followed by the loss of trimethylsilanol.[50]

Regarding the EI mass spectra of Δ^5-sterols with unsaturated side chains, it is also possible to recognize product ions formed by the cleavage of the side chain in the beta-position to the double bond.[50] The EI fragmentation of 24-methylenecholesterol (**49**) TMS ether (Table 8) generated an abundant product ion at *m/z* 386, due to the loss of 2,3-dimethylbutene (-84 Da). Following the same trend, the base peak of EI mass spectrum of Δ^5-avenasterol (**56**) TMS ether derivative was detected at *m/z* 386, arising from the loss of 3,4-dimethylpent-2-ene (-98 Da).

3.2.6 Pentacyclic triterpenes

Ten pentacyclic triterpenes were identified in the *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts (Table 2 and Figure 18): *β*-amyrin (**53**), *α*-amyrin (**57**), lupeol (**58**), *β*-amyrin acetate (**59**), *α*-amyrin acetate (**60**), lupenyl acetate (**61**), *ψ*-taraxasterol (**62**), taraxasterol (**63**), *ψ*-taraxasteryl acetate (**64**) and taraxasteryl acetate (**65**). These can be grouped into four classes, namely oleananes (**53** and **59**), ursanes (**57** and **60**), lupanes (**58** and **61**) and taraxastanes (**62**-**65**).

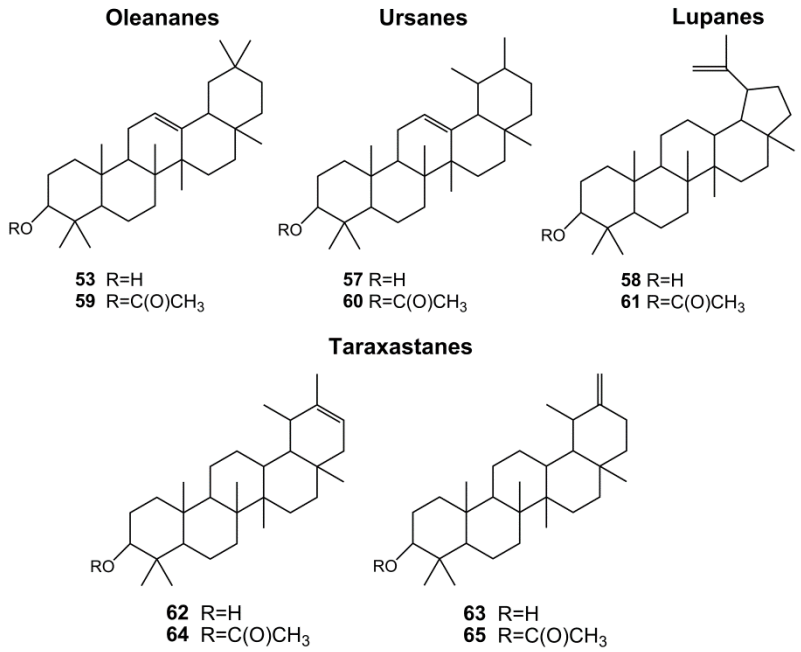


Figure 18: Structures of pentacyclic triterpenes identified in the *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts.

Lupenyl acetate (**61**) was cited here for the first time as *C. cardunculus* L. component. The remaining pentacyclic triterpenes were described in artichoke.[24,38] Some pentacyclic triterpenes (**53**, **57**, **58**, **62, 63**, **65**) were also found in cardoon,[26,43] although without citing the cultivated or wild variety. In this manner, the acetates of *β*-amyrin (**59**) and *α*-amyrin (**60**) and *ψ*-taraxasteryl acetate (**64**) were identified here for the first time as *C. cardunculus* L. var. *altilis* (DC) components.

Pentacyclic triterpenes were identified by comparing the EI-MS data of the corresponding TMS derivatives (Table 9) with the literature and, in some cases, with the injection of standards, under the same experimental conditions, as explained bellow.

Table 9: EI mass spectroscopic data of the pentacyclic triterpene trimethylsilyl ethers identified in the C. cardunculus L. var. altilis (DC) dichloromethane extracts.

No.	Pentacyclic triterpene TMS derivative	$[M]^{+\cdot}$ (*m/z*)	EI-MS product ions *m/z* (relative intensity %)	Id.
53	*β*-Amyrin	498	498 $[M]^{+\cdot}$ (1), 483 $[M\text{-}CH_3]^+$ (1), 408 $[M\text{-}TMSOH]^+$ (1), 393 $[M\text{-}TMSOH\text{-}CH_3]^+$ (2), 279 $[M\text{-}(D+E)]^+$ (3), 218 $[M\text{-}(A+B)]^+$ (100), 205 $[M\text{-}(A+B+C11)]^+$ (2), 203 $[M\text{-}(A+B+C28)]^+$ (45), 75 $[(CH_3)_2SiOH]^+$ (6), 73 $[(CH_3)_3Si]^+$ (9)	Co
57	*α*-Amyrin	498	498 $[M]^{+\cdot}$ (3), 483 (1), 408 (1), 393 (2), 279 (5), 218 (100), 205 (2), 203 (21), 75 (8), 73 (13)	[53]
58	Lupeol	498	498 $[M]^{+\cdot}$ (16), 483 $[M\text{-}CH_3]^+$ (7), 456 $[M\text{-}(C20+C29+C30)]^+$ (1), 408 $[M\text{-}TMSOH]^+$ (8), 279 $[M\text{-}(C+D+E)]^+$ (15), 218 $[M\text{-}(A+B)]^+$ (31), 203 $[218\text{-}CH_3]^+$ (54), 190 $[279\text{-}TMSO]^+$ (63), 189 $[M\text{-}(C+D+E)]^+$ (100), 175 $[279\text{-}CH_3\text{-}TMSOH]^+$ (32), 161 $[175\text{-}CH_2]^+$ (29), 135 $[203\text{-}C_5H_8]^+$ (47), 75 (45), 73 (52)	Co
59	*β*-Amyrin acetate	468	468 $[M]^{+\cdot}$ (2), 453 $[M\text{-}CH_3]^+$ (1), 408 $[M\text{-}CH_3CO_2H]^+$ (1), 249 $[M\text{-}(C+D+E)]^+$ (1), 218 $[M\text{-}(A+B)]^+$ (100), 203 $[218\text{-}CH_3]^+$ (57), 189 [218-(C11+C28), 249-$CH_3CO_2H]^+$ (19)	[32]
60	*α*-Amyrin acetate	468	468 $[M]^{+\cdot}$ (4) 453 (2), 408 (2), 249 (2), 218 (100), 203 (24), 189 (28)	[32]
61	Lupenyl acetate	468	468 $[M]^{+\cdot}$ (18), 453 $[M\text{–}CH_3]^+$ (11), 408 $[M\text{–}CH_3CO_2H]^+$ (6), 249 $[M\text{–}(C+D+E)]^+$ (13), 218 $[M\text{–}(A+B)]^+$ (30), 204 $[M\text{–}(A+B+C)]^+$ (32), 203 $[204\text{–}H]^+$ (40), 189 [M–(A+B+C), 249–$CH_3CO_2H]^+$ (100)	Co
62	*ψ*-Taraxasterol	498	498 $[M]^{+\cdot}$ (6), 483 $[M\text{-}CH_3]^+$ (2), 408 $[M\text{-}TMSOH]^+$ (12), 279 $[M\text{-}(C+D+E)]^+$ (5), 218 $[M\text{-}(A+B)]^+$ (10), 203 $[218\text{-}CH_3]^+$ (16), 190 $[279\text{-}TMSO]^+$ (30), 189 $[M\text{-}(A+B+C)]^+$ (100), 175 $[279\text{-}CH_3\text{-}TMSOH]^+$ (19), 161 $[175\text{-}CH_2]^+$ (15), 135 (27), 75 (14), 73 (23)	[54,55]

No.	Pentacyclic triterpene TMS derivative	$[M]^{+\cdot}$ (*m/z*)	EI-MS product ions *m/z* (relative intensity %)	Id.
63	Taraxasterol	498	498 $[M]^{+\cdot}$ (8), 483 (3), 456 (<1), 408 (8), 279 (8), 218 (9), 203 (21), 190 (49), 189 (100), 175 (21), 161 (18), 135 (28), 75 (22), 73 (34)	[54,55]
64	*ψ*-Taraxasteryl acetate	468	468 $[M]^{+\cdot}$ (10), 453 $[M\text{-}CH_3]^+$ (1), 408 $[M\text{-}CH_3COOH]^+$ (9), 393 $[M\text{-}CH_3\text{-}CH_3COOH]^+$ (6), 249 $[M\text{–}C_{16}H_{27}]^+$ (8), 218 $[M\text{-}C_{16}H_{26}O_2]^+$ (3), 205 $[M\text{-}C_{17}H_{29}O_2]^+$ (6), 204 $[M\text{-}C_{17}H_{28}O_2]^+$ (10), 203 $[218\text{-}CH_3]^+$ (12), 190 $[205\text{-}CH_3]^+$ (27), 189 [249-CH_3COOH, 204-CH_3]$^+$ (100)	[56]
65	Taraxasteryl acetate	468	468 $[M]^{+\cdot}$ (10), 453 (1), 408 (8), 393 (1), 249 (11), 218 (8), 205 (15), 204 (17), 203 (20), 190 (37), 189 (100)	Co

Abbreviations: A, loss of A-ring; B, loss of B-ring; C, loss of C-ring; Co, Co-injection of authentic standard; Id., identification; TMSOH, trimethylsilanol.

3.2.6.1 Oleananes and ursanes

β-amyrin (**53**) and *α*-amyrin (**57**) (Figure 18) were identified by comparing the EI-MS fragmentation of their TMS derivatives[53] (Table 9), retention time[29] and elution order[57] with the literature data. The EI mass spectra of both *β*- and *α*-amyrin TMS ethers, with the molecular ion at *m/z* 498, revealed a product ion at *m/z* 218 (base peak), due to the cleavage of C9-C11 and C8-C14 bonds, containing the D and E rings. An intense peak was also detected at *m/z* 203 which was generated by the loss of A and B rings and methyl radical (-15 Da) at C28. Other characteristic product ions were found at *m/z* 408 (loss of TMSOH (-90 Da)), 393 (loss of TMSOH and a methyl radical), 279 (loss of D and E rings) and 205 (loss of A and B rings and C11).[53]

β-amyrin acetate (**59**) and *α*-amyrin acetate (**60**) (Figure 18) were identified in the cultivated cardoon dichloromethane extracts, through the EI mass data[32] (Table 9), retention time[29] and elution order.[57] The EI mass spectra of both *β*- and *α*-amyrin acetate derivatives (molecular ion at *m/z* 468) displayed the base peak at *m/z* 218 whose product ion contained the D and E rings, resulting from C9-C11 and C8-C14 bonds cleavages.[32] A low intense fragmentation peak was observed at *m/z* 189, which may be assigned to two product ions, afforded by: (i) the loss of a C_2H_5 unit from the product ion at *m/z* 218, and (ii) the loss of the acetate group from the product ion at *m/z* 249, containing the A and B rings.[32]

3.2.6.2 Lupanes

The EI mass spectrum of the lupeol (**58**) TMS ether (Table 9 and Figure 19) displayed the molecular ion at *m/z* 498 (*I*), and the base peak at *m/z* 189 (*VI*) which contained the D and

E rings, after C12-C13 and C8-C14 bonds cleavages.[58] The loss of the propenyl moiety from molecular ion yielded a product ion at *m/z* 456 (*II*). Two product ions were formed due to the cleavage of C9-C11 and C8-C14 bonds, namely at *m/z* 279 (*IV*), containing the A and B rings, and at *m/z* 218 (*V*), presenting the D and E rings. An intense peak was noted at *m/z* 161 (*VII*), whose product ion was due to the loss of CH_2 from the ion at *m/z* 175 (*VIII*). Cleavages of the C18-C19 and C21-C22 bonds in the ion at *m/z* 203 (*IX*) gave origin to the product ion at *m/z* 135 (*X*).[58] The identification of lupeol was further confirmed with the injection of the standard compound.

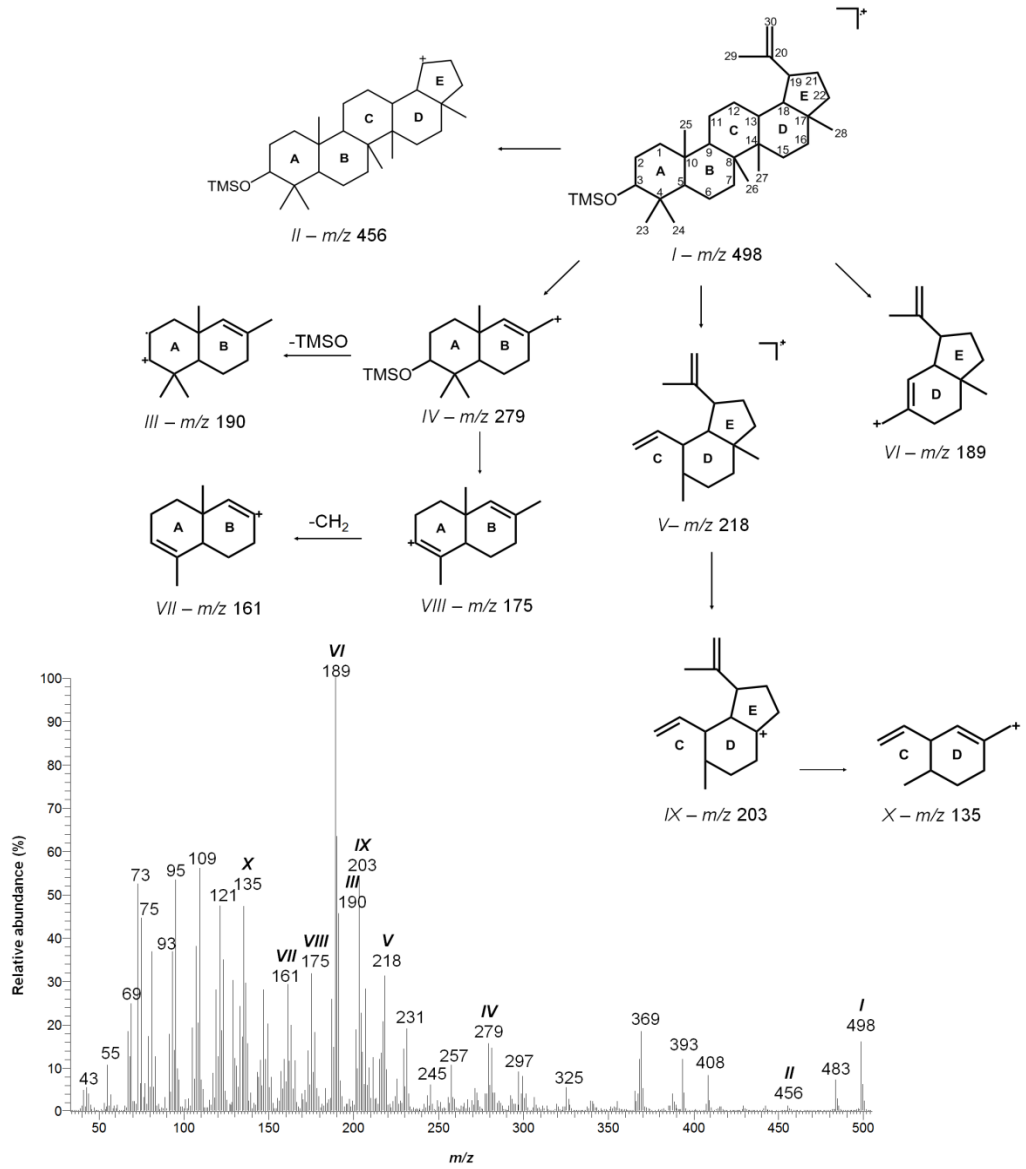


Figure 19: EI mass spectrum and the main fragmentation product ions of lupeol (**58**) trimethylsilyl (TMS) ether.

The EI-MS spectrum of lupenyl acetate (**61**) (Table 9 and Figure 20) exhibited the molecular ion at *m/z* 468 (*I*) and the base peak at *m/z* 189 (*III*). The base peak may represent two product ions with *m/z* 189 (*III*), such as: (i) a product ion containing the D and E rings, caused by cleavages of C8-C14 and C12-C13 bonds; and (ii) a product ion containing the A and B rings, due to the loss of an acetate from the product ion at *m/z* 249 (*VII*).[32] The product ion at *m/z* 249 resulted from the C9-C11 and C13-C14 bonds cleavages.[32] Two intense peaks were also noticed in the EI mass spectrum of lupenyl acetate, namely at *m/z* 204 (*V*) and 218 (*VI*): the first was yielded by C11-C12 and C8-C14 bonds cleavages, while the latter was afforded by C9-C11 and C8-14 bonds cleavages. Other characteristic product ion was observed at *m/z* 408 (*II*), due to the loss of an acetate fragment (-60 Da). The identification of lupenyl acetate was further confirmed with the injection of a sample previously prepared in this work.

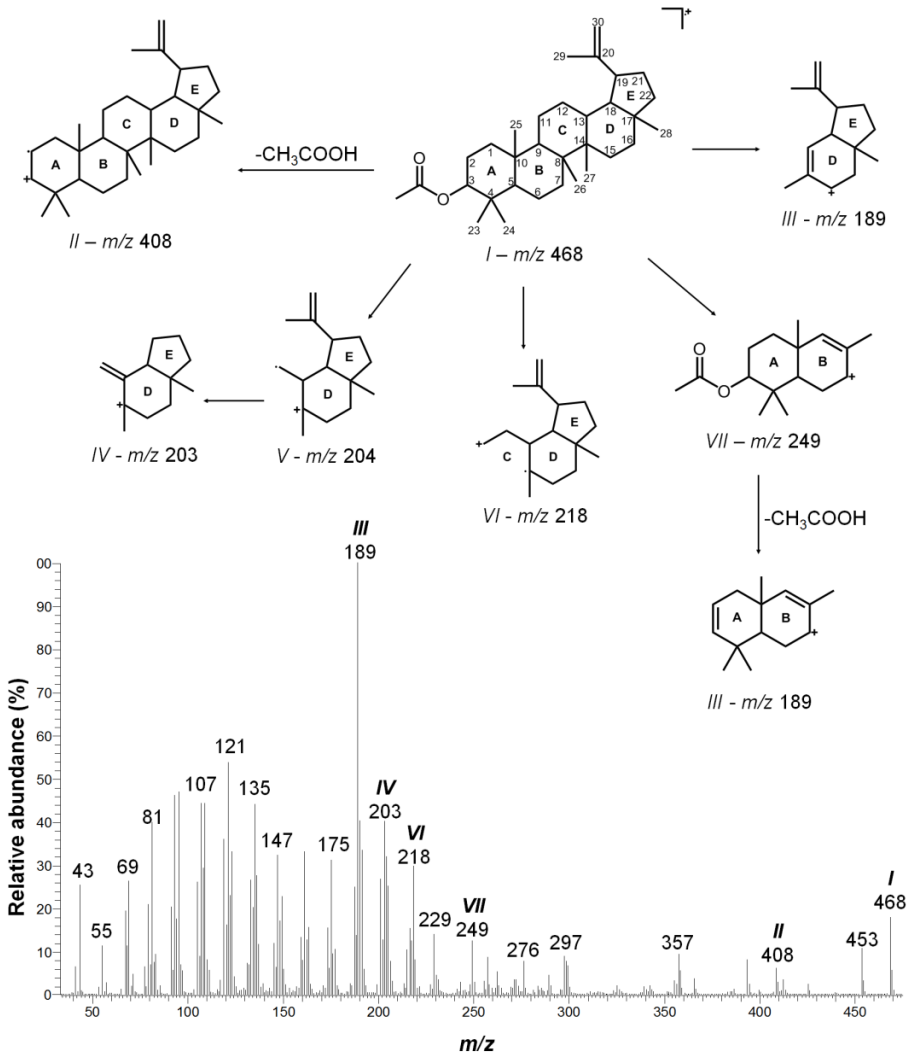


Figure 20: EI mass spectrum and the main fragmentation product ions of lupenyl acetate (**61**).

3.2.6.3 Taraxastanes

Both *ψ*-taraxasterol (**62**) and taraxasterol (**63**) TMS ethers presented similar fragmentation profiles to the one of lupeol (**58**) TMS ether. Figure 21 highlights the EI mass spectrum and the main fragmentation product ions of taraxasterol TMS ether. The EI mass spectra of *ψ*-taraxasterol and taraxasterol TMS ethers demonstrated the molecular ion at *m/z* 498 (*I*) and the base peak at *m/z* 189 (*III*) (Table 9 and Figure 21).[55] The main product ions were generated by C8-C14 and C9-C11 bonds cleavages, containing either the A and B rings (*IV*, *V*, *VI* and *VII*) or the D and E rings (*VIII* and *IX*) (Figure 21).[58] The unambiguous identification of *ψ*-taraxasterol and taraxasterol was complemented by comparing the elution order of their TMS ethers with the literature data.[54,57,59]

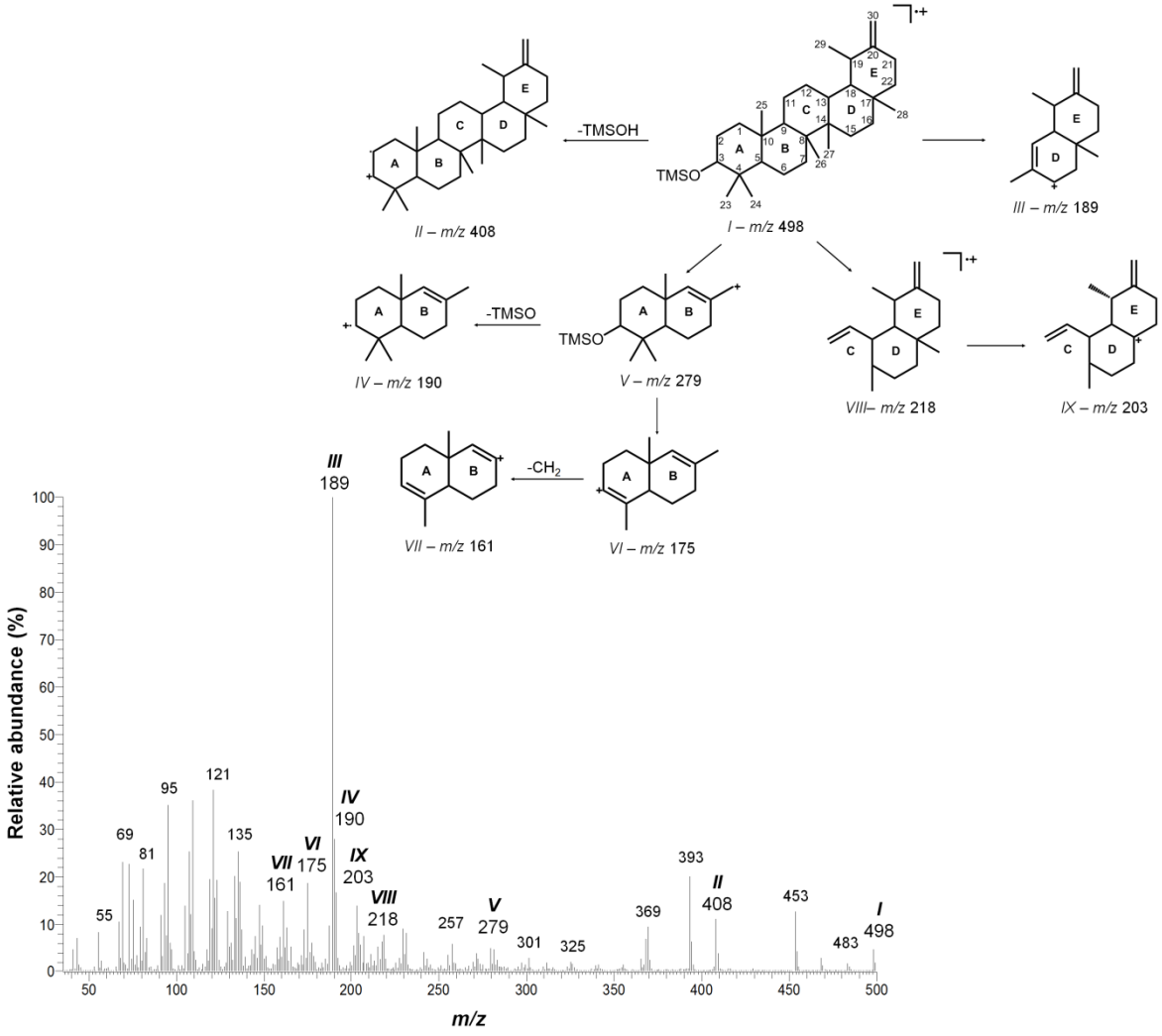


Figure 21: EI mass spectrum and the main fragmentation product ions of taraxasterol (**62**) trimethylsilyl (TMS) ether.

The EI mass spectra of *ψ*-taraxasteryl acetate (**64**) and taraxasteryl acetate (**65**) were similar to the one of lupenyl acetate (**61**). Figure 22 indicates the EI mass spectrum and the main fragmentation product ions of taraxasteryl acetate. The EI ionization of taraxasteryl acetate yielded the molecular ion at *m/z* 468 (*I*), as well as two ion products at *m/z* 249 (*II*) and at *m/z* 218 (*VII*) due to the cleavage of C8-C14 and C9-C11 bonds (Table 9 and Figure 22). The former product ion (*m/z* 249) contained the A and B rings, whereas the last one (*m/z* 218) enclosed the D and E rings and part of C ring. Additionally, the base peak was detected at *m/z* 189 (*IV*) which was afforded by the loss of acetate (-60 Da) from the ion at *m/z* 249 (*II*).[32,56] Other characteristic product ions were detected at *m/z* 204 (*V*), owing to C8-C14 and C11-C12 bonds cleavages.[32] The identification of taraxasteryl acetate was further confirmed by injection of the standard compound, in the same experimental conditions. In the case of *ψ*-taraxasteryl acetate, its identification was based on the similar EI-MS mass and the elution order relatively to taraxasteryl acetate.[57]

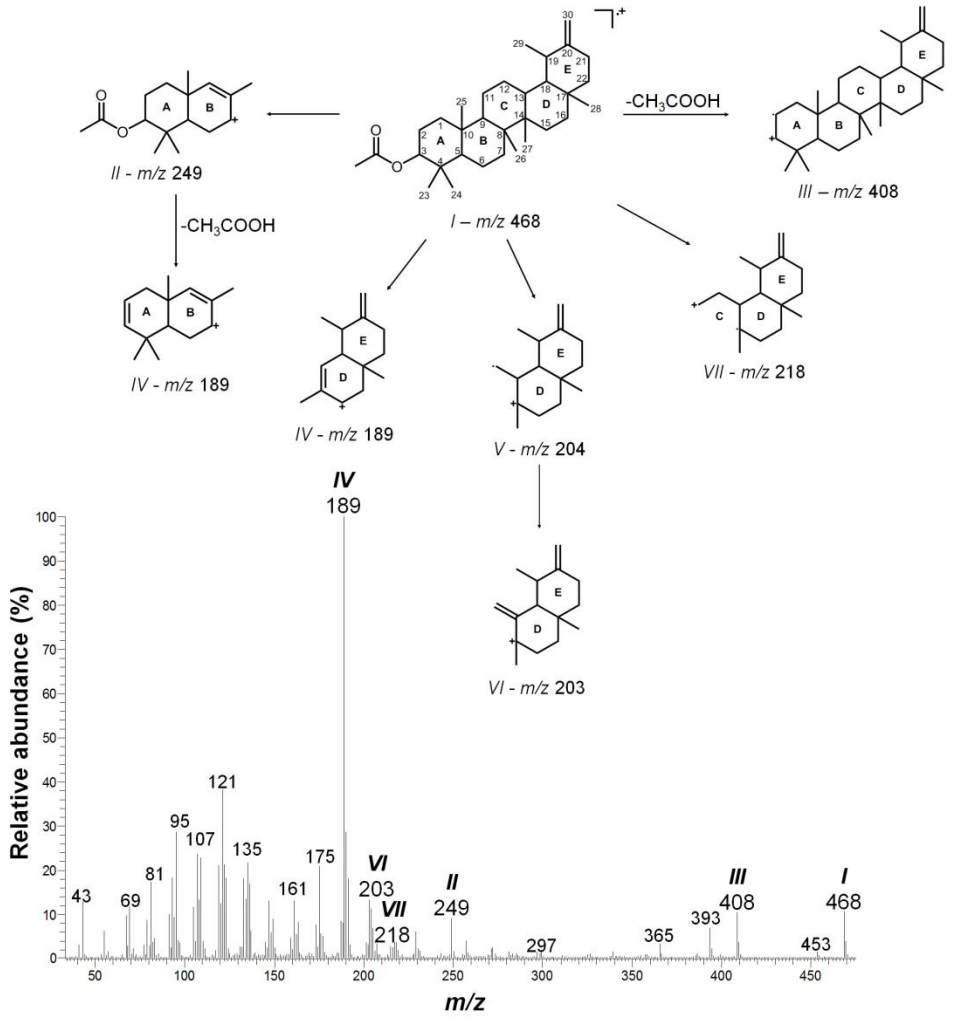


Figure 22: EI mass spectrum and the main fragmentation product ions of taraxasteryl acetate (**65**).

3.2.6.4 Triterpenyl fatty acid esters

The presence of triterpenyl fatty acid esters in *C. cardunculus* L. was reported in this work for the first time. These compounds were detected in the derivatized dichloromethane extracts of *C. cardunculus* L. var. *altilis* (DC), by using a short lenght GC column (15 m). Figure 23 illustrates the GC-MS chromatogram of the derivatized dicloromethane extract of stalks outer part from cultivated cardoon, obtained under these conditions.

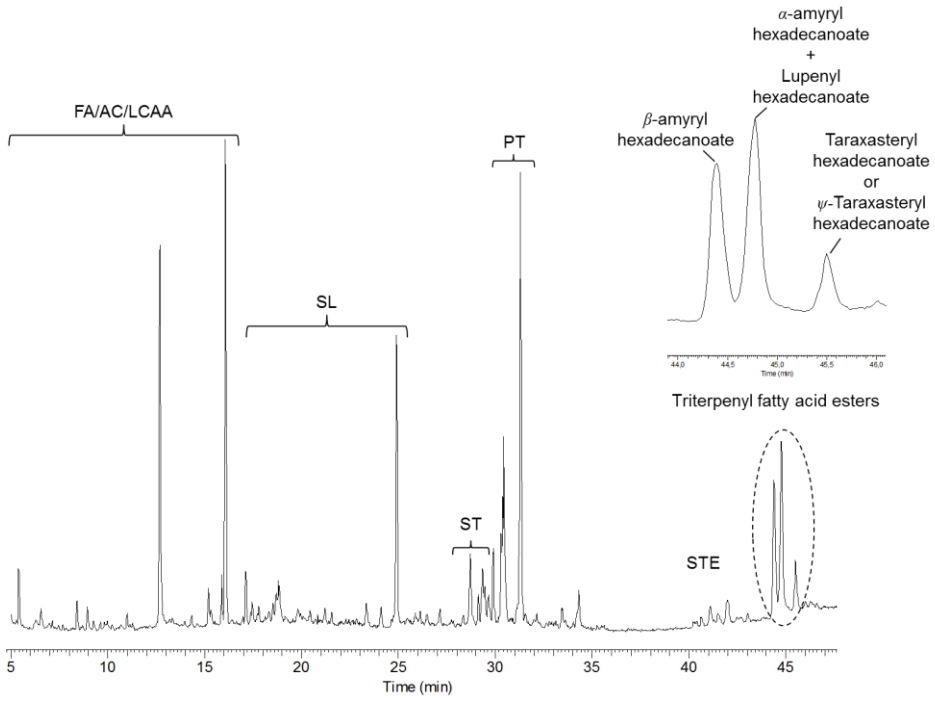


Figure 23: GC-MS chromatogram obtained with a short column (DB-1 15 m) of the derivatized dichloromethane extract of stalks outer part from *C. cardunculus* L. var. *altilis* (DC), before alkaline hydrolysis. Abbreviations: AC, aromatic compounds; FA, fatty acids; PT, pentacyclic triterpenes; ST, sterols; STE, steryl esters.

Four triterpenyl fatty acid esters were identified in the cultivated cardoon dichloromethane extracts, by comparing their EI mass data with those earlier described in the literature[60] (Table 10).

The EI-MS of the peak at RT = 44.39 min (Table 10) showed the molecular ion at *m/z* 664 and two characteristic product ions at *m/z* 409 and *m/z* 408, owing the loss of hexadecanoic acid, through either direct cleavage of the ester bond, or the 1,5 H-transfer rearrangement, respectively.[60] Furthermore, two characteristic product ions were observed, due to the EI fragmentation of *β*-amyryl ion, namely at *m/z* 218 (base peak) and at *m/z* 189.[32] Comparing EI-MS data and elution order with those reported in the literature data, this compound was tentatively assigned as *β*-amyryl hexadecanoate.[60]

Table 10: EI mass spectroscopic data of the triterpenyl fatty acid esters identified in the *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts.

RT (min)	Triterpenyl fatty acid esters	$[M]^{+\cdot}$ (*m/z*)	EI-MS product ions *m/z* (relative intensity %)	Id.
44.39	*β*-Amyryl hexadecanoate	664	664 $[M]^{+\cdot}$ (<1), 649 $[M\text{-}CH_3]^+$ (<1), 409 $[M\text{-}CH_3(CH_2)_{14}CO_2]^+$ (1), 408 $[M\text{-}CH_3(CH_2)_{14}CO_2H]^+$ (<1), 218 $[408\text{-}(A+B)]^+$ (100), 203 $[218\text{-}C28]^+$ (39), 189 $[218\text{-}(C11+C28)]^+$ (16)	[32,60]
45.50	Taraxastarane hexadecanoate derivative	664	664 $[M]^{+\cdot}$ (2), 649 $[M\text{-}CH_3]^+$ (<1), 409 $[M\text{-}CH_3(CH_2)_{14}CO_2]^+$ (3), 408 $[M\text{-}CH_3(CH_2)_{14}CO_2H]^+$ (7), 218 $[408\text{-}(A+B)]^+$ (7), 204 $[408\text{-}(A+B+C11)]^+$ (9), 203 $[218\text{-}C28]^+$ (13), 189 $[204\text{-}C28]^+$ (100)	[56,60]

A, loss of A-ring; B, loss of B-ring; C11, loss of C11; C28, loss of C28; Id., identification.

The chromatographic peak at RT = 44.78 min seems to result from the co-elution of two triterpenyl hexadecanoates. Their EI-MS spectra displayed the molecular ion at *m/z* 664 and characteristic product ions at *m/z* 649 $[M\text{-}CH_3]^+$ and 409 $[M\text{-}CH_3(CH_2)_{14}COO]^+$. However, two fragmentation peaks at *m/z* 189 and 218 with 100% relative abundance were observed; these are generally the base peaks of distinct triterpenic classes, namely lupanes and ursanes. Considering the pentacyclic triterpenols identified here, this peak could be attributed to lupenyl hexadecanoate (for *m/z* 189) and *α*-amyryl hexadecanoate (for *m/z* 218).[61] Despite the base peaks of taraxasterol, *ψ*-taraxasterol and lupeol EI mass spectra being found at *m/z* 189, it is not probable that taraxasteryl or *ψ*-taraxasteryl hexadecanoate (or a mixture) were co-eluting with *α*-amyryl hexadecanoate, since it is known that taraxasteryl hexadecanoate elutes after *α*-amyryl hexadecanoate.[62]

Figure 24 illustrates the main fragmentation product ions of the compound observed at RT = 45.50 min (Table 10). Its molecular ion was assigned at *m/z* 664. Moreover, characteristic product ions of a triterpenyl hexadecanoate were detected at *m/z* 649 ($[M\text{-}CH_3]^+$) and at *m/z* 408 ($[M\text{-}CH_3(CH_2)_{14}COOH]^+$). Other product ions were noticed at *m/z* 218, 204 and 189 (base peak), arising from the EI fragmentation of the taraxastanediene ion (*m/z* 408).[56,60] Furthermore, this compound eluted after *α*-amyryl hexadecanoate. Therefore, it is most likely that this peak corresponds to taraxasteryl hexadecanoate or *ψ*-taraxasteryl hexadecanoate, or a mixture of both.

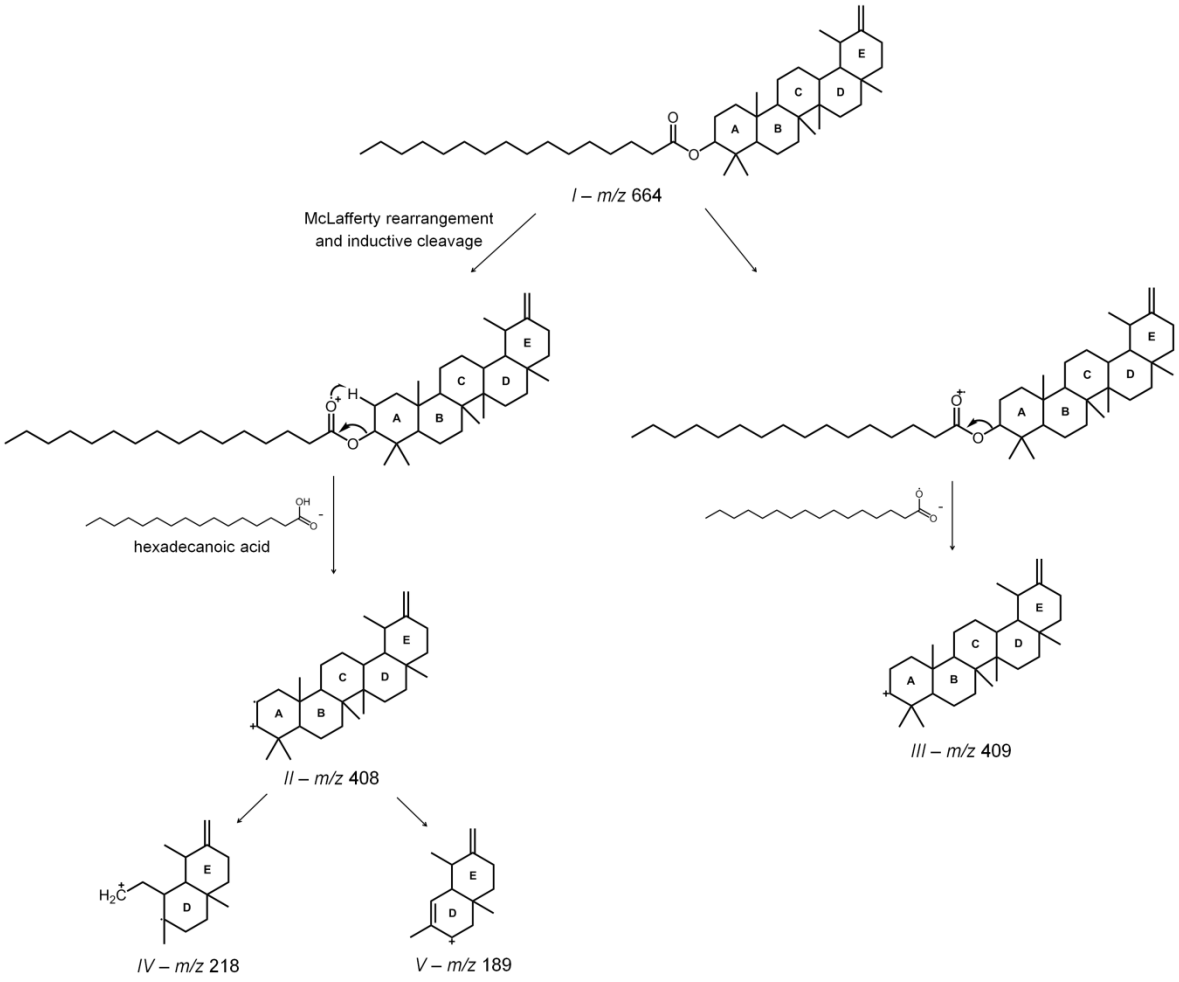


Figure 24: The main fragmentation product ions of taraxasteryl hexadecanoate.

3.2.7 Other compounds

Besides the above families, other compounds were identified in dichloromethane extracts of *C. cardunculus* L. var. *altilis* (DC), namely inositol (**17**), 2,3-dihydroxypropyl hexadecanoate (**35**), *E*-squalene (**39**) and α-tocopherol (**46**) (Figure 25). With the exception of *α*-tocopherol,[25] these compounds were detected here for the first time as *C. cardunculus* L. components.

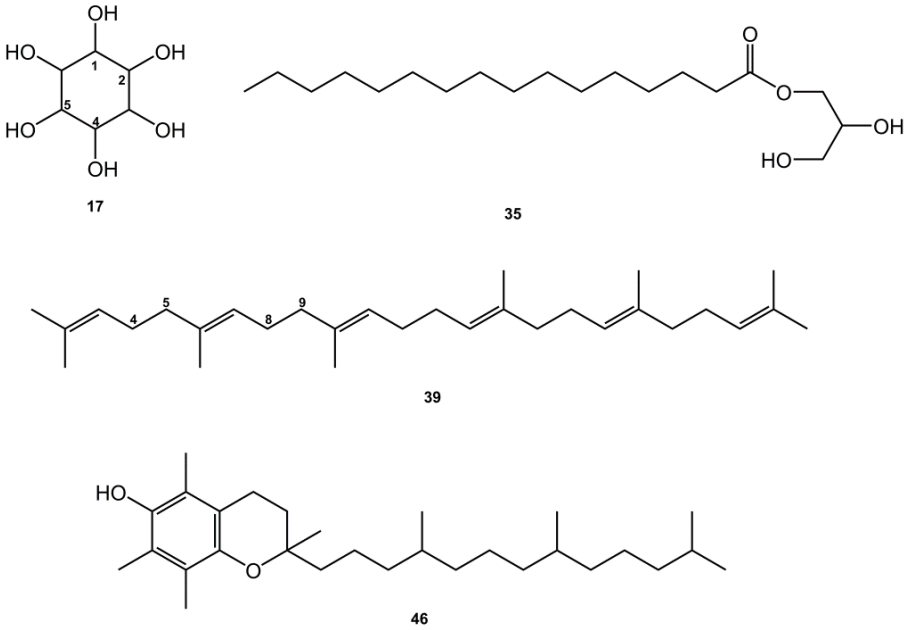


Figure 25: Structures of inositol (**17**), 2,3-dihydroxypropyl hexadecanoate (**35**), *E*-squalene (**39**) and *α*-tocopherol (**46**).

The identification of compounds **17**, **35**, **39** and **46** was performed by comparing the EI mass spectroscopic data with the literature (Table 11).

Table 11: EI mass spectroscopic data of the trimethylsilyl ether derivatives of compounds **17**, **35**, **39** and **46** identified in the *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts.

No.	Compound TMS derivative	$[M]^{+\cdot}$ (*m/z*)	EI-MS product ions *m/z* (relative intensity %)	Id.
17	Inositol	612	367 (7), 318 (65), 305 $[C_3H_2((CH_3)SiO)_3]^+$ (49), 265 (17), 217 (49), 191 (24), 147 (30), 129 (14), 117 (12), 103 (5), 75 $[(CH_3)_2SiOH]^+$ (55), 73 $[(CH_3)_3Si]^+$ (100), 45 (7)	[63]
35	2,3-Dihydroxypropyl hexadecanoate	474	459 $[M\text{-}CH_3]^+$ (6), 371 $[M\text{-}CH_2OSi(CH_3)_3]^+$ (100), 313 $[C_{16}H_{31}O_2Si(CH_3)_2]^+$ (7), 239 $[C_{16}H_{31}O]^+$ (8), 218 $[M\text{-}C_{16}H_{32}O_2]^+$ (5), 205 $[M\text{-}C_{17}O_2H_{33}]^+$ (17), 203 $[M\text{-}C_{16}H_{32}O_2\text{-}CH_3]^+$ (14), 191 $[((CH_3)_3Si)_2O_2CH]^+$ (3), 147 $[C_5H_{15}Si_2O]^+$ (35), 129 $[(CH_3)_3SiOC_3H_4]^+$ (16), 103 $[(CH_3)_3SiOCH_2]^+$ (9), 75 (19), 73 (39)	[64]
39	*E*-Squalene	410	410 (1), 341 (3), 299 (1), 231 (2), 191 (3), 175 (4), 161 (4), 149 (10), 137 $[C_{10}H_{17}]^+$ (16), 121 (16), 109 (9), 95 (20), 81 (70), 69 $[C_5H_9]^+$ (100), 41 (11)	Wiley NIST MS library
46	*α*-Tocopherol	502	502 $[M]^{+\cdot}$ (100), 277 $[C_6(CH_3)_3CH_2OSi(CH_3)_3OCCH_2CH_3]^+$ (8), 237 $[C_6(CH_3)_3CH_2OSi(CH_3)_3OH]^+$ (76), 221 (7), 193 (3), 75 (4), 73 (35)	[65]

The EI mass spectrum of the inositol (**17**) TMS derivative ($[M]^{+\cdot}$ at *m/z* 612) depicts the base peak at *m/z* 73 due to the formation of the $[(CH_3)_3Si]^+$ ion. The peak at *m/z* 75, regarding the $[(CH_3)_2SiOH]^+$ ion, also exhibited high relative abundance. The product ion at *m/z* 305 also revealed high relative abundance, resulting from cleavages on the C1-C2 and C4-C5 bonds.[63]

The EI mass spectrum of 2,3-dihydroxypropyl hexadecanoate (**35**) (monoglyceride) TMS diether ($[M]^{+\cdot}$ at *m/z* 612) displayed the base peak at *m/z* 371 arising from the loss of trimethylsilyloxymethyl radical (103 Da), which is characteristic of the EI fragmentation of monoglyceride TMS ethers. Other abundant product ion was found at *m/z* 205, generated by the loss of the hexadecanoyl radical.[45] Characteristic product ions of the EI fragmentation of monoglyceride TMS ethers were also observed at *m/z* 313, 239, 218 and 203, in what concerns to product ions originated by the loss of hexadecanoyl derivates.[45]

The EI mass spectrum of *E*-squalene (**39**) ($[M]^{+\cdot}$ at *m/z* 410) demonstrated the base peak at m/z 69, due to the cleavage of C4-C5 bond. A product ion at *m/z* 137 resulted from the C8-C9 bond cleavage.

Finally, the EI fragmentation of *α*-tocopherol (**46**) TMS ether yielded the molecular ion at *m/z* 502, also being the base peak. An intense peak was observed at *m/z* 237 whose product ion arised from the loss of the side aliphatic chain and part of the epoxide. Moreover, a low relative abundance peak was detected at *m/z* 277 due to the loss of side aliphatic chain.[65]

3.3 Quantitative analysis of the identified compounds in *C. cardunculus* L. var. *altilis* (DC) dichloromethane extracts

The contents of the several families of lipophilic compounds identified in stalks (outer and inner parts), capitula (receptacles, bracts and florets) and leaves from *C. cardunculus* L. var. *altilis* (DC) are given in Figure 26. Sesquiterpene lactones and pentacyclic triterpenes were the main families of cultivated cardoon lipophilic components, whereas fatty acids, sterols, long chain aliphatic alcohols and aromatic compounds were present in smaller abundances.

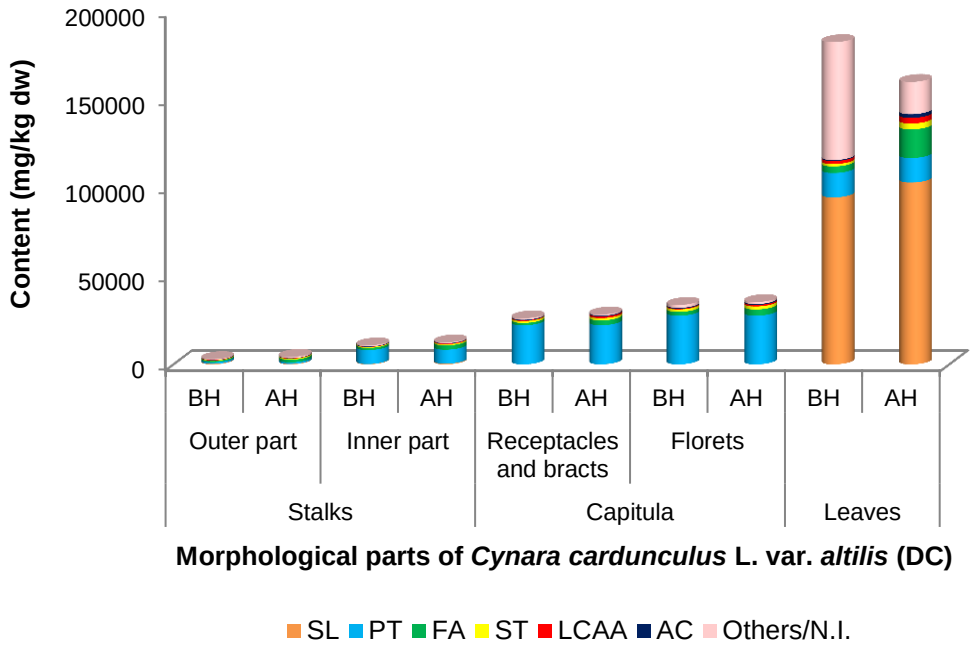


Figure 26: Major families of lipophilic compounds identified in the dichloromethane extracts of *C. cardunculus* L. var. *altilis* (DC), before (BH) and after (AH) alkaline hydrolysis. Abbreviations: AC, aromatic compounds; FA, fatty acids; LCAA, long chain aliphatic alcohols; N.I. – non-identified compounds; PT, pentacyclic triterpenes; SL, sesquiterpene lactones; ST, sterols.

The abundances of specific compounds were quite different between the different parts of *C. cardunculus* L. var. *altilis* (DC). The concentrations of the different families and individual compounds identified in the dichloromethane extracts of cultivated cardoon, before and after alkaline hydrolysis, are given in Table 12. The total content of identified compounds varied from 2985 mg/kg dw in stalks outer part to 182544 mg/kg dw in leaves. After alkaline hydrolysis, the total content of identified compounds decreased in leaves extracts, while it increased in the remaining cultivated cardoon extracts.

Table 12: Composition (mg of compound/kg dry weight) of dichloromethane extracts from different morphological parts of *C. cardunculus* L. var. *altilis* (DC) obtained before (BH) and after (AH) alkaline hydrolysis.

No.	Compound	Content (mg/kg dw)									
		Stalks				Capitula				Leaves	
		Outer part		Inner part		Receptacles and bracts		Florets			
		BH	AH	BH	AH	BH	AH	BH	AH	BH	AH
	Aromatic compounds	**114** ^{e}	**112** ^{e}	**223** ^{d}	**248** ^{d}	**373** ^{c}	**460** ^{b}	**487** ^{b}	**477** ^{b}	**516** ^{b}	**2084** ^{a}
1	Benzoic acid	16 ^{f}	13 ^{f}	32 ^{e}	31 ^{e}	53 ^{d}	63 ^{c,d}	63 ^{c,d}	66 ^{c}	254 ^{b}	282 ^{a}
3	Vanillin	17 ^{d}	15 ^{d}	32 ^{c}	31 ^{c}	53 ^{b}	63 ^{b}	–	–	–	279 ^{a}
4	Syringaldehyde	16 ^{f}	16 ^{f}	32 ^{e}	31 ^{e}	54 ^{d}	64 ^{c,d}	83 ^{b}	76 ^{b,c}	–	277 ^{a}
5	2,6-Dimethoxyhydroquinone	16 ^{d}	–	32 ^{c}	30 ^{c}	52 ^{b}	–	63 ^{a}	–	–	–
7	Vanillic acid	16 ^{e}	13 ^{e}	32 ^{d}	31 ^{d}	53 ^{c}	63 ^{b,c}	63 ^{b,c}	66 ^{b}	–	278 ^{a}
8	3-Vanillylpropanol	–	13 ^{d}	–	–	–	–	–	66 ^{c}	262 ^{b}	360 ^{a}
10	Vanillylpropanoic acid	–	–	–	–	–	–	64 ^{a}	67 ^{a}	–	–
11	Syringic acid	16 ^{d}	13 ^{d}	32 ^{c}	31 ^{c}	52 ^{b}	64 ^{a}	65 ^{a}	68 ^{a}	–	–
12	*Z*-Ferulic acid	–	13 ^{d}	–	31 ^{c}	–	63 ^{b}	–	–	–	281 ^{a}
19	*E*-Ferulic acid	16 ^{e}	16 ^{e}	32 ^{d,e}	34 ^{d}	56 ^{c}	79 ^{b}	–	69 ^{b,c}	–	328 ^{a}
48	Scopolin	–	–	–	–	–	–	87	–	–	–
	Fatty acids	**712** ^{g}	**1499** ^{f}	**1200** ^{e}	**2391** ^{d}	**1354** ^{e,f}	**2748** ^{c}	**2164** ^{d}	**3542** ^{b}	**3721** ^{b}	**16220** ^{a}
	Saturated	*340* ^{f}	*913* ^{d,e}	*559* ^{e,f}	*1244* ^{d}	*960* ^{d}	*1852* ^{c}	*1186* ^{d}	*2153* ^{c}	*2753* ^{b}	*8489* ^{a}
9	Tetradecanoic acid	16 ^{f}	18 ^{e,f}	27 ^{e}	31 ^{e}	101 ^{c}	63 ^{d}	54 ^{d}	64 ^{d}	210 ^{b}	306 ^{a}
13	Pentadecanoic acid	15 ^{e}	28 ^{d,e}	30 ^{d}	44 ^{c,d}	44 ^{c}	61 ^{b}	55 ^{b,c}	65 ^{b}	–	311 ^{a}
18	Hexadecanoic acid	174 ^{f}	604 ^{d,e}	266 ^{f}	765 ^{c,e}	490 ^{d,e}	1038 ^{b}	545 ^{d,e}	1009 ^{b,c}	638 ^{d,e}	4011 ^{a}
21	Heptadecanoic acid	15 ^{f}	23 ^{e,f}	26 ^{e,f}	32 ^{d,e,f}	44 ^{d,e}	88 ^{c}	52 ^{d}	79 ^{c}	203 ^{b}	290 ^{a}
27	Octadecanoic acid	31 ^{g}	67 ^{e,f}	47 ^{f}	92 ^{e,f}	90 ^{d,f}	179 ^{b,c}	98 ^{d,e}	162 ^{c}	222 ^{b}	765 ^{a}
28	Nonadecanoic acid	13 ^{d}	11 ^{d}	24 ^{c}	24 ^{c}	–	49 ^{b}	48 ^{b}	53 ^{b}	–	237 ^{a}
31	Eicosanoic acid	21 ^{f}	39 ^{e,f}	29 ^{e,f}	41 ^{e,f}	50 ^{d,e}	72 ^{d}	72 ^{d}	101 ^{c}	224 ^{b}	544 ^{a}
33	Heneicosanoic acid	13 ^{e}	18 ^{d,e}	25 ^{d}	25 ^{d}	41 ^{c}	51 ^{b}	52 ^{b}	56 ^{b}	–	238 ^{a}

No.	Compound	Content (mg/kg dw)									
		Stalks				Capitula				Leaves	
		Outer part		Inner part		Receptacles and bracts		Florets			
		BH	AH	BH	AH	BH	AH	BH	AH	BH	AH
36	Docosanoic acid	14 ^{f}	23 ^{e,f}	27 ^{e,f}	34 ^{e,f}	48 ^{d,e}	69 ^{c,d}	91 ^{c}	191 ^{b}	221 ^{b}	408 ^{a}
37	Tricosanoic acid	14 ^{f}	16 ^{f}	29 ^{e}	28 ^{e}	–	57 ^{d}	54 ^{d}	67 ^{c}	209 ^{b}	255 ^{a}
40	Tetracosanoic acid	15 ^{g}	21 ^{f,g}	28 ^{f,g}	34 ^{f}	51 ^{e}	72 ^{d}	65 ^{d,e}	104 ^{c}	251 ^{b}	347 ^{a}
42	Pentacosanoic acid	–	13 ^{c}	–	25 ^{b}	–	53 ^{a}	–	57 ^{a}	–	–
44	Hexacosanoic acid	–	17 ^{c}	–	38 ^{c}	–	–	–	71 ^{c}	345 ^{b}	430 ^{a}
51	Octacosanoic acid	–	17 ^{c}	–	31 ^{c}	–	–	–	76 ^{c}	230 ^{b}	347 ^{a}
	Unsaturated	*360* ^{e}	*573* ^{e}	*616* ^{e}	*1122* ^{c}	*394* ^{e}	*896* ^{c}	*917* ^{c}	*1389* ^{b}	*968* ^{c}	*7731* ^{a}
15	(9*Z*)-Hexadec-9-enoic acid	13 ^{e}	11 ^{e}	25 ^{d}	25 ^{d}	41 ^{c}	52 ^{b}	48 ^{b,c}	52 ^{b}	–	220 ^{a}
16	(9*E*)-Hexadec-9-enoic acid	13 ^{d} (16+17)	12 ^{d}	26 ^{c} (16+17)	25 ^{c}	–	51 ^{b}	49 ^{b} (16+17)	54 ^{b}	–	233 ^{a}
23	(9*Z*,12*Z*)-Octadeca-9,12-dienoic acid	181 ^{g,h}	349 ^{d,g,h}	374 ^{d,h}	749 ^{c}	163 ^{f,g}	420 ^{d,e}	511 ^{d}	887 ^{b}	233 ^{e,f,h}	1358 ^{a}
24	(9*Z*,12*Z*,15*Z*)-Octadeca-9,12,15-trienoic acid	47 ^{c,f}	64 ^{e,f}	108 ^{c,d,f}	169 ^{b,c,d}	81 ^{d,e}	190 ^{b,c}	175 ^{b}	214 ^{b}	260 ^{b}	4878 ^{a}
25	(9*Z*)-Octadec-9-enoic acid	88 ^{c}	121 ^{c}	53 ^{d}	112 ^{c}	63 ^{c}	121 ^{c}	71 ^{c}	106 ^{c}	232 ^{b}	683 ^{a}
26	(9*E*)-Octadec-9-enoic acid	17 ^{e}	17 ^{e}	30 ^{d,e}	41 ^{c,d,e}	45 ^{c,d}	62 ^{c,d}	62 ^{c,d}	76 ^{c}	243 ^{b}	359 ^{a}
	Hydroxyfatty acids	*13* ^{c}	*13* ^{c}	*25* ^{b}	*26* ^{b}	–	–	*61* ^{a}	–	–	–
2	2-Hydroxyheptanoic acid	13 ^{b}	13 ^{b}	25 ^{a}	26 ^{a}	–	–	–	–	–	–
6	2-Hydroxyundecanoic acid	–	–	–	–	–	–	61	–	–	–
	Long chain aliphatic alcohols	**181** ^{e,f,g}	**182** ^{e,f,g}	**146** ^{g}	**305** ^{d,f}	**332** ^{d,e}	**977** ^{c}	**385** ^{d}	**866** ^{c}	**1764** ^{b}	**3170** ^{a}
14	Hexadecan-1-ol	26 ^{e}	23 ^{e}	49 ^{d,e}	51 ^{d,e}	–	190 ^{c}	96 ^{d}	154 ^{c}	391 ^{b}	470 ^{a}
20	(9*Z*)-Octadec-9-en-1-ol	28 ^{b}	24 ^{b}	–	–	–	238 ^{a}	–	–	–	–
22	Octadecan-1-ol	25 ^{e}	22 ^{e}	49 ^{d,e}	49 ^{d,e}	81 ^{c,d}	147 ^{b}	96 ^{c}	143 ^{b}	–	456 ^{a}
30	Eicosan-1-ol	–	21 ^{c}	–	48 ^{b}	–	98 ^{a}	–	104 ^{a}	–	–
34	Docosan-1-ol	26 ^{e}	23 ^{e}	–	48 ^{d}	83 ^{c}	99 ^{b,c}	96 ^{b,c}	107 ^{b}	–	494 ^{a}
38	Tetracosan-1-ol	25 ^{f}	21 ^{f}	48 ^{e}	51 ^{e}	85 ^{d}	103 ^{c}	97 ^{c,d}	111 ^{c}	442 ^{b}	550 ^{a}
43	Hexacosan-1-ol	26 ^{d}	24 ^{d}	–	–	83 ^{c}	102 ^{c}	–	124 ^{c}	509 ^{b}	588 ^{a}
47	Octocosan-1-ol	25 ^{d}	24 ^{d}	–	58 ^{d}	–	–	–	123 ^{c}	422 ^{b}	612 ^{a}

No.	Compound	Content (mg/kg dw)									
		Stalks				Capitula				Leaves	
		Outer part		Inner part		Receptacles and bracts		Florets			
		BH	AH	BH	AH	BH	AH	BH	AH	BH	AH
	Sesquiterpene lactones	**301** ^{b}	**244** ^{b}	**184** ^{b}	**397** ^{b}	**16** ^{b}	**46** ^{b}	–	**9** ^{b}	**94571** ^{a}	**103028** ^{a}
29	Grosheimin	13 ^{b}	–	20 ^{b}	–	–	–	–	–	6248 ^{a}	819 ^{b}
32	Deacylcynaropicrin	9 ^{b}	244 ^{b}	10 ^{b}	397 ^{b}	–	46 ^{b}	–	9 ^{b}	841 ^{b}	102208 ^{a}
41	Cynaropicrin	279 ^{b}	–	154 ^{b}	–	16 ^{b}	–	–	–	87482 ^{a}	–
	Sterols	**320** ^{e}	**591** ^{e}	**470** ^{e}	**694** ^{d,e}	**1102** ^{c,d}	**1295** ^{b,c}	**1441** ^{b,c}	**1727** ^{b}	**1328** ^{b,c}	**3346** ^{a}
45	Cholesterol	10 ^{c}	13 ^{c}	–	–	–	–	–	63 ^{b}	–	276 ^{a}
49	24-Methylenecholesterol	–	7 ^{c}	–	17 ^{c}	–	–	54 ^{b}	65 ^{b}	–	193 ^{a}
50	Campesterol	26 ^{f}	46 ^{e,f}	41 ^{e,f}	56 ^{e,f}	81 ^{c,e}	117 ^{c,d}	150 ^{b}	190 ^{b}	151 ^{b,d}	248 ^{a}
52	Stigmasterol	129 ^{e}	221 ^{d}	252 ^{d}	324 ^{b,d}	523 ^{b,c}	542 ^{b,c}	459 ^{b,c}	461 ^{b,c}	338 ^{b,d}	588 ^{a,c}
54	*β*-Sitosterol	131 ^{d}	275 ^{c,d}	140 ^{d}	257 ^{c,d}	392 ^{b,d}	637 ^{b}	498 ^{b,c}	708 ^{b}	639 ^{b}	1716 ^{a}
55	*β*-Sitostanol	25 ^{c}	30 ^{b,c}	38 ^{b,c}	40 ^{b}	106 ^{a}	–	–	–	–	–
56	Δ^{5}-Avenasterol	–	–	–	–	–	–	280 ^{b,c}	239 ^{b,d}	200 ^{d}	325 ^{a,c}
	Pentacyclic triterpenes	**933** ^{d}	**937** ^{d}	**7934** ^{c}	**7941** ^{c}	**22107** ^{a}	**22114** ^{a}	**27556** ^{a}	**27558** ^{a}	**13851** ^{b}	**13857** ^{b}
53	*β*-Amyrin	33 ^{f}	100 ^{f}	81 ^{f}	1090 ^{e}	2348 ^{c}	3520 ^{b}	1197 ^{e}	6041 ^{a}	290 ^{f}	1828 ^{d}
57	*α*-Amyrin	41 ^{f}	81 ^{e,f}	63 ^{d,f}	1144 ^{c}	1886 ^{b}	2796 ^{a}	757 ^{c,d,e}	3030 ^{a}	80 ^{e,f}	300 ^{e,f}
58	Lupeol	62 ^{e,g}	136 ^{e,g}	48 ^{d,g}	774 ^{d,f,g}	1397 ^{c,f}	2265 ^{b,a}	372 ^{d,e}	1892 ^{b,c}	524 ^{e,g}	2853 ^{a}
59	*β*-Amyrin acetate	63 ^{c}	traces	1003 ^{b}	traces	1164 ^{b}	traces	4844 ^{a}	traces	1538 ^{b}	traces
60	*α*-Amyrin acetate	40 ^{c}	traces	1081 ^{b}	traces	910 ^{b}	traces	2271 ^{a}	traces	214 ^{c}	traces
61	Lupenyl acetate	74 ^{d}	traces	727 ^{c,d}	traces	868 ^{b,c}	traces	1521 ^{b}	traces	2330 ^{a}	traces
62	*ψ*-Taraxasterol	46 ^{e}	105 ^{e}	143 ^{e}	1331 ^{d}	2530 ^{c}	3962 ^{b}	1351 ^{d}	6014 ^{a}	705 ^{d,e}	2925 ^{c}
63	Taraxasterol	178 ^{d}	515 ^{d}	146 ^{d}	3601 ^{c}	5306 ^{b,c}	9571 ^{a}	1638 ^{d}	10581 ^{a}	1144 ^{d}	5950 ^{b}
64	*ψ* -Taraxasteryl acetate	59 ^{d}	traces	1188 ^{c}	traces	1432 ^{c,b}	traces	4663 ^{a}	traces	2221 ^{b}	traces
65	Taraxasteryl acetate	336 ^{c}	traces	3455 ^{b}	traces	4265 ^{b}	traces	8942 ^{a}	traces	4806 ^{b}	traces

No.	Compound	Content (mg/kg dw)									
		Stalks				Capitula				Leaves	
		Outer part		Inner part		Receptacles and bracts		Florets			
		BH	AH	BH	AH	BH	AH	BH	AH	BH	AH
	Others	**4** ^{c}	**–**	**11** ^{c}	**2** ^{c}	**8** ^{c}	**–**	**12** ^{c}	**–**	**1221** ^{a}	**528** ^{b}
17	Inositol	(16+17)	–	(16+17)	–	–	–	(16+17)	–	34	–
35	2,3-Dihydroxypropyl hexadecanoate	4 ^{c,d}	–	11 ^{b}	2 ^{d}	8 ^{b,c}	–	12 ^{b}	–	23 ^{a}	–
39	*E*-Squalene	–	–	–	–	–	–	–	–	158 ^{a}	130 ^{a}
46	*α*-Tocopherol	–	–	–	–	–	–	–	–	1007 ^{a}	399 ^{b}
	Non-identified compounds	**419** ^{c}	**714** ^{c}	**609** ^{c}	**515** ^{c}	**825** ^{c}	**643** ^{c}	**1648** ^{c}	**1152** ^{c}	**65572** ^{a}	**17773** ^{b}
	Total detected compounds	**2985** ^{d}	**4280** ^{c,d}	**10776** ^{d,e}	**12492** ^{b,d,e}	**26116** ^{b,c,e}	**28283** ^{b,e}	**33692** ^{b,e}	**35331** ^{b}	**182544** ^{a}	**160007** ^{a}

Results represent the means estimated from the analysis of two extracts injected in duplicate (standard deviation less than 5%). Means with different letters within the same row are significantly different ($p < 0.05$). Numbers in parenthesis correspond to compounds whose peaks were overlapped.

3.3.1 Sesquiterpene lactones

Leaves demonstrated the highest sesquiterpene lactones content (Table 12), accounting for 94571 mg/kg dw which represented 52% of the total detected compounds content. The remaining morphological parts of cultivated cardoon contained considerably lower sesquiterpene lactone contents, ranging from 16 mg/kg dw in receptacles and bracts, to 301 mg/kg dw in stalks outer part. These compounds were absent in capitula florets.

Cultivated cardoon leaves exhibited the highest amount of cynaropicrin (**41**), contributing for 87482 mg/kg dw (Table 12). Cynaropicrin was present at much lower contents in stalks (154-279 mg/kg dw), and in receptacles and bracts (16 mg/kg dw). Previously, Schneider and Thiele[66] demonstrated that the artichoke bitter principle, quantified as cynaropicrin, was mainly retained in leaf laminas (0.5-4.5% dw), and absent in the roots and developed capitula. Menin, *et al.*[67] also noticed much higher cynaropicrin content in mature and old leaves than in receptacles of artichoke (23- and 33-fold, respectively). It is worthy to refer that cultivated cardoon cynaropicrin content, shown in this study, was 3.9-fold higher relatively to the commercial artichoke leaves extracts, available in the European market.[6]

Grosheimin (**29**) and deacylcynaropicrin (**32**) represented respectively 4-11% and 1-5% of the total sesquiterpene lactones contents (Table 12). Grosheimin was detected in considerable quantities in the leaves (6248 mg/kg dw), and at minor amounts in both parts of stalks. Deacylcynaropicrin was also concentrated in cultivated cardoon leaves (841 mg/kg dw).

After alkaline hydrolysis, slightly higher amounts of sesquiterpene lactones were observed (Table 12). This may be related to the deacylcynaropicrin concentration in the hydrolyzed extracts, which was much higher than the sum of cynaropicrin and deacylcynaropicrin contents in the dichloromethane extracts. Deacylcynaropicrin or cynaropicrin may be esterified to high molecular weight compounds. On the contrary, the grosheimin abundance decreased 7.6-fold after alkaline hydrolysis (Table 12), probably being retained in the aqueous phase.

3.3.2 Pentacyclic triterpenes

Capitula exhibited the highest contents of pentacyclic triterpenes, accounting for 27556 mg/kg dw in florets and 22107 mg/kg dw in receptacles and bracts (Table 12). These compounds were also found in the other morphological parts of cultivated caroon, varying between 933 mg/kg dw in stalks outer part and 13851 mg/kg dw in leaves. This family of lipophilic compounds ranged from 8% in the leaves to 84% in the receptacles and bracts, regarding the total amount of detected compounds.

Taraxastanes (**62**-**65**) revealed the highest abundance, among pentacyclic triterpenes, representing 60-66% of the total pentacyclic triterpene content. Florets presented the highest

amount of taraxasteryl acetate (**65**) (8942 mg/kg dw), while receptacles and bracts showed the highest taraxasterol (**63**) content (5306 mg/kg dw). Oleananes, such as *β*-amyrin (**53**) and its acetate derivative (**59**), were noted at considerable amounts (10-22% of the total pentacyclic triterpenic content), followed by lupanes, like lupeol (**58**) and lupenyl acetate (**61**) (7-21% of total pentacyclic triterpene contents), and ursanes, such as *α*-amyrin (**57**) and its acetate derivative (**60**) (2-14% of the total pentacyclic triterpenic content).

The content of pentacyclic triterpene alcohols slightly increased in all hydrolyzed extracts of cultivated cardoon. This variation can be related to alkaline hydrolysis of either the corresponding acetates or of the triterpenyl fatty acid esters.

3.3.3 Fatty acids

Fatty acids (Table 12) were mainly concentrated in leaves (3721 mg/kg dw). In the remaining parts of cultivated cardoon, the contents of these compounds varied from 712 mg/kg dw in stalks outer part to 2164 mg/kg dw in florets. Moreover, this group represented 12-24% of total amount of detected compounds, in the inner and outer parts of stalks, respectively. Both saturated and unsaturated fatty acids were identified in *C. cardunculus* L. var. *altilis* (DC) at appreciable amounts, accounting respectively for 44-70% and 25-49% of the total fatty acids content.

Saturated fatty acids were predominantly observed in leaves (2753 mg/kg dw), while unsaturated fatty acids were mainly found in leaves and florets (968 and 917 mg/kg dw, respectively). Hexadecanoic acid (**18**) and 9*Z*,12*Z*-octadeca-9,12-dienoic acid (**23**) were respectively the predominant saturated and unsaturated fatty acid (638 mg/kg dw in leaves and 511 mg/kg dw in florets, respectively). After alkaline hydrolysis, fatty acids contents increased in all extracts, particularly in what regards to leaves (4-fold). 9*Z*,12*Z*,15*Z*-Octadeca-9,12,15-trienoic acid (**24**) was the major fatty acid present in hydrolyzed extracts of leaves (4878 mg/kg dw), followed by hexadecanoic acid (4011 mg/kg dw). Furthermore, higher contents of odd-numbered saturated fatty acids were found in the hydrolyzed extracts.

3.3.4 Long chain aliphatic alcohols

Long chain aliphatic alcohols (Table 12) were present at quite low amounts, varying from 146 mg/kg dw in stalks inner part to 1764 mg/kg dw in leaves. These compounds ranged from 1% in leaves to 6% in stalks outer part, relatively to the total detected compounds content. Hexacosan-1-ol (**14**) was the major compound present in leaves (509 mg/kg dw), before alkaline hydrolysis. After alkaline hydrolysis, long chain aliphatic alcohols contents increased in cultivated cardoon extracts (with the exception of stalks outer part), principally in those of receptacles and bracts (2.9-fold). Moreover, eicosan-1-ol (**30**) was only detected after alkaline hydrolysis in all morphological parts, with the exception of leaves.

3.3.5 Sterols

Sterols were detected in all morphological parts of cultivated cardoon at low abundance (Table 12), ranging from 320 mg/kg dw in stalks outer part to 1441 mg/kg dw in florets. This family of compounds represented between 1% in leaves and 11% in stalks outer part, regarding the total detected compounds content. Stigmastanes (**52**, **54**-**56**) were the main sterol class (86-93% of the total sterols content). *β*-Sitosterol (**54**) was mainly found in leaves (639 mg/kg dw), florets (498 mg/kg dw) and in receptacles and bracts (392 mg/kg dw). Stigmasterol (**52**) was also found in the same content order. Campesterol (**50**) was found at lower content (26-151 mg/kg dw). 24-methylenecholesterol (**49**) and cholesterol (**45**) were observed in very low contents in florets and in stalks outer part, respectively.

After alkaline hydrolysis, the sterol content increased in leaves hydrolyzed extracts (2.5-fold). This is in agreement with the presence of esterified sterols structures in the GC-MS chromatograms obtained with a short length column. It is also worth mentioning that the sterol content variation with hydrolysis is not related to the presence of steryl glycosides.[68]

3.3.6 Aromatic and other compounds

Aromatic compounds (Table 12) varied from 114 mg/kg dw in stalks outer part to 516 mg/kg dw in leaves. This group corresponded to 0.3% in leaves and 4% in stalks outer part, relatively to total detected compounds content. 3-Vanillylpropanol (**8**) was mainly found in leaves (262 mg/kg dw). Scopolin (**48**) was only detected in capitula florets (87 mg/kg dw).

Hydrolyzed extracts of receptacles and bracts, and leaves demonstrated higher contents of aromatic compounds (1.2- and 4-fold, respectively). *Z*-ferulic acid (**12**) was only noted in hydrolyzed extracts, with the exception of florets, which can be related with the fact that hydroxycinnamic acids tend to be esterified with lignin- and polysaccharides-like components.[27,68,69]

Finally, 2,3-dihydroxypropyl hexadecanoate and *E*-squalene were also found in *C. cardunculus* L. var. *altilis* (DC) in reduced amounts; *α*-tocopherol was only detected in leaves (1007 mg/kg dw).

4. Conclusions

This chapter reflects one of the first detailed studies about the lipophilic composition of stalks (outer and inner parts), capitula (receptacles, bracts and florets) and leaves of *C. cardunculus* L. var. *altilis* (DC) from the south of Portugal. Sixty five compounds were identified in the dichloromethane extracts of cultivated cardoon, before and after alkaline hydrolysis, by GC-MS analysis.

Sesquiterpene lactones were the main lipophilic compounds of cultivated cardoon leaves, accounting for ≈94.5 g/kg dw. Cynaropicrin (**41**) was the most abundant sesquiterpene lactone in cultivated cardoon leaves, accounting for ≈87.4 g/kg dw. Recently, Eljournadi, *et al*.[70] quantified cynaropicrin in artichoke tissues, at different developmental stages. They observed that cynaropicrin was mainly retained in leaves (9.6 g/kg dw), at the earliest stage of plant development (6 weeks old). They also reported that wild cardoon leaves contained approximately 7 g/kg dw of cynaropicrin. In this manner, the studied cultivated cardoon leaves revealed much higher cynaropicrin content, regarding artichoke (9.1-fold) and wild cardoon (≈12.5-fold).[70] According to Eljournadi, *et al.,*[70] the cynaropicrin content decreased 87.5% in the receptacles during its development, until it reached the commercial stage. Cynaropicrin content of the studied cultivated cardoon receptacles and bracts, collected in the florescence stage, was 3.1-fold lower relatively to artichoke edible capitula (0.05 g/kg dw).[70] Moreover, deacylcynaropicrin (**32**) was identified for the first time in the *C. cardunculus* L. var. *altilis* (DC) lipophilic fraction. Meanwhile, Rial, *et al.*[71] isolated grosheimin and cynaropicrin from wild cardoon leaves.

Pentacyclic triterpenes were the main lipophilic components of stalks and capitula, accounting for ≈27.5 g/kg dw of florets. Taraxasteryl acetate (**65**) was the main compound of this family (≈8.9 g/kg dw in florets), followed by taraxasterol (**63**) (≈5.3 g/kg dw in receptacles and bracts), particularly concentrated in the capitula parts. Receptacles, bracts and florets of cultivated cardoon showed considerable taraxasterol and *ψ*-taraxasterol contents (≈7.8 and ≈2.9 g/kg dw, respectively), being comparable to other Asteraceae plants, for instance flowers of *Calendula officinalis* (≈2.1-4.1 g/kg dw).[72] Lupenyl acetate (**61**) was detected here for the first time in *C. cardunculus* L. species. Besides this pentacyclic triterpene, two more were identified in this work, for the first time, as cultivated cardoon components, namely *β*-amyrin acetate (**59**), *α*-amyrin acetate (**60**) and *ψ*-taraxasteryl acetate (**64**).

Low contents of other families of lipophilic compounds were also noted in the morphological parts of cultivated cardoon, namely fatty acids (≈3.7 g/kg dw in leaves), long chain aliphatic alcohols (≈1.7 g/kg dw in leaves), sterols (≈1.4 g/kg dw in florets) and aromatic compounds (≈0.5 g/kg dw in leaves). Meanwhile, Farag, *et al.*[73] identified 9*Z*,12*Z*,15*Z*-octadeca-9,12,15-trienoic acid and two isomers of hydroxyoctadecadienoic and hydroxyoctadecatrienoic acids in artichoke leaves.

Since sesquiterpene lactones and pentacyclic triterpenes, namely cynaropicrin and taraxasteryl acetate, are known, respectively, for tumor antiproliferative[30] and anti-inflammatory[24] properties, this study is an important contribution for *C. cardunculus* L. var. *altilis* (DC) valorization as a promising source of bioactive compounds. Finally, the extraction of high-value bioactive compounds can be perfectly integrated within the potential large-scale

applications of cultivated cardoon, as raw material for energy conversion and for pulp fibers production.

5. References

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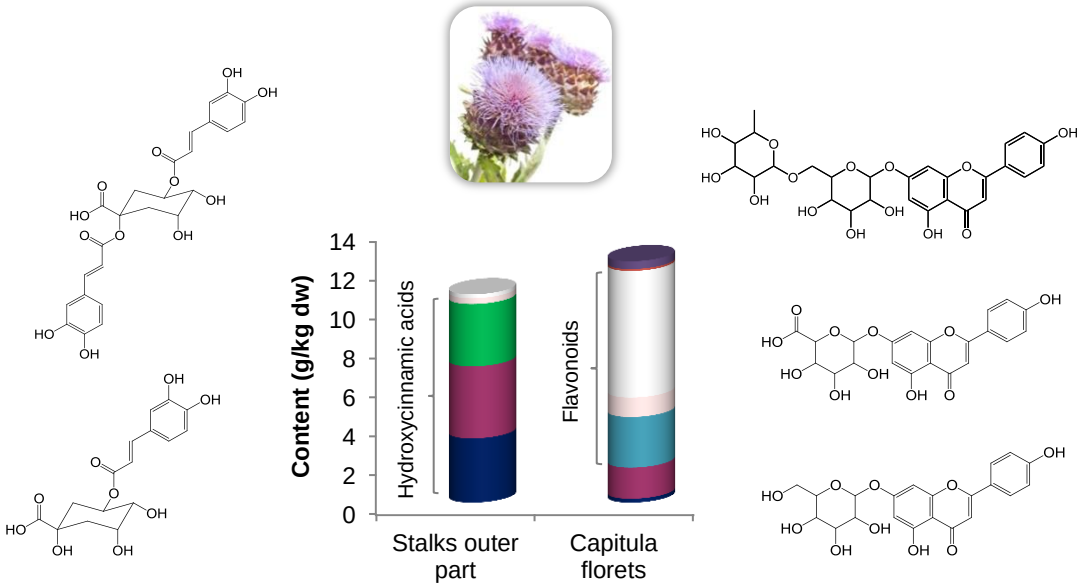
Chapter IV

Chemical characterization of *Cynara cardunculus* L. var. *altilis* (DC) phenolic-rich fraction



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Abstract

The phenolic composition of *Cynara cardunculus* L. var. *altilis* (DC) from the south of Portugal was determined by analyzing the methanol/water/acetic acid (49.5:49.5:1) extracts of the different morphological parts of the plant, through high temperature-ultra high pressure liquid chromatography-tandem mass spectrometry. Among the 28 phenolic compounds identified, eriodictyol hexoside was described for the first time in *C. cardunculus* L., and 1,4-di-*O*-caffeoylquinic acid, naringenin 7-*O*-glucoside, naringenin rutinoside, naringenin, luteolin acetylhexoside and apigenin acetylhexoside as cultivated cardoon components. Phenolic compounds were essentially retained in the capitula florets, accounting for ≈12.6 g/kg dw. Both outer and inner parts of stalks showed the highest hydroxycinnamic acids content (≈10.3 g/kg dw and ≈9.9 g/kg dw, respectively), and capitula florets demonstrated the major flavonoids concentration (≈10.3 g/kg dw). Receptacles and bracts also evidenced considerably high hydroxycinnamic acids contents, while flavonoids were the main phenolic compounds found in leaves. The 5-*O*-caffeoylquinic and 1,5-di-*O*-caffeoylquinic acids represented the main mono and dicaffeoylquinic acids, whereas apigenin glucuronide, 7-*O*-glucoside and 7-*O*-rutinoside were the major flavonoids of cultivated cardoon.

1. Introduction

Cynara cardunculus L. (Asteraceae) is a Mediterranean species that includes the ancestor wild cardoon (var. *sylvestris* (Lamk) Fiori) and its two cultivated forms, named as globe artichoke (var. *scolymus* (L.) Fiori) and cultivated cardoon (var. *altilis* (DC)).[1] Wild cardoon grows naturally in the Iberian Peninsula, north Africa, west Turkey and Macaronesia (Madeira and Canary Islands), while artichoke is produced all over the world, with special relevance in Italy, Spain, France and Turkey, owing to the edible immature capitula.[2] The production of cultivated cardoon is located in Spain, Italy and France,[3] once the blanched fleshy stems and leaf petioles are much enjoyed in the regional plates. Cultivated cardoon is very resistant to the hot summers and dry soils of the Mediterranean countries, yielding biomass productivity which ranges from 15.2 to 24.2 t/ha/year.[4] Given this productivity and the high contents in cellulose and hemicelluloses, this plant has been also regarded as a multipurpose crop for several industrial applications, namely pulp fibers production and as solid biofuel.[4]

Traditionally, water extracts of wild cardoon capitula are utilized in the Iberian Peninsula for manufacturing of protected designation of origin cheeses, once aspartic proteinases (cardosins A and B) are implicated in the milk clotting.[5] Since the ancient times, infusions of artichoke and cardoon leaves have been used in the folk medicine, owing to their hepatoprotective,[6] choleretic and anticholestatic actions.[7] These physiological effects have been mostly attributed to phenolic compounds, in particular to caffeoylquinic acids and flavones, such as 5-*O*-caffeoylquinic and 1,3-di-*O*-caffeoylquinic acids,[6] luteolin and luteolin 7-*O*-glucoside.[7] Moreover, several studies also proved other biological activities of artichoke and cardoon extracts, containing phenolic compounds, namely antioxidant,[8–10] antimicrobial,[8,11,12] anti-inflammatory,[13] and antitumor[9,14] properties.

Several studies have been carried out on the phenolic composition of both varieties of cardoon.[8,15–18] Nevertheless, there is still missing a systematic detailed chemical characterization of the phenolic compounds, regarding all morphological parts of cultivated cardoon. The knowledge about the chemical composition of cultivated cardoon is essential to increase its economic value, and consequently to host its production in the Mediterranean countries. After the chemical characterization of the lipophilic fraction,[19] the objective of this work is to determine the phenolic composition of stalks (outer and inner parts), capitula (receptacles, bracts and florets) and leaves of *C. cardunculus* L. var. *altilis* (DC) from Baixo Alentejo region (south of Portugal), by analyzing the chemical composition of their methanol/water/acetic acid (49.5:49.5:1) extracts, through high temperature-ultra high-performance liquid chromatography (HT-UHPLC) coupled to diode array detector (DAD) and tandem mass spectrometry analysis (MS^n). Moreover, the total phenolic contents of cultivated cardoon extracts are also determined by the Folin Ciocalteu assay.

2. Materials and Methods

2.1 Chemicals

Dichloromethane (p.a., ≥99% purity), methanol (p.a., ≥99.8% purity), HPLC-grade methanol, water and acetonitrile were supplied by Fisher Scientific (Pittsburgh, Pennsylvania, USA). Glacial acetic acid (p.a., ≥99.7% purity) was purchased from Panreac (Castellar del Vallès, Spain). Sodium carbonate (p.a., ≥99.9% purity) was obtained from Pronalab (Lisbon, Portugal). Gallic acid (≥97.5% purity), Folin Ciocalteu's phenol reagent (2N), 3- and 5-*O*-caffeoylquinic acids (≥98% and ≥95% purity, respectively) and luteolin 7-*O*-glucoside (≥98% purity) were supplied from Sigma Chemicals Co. (Madrid, Spain). Formic acid (≥98% purity) was purchased from Fluka Chemie (Madrid, Spain). 1,5-Di-*O*-caffeoylquinic acid (≥98 purity) was obtained from Biopurify (Chengdu Biopurify Phytochemicals Ltd., Chengdu, China). Scopoletin (≥95% purity), naringenin 7-*O*-glucoside (≥99% purity), apigenin 7-*O*-glucoside (≥99% purity) and apigenin 7-*O*-rutinoside (≥98.5% purity) were supplied by Extrasynthese (Lyon, France). Solvents were filtered using a Solvent Filtration Apparatus 58061 from Supelco (Bellefonte, Pennsylvania, USA).

2.2 Samples preparation

C. cardunculus L. var. *altilis* (DC) was collected at the flowering stage, in June 2010, at the Experimental Center of the Agriculture School from Instituto Politécnico de Beja, south of Portugal. Plants were separated in stalks, capitula and leaves, and preserved at -20 ºC until analysis. Before extraction, samples were freeze-dried. Then, stalks were separated in outer and inner parts and capitula in receptacles, bracts and florets.

2.3 Extraction

All plant fractions were grounded to a granulometry of 40–60 mesh prior to extraction. Each sample (6 g dw) was submitted to Soxhlet extraction with dichloromethane (150 mL) for 7 h to remove the lipophilic fraction, as explained elsewhere.[19] Then, the extraction of phenolic compounds was carried out as previously cited.[20] Briefly, 2 g of the dry leftover solid residue was extracted with 200 mL of methanol/water/acetic acid (49.5:49.5:1) under constant stirring, protected from the light, for 24 h at room temperature. The suspensions were then filtered; methanol and acetic acid were removed by low pressure evaporation (37-40 ºC) and water by freeze-drying. The dried extracts were weighted, and the extraction yield determined as the percentage of dry biomass material (w/w, %). The extracts were kept at room temperature protected from light until analysis. Two extracts were prepared for each morphological part of cultivated cardoon.

2.4 Total phenolic content

The total phenolic content (TPC) was determined using the Folin Ciocalteu assay, according to the procedure employed by Dewanto, *et al.*[21] Dry extracts were firstly dissolved in methanol/water (1:1), with concentrations ranging from 2 to 3 mg/mL. Then, 0.125 mL of each extract was mixed with 0.625 mL of Folin Ciocalteu's reagent, previously diluted with water (1:5, v/v). After 6 min, 1.25 mL of 7% sodium carbonate aqueous solution and 1 mL of water were added. The reaction mixtures were allowed to stand in the dark for 60 min at room temperature. The absorbance was then read against a blank at 760 nm, using a UV/Vis V-530 spectrophotometer (Jasco, Tokyo, Japan). The TPC was calculated as gallic acid equivalents (GAE) from the gallic acid standard curve (40-180 μg/mL), and expressed as mg GAE/g dry extract and as mg GAE/kg dw. The analyses were carried out in triplicate for each extract and the average value from the two extracts was calculated for each morphological part.

2.5 HT-UHPLC-UV analysis

The extracts of cultivated cardoon were previously dissolved in HPLC grade methanol/water mixture (1:1) at 10 mg/mL, and then filtered through a 0.2 μm polytetrafluoroethylene syringe filter. Methanol/water extracts (5 μL) were injected in the HPLC system equipped with an Accela 600 LC pump, an Accela autosampler (set at 16 ºC) and an Accela 80 Hz photo DAD. The separation of compounds was carried out on a Hypersil Gold RP C18 column (100 x 2.1 mm; 1.9 μm particle size) supplied by Thermo Fisher Scientific (San Jose, California, USA), maintained at 45 ºC. The mobile phase was constituted by (A) water:acetonitrile (99:1, v/v) and (B) acetonitrile, both with 0.1% of formic acid. A gradient elution program was applied at a flow rate of 0.48 mL/min during a 38 min period. The percentage of B was kept at 3% from 0 to 5 min, and then reached 12.0% from 5 to 14 min, 12.8% from 14 to 14.50 min, 13.3% from 14.5 to 18.5 min, 14.5% from 18.5 to 24 min, 30% from 24 to 34 min and, finally 100% from 34 to 38 min. Before the next run, the percentage of B decreased from 100% to 3% during 4.5 min and it was maintained at 3% for 4.5 min. The chromatograms were recorded at 280, 330 and 350 nm and UV-Vis spectra recorded from 210 to 600 nm.

2.6 HT-UHPLC-MS^n analysis

The HT-UHPLC-MS^n analysis was performed following previously optimized conditions.[20] The HPLC system was coupled to a LCQ Fleet ion trap mass spectrometer (ThermoFinnigan, San Jose, California, USA), equipped with an electrospray ionization (ESI) source. The ESI-MS was operated under the negative ionization mode with a spray voltage of 5 kV and capillary temperature of 360 ºC. The flow rate of nitrogen sheath and auxiliary gas

were 50 and 10 (arbitrary units), respectively. The capillary and tube lens voltages were set at -28 V and -115 V, respectively. CID–MS^n experiments were performed on mass-selected precursor ions in the range of *m/z* 100–2000. The isolation width of precursor ions was 1.0 mass units. The scan time was equal to 100 ms and the collision energy was 35%, using helium as collision gas. The data acquisition was carried out by using Xcalibur® data system (ThermoFinnigan, San Jose, California, USA).

2.7 Quantification of phenolic compounds by HT-UHPLC-UV

Standard curves were obtained by HPLC injection of 5-*O*-caffeoylquinic and 1,5-di-*O*-caffeoylquinic acids, scopoletin, naringenin 7-*O*-glucoside, luteolin 7-*O*-glucoside and apigenin 7-*O*-glucoside standard solutions in HPLC grade methanol/water (1:1), with six different concentrations ranging from 0.1 to 350 µg/mL. The quantification of individual compounds was done by using the linear equation (Table 1), obtained with the most similar standard compound. Accordingly, monocaffeoylquinic acids were quantified by using the 5-*O*-caffeoylquinic acid standard curve, while dicaffeoylquinic and dicaffeoylsuccinoylquinic acids were quantified with the 1,5-di-*O*-caffeoylquinic acid standard curve. The scopoletin standard curve was used for the quantification of coumarins. The concentrations of naringenin, luteolin and apigenin derivatives were determined by using, respectively, the standard curve of naringenin 7-*O*-glucoside, luteolin 7-*O*-glucoside and apigenin 7-O-glucoside. Concentrations were assessed in triplicate for each extract, and the mean value of two extracts was calculated for each morphological part.

Table 1: Standard data used for the HT-UHPLC-UV quantification of phenolic compounds of methanol/water/acetic acid extracts from *C. cardunculus* L. var. *altilis* (DC).

Standard compound	λ [(I)] (nm)	Conc. range (µg/mL)	Linear equation [(II)]	r^2	LOD [(III)] (µg/mL)	LOQ [(IV)] (µg/mL)
5-*O*-Caffeoylquinic acid	330	0.5-350	$y = 115495x + 316815$	0.9992	13.26	44.18
1,5-Di-*O*-caffeoylquinic acid	330	0.5-350	$y = 118780x + 330317$	0.9992	13.20	43.99
Scopoletin	330	0.5-20	$y = 188470x - 25906$	0.9991	0.76	2.54
Naringenin 7-*O*-glucoside	280	0.5-100	$y = 100708x + 108220$	0.9988	4.42	14.72
Luteolin 7-*O*-glucoside	350	0.1-40	$y = 193015x - 50750$	0.9998	0.71	2.38
Apigenin 7-*O*-glucoside	330	0.5-100	$y = 95591x + 14374$	0.9987	4.66	15.52

[(I)] Detection wavelength. [(II)] y = peak area, x = concentration in µg/mL. [(III)] LOD, limit of detection. [(IV)] LOQ, limit of quantification.

2.8 Statistical analysis

The quantitative analysis data were analyzed using the MIXED procedure option of SAS (SAS Institute Inc., Cary, North Carolina, USA), considering morphological parts of *C. cardunculus* L. var. *altilis* (DC) and extracts as fixed and random effects, respectively. Where differences existed, the source of the differences at $p < 0.05$ of significance level was identified by all pairwise multiple comparison procedure. The Tukey's test was used for pairwise comparisons.

3. Results and Discussion

3.1 Extractives yield and total phenolic content

The extractives yields for the different parts of *C. cardunculus* L. var. *altilis* (DC), using methanol/water/acetic acid (49.5:49.5:1), as well as the corresponding TPC, determined through the Folin Ciocalteu assay, are shown in Table 2.

The highest extractives yield was obtained from the capitula florets, being almost the double of the one provided by stalks outer part (Table 2). Comparing with the literature data, the extractives yields obtained in this work with both parts of capitula were higher than those previously indicated for methanol extracts of artichoke capitula (13.2%).[22] Nonetheless, the extractives yield of cultivated cardoon leaves was slightly lower than the one earlier reported for methanol extracts of wild cardoon leaves (34.72%).[8] These differences can be related to the geographical location, life cycle phase, agricultural methodologies, collection year, as well as extraction methodologies.

Table 2: Extractives yield and total phenolic content of several morphological parts of *C. cardunculus* L. var. *altilis* (DC) by using methanol/water/acetic acid (49.5:49.5:1).

***C. cardunculus* L. var. *altilis* (DC)**		**Extractives yield (% w/w)**	**Total phenolic content**	
			mg GAE/kg DW	**mg GAE/g extract**
Stalks	Outer part	17.5 ± 0.4	9243 ± 277 ^{b}	52.9 ± 1.1 ^{b}
	Inner part	20.2 ± 0.1	7503 ± 704 ^{c}	37.4 ± 3.5 ^{c}
Capitula	Receptacles and bracts	18.7 ± 1.8	11116 ± 707 ^{a}	59.4 ± 2.5 ^{a}
	Florets	33.9 ± 0.7	8499 ± 194 ^{b}	25.1 ± 1.0 ^{d}
Leaves		28.0 ± 0.1	6310 ± 461 ^{d}	22.6 ± 1.7 ^{d}

Results correspond to the means±standard deviation estimated from three aliquots of two extracts. Means with different superscript letters within the same column are statistically different ($p < 0.05$).

The TPCs varied from 6310 mg/kg dw (22.6 mg/g extract) in leaves to 11116 mg/kg dw (59.4 mg/g extract) in receptacles and bracts (Table 2). When compared to literature data,

TPCs of both parts of capitula were higher than those previously cited for capitula of cultivated and wild cardoon,[8,9] but lower than those noted for artichoke capitula.[22] Moreover, TPCs of both parts of stalks were much higher relatively to those earlier noticed for cultivated and wild varieties of cardoon from south of Portugal.[9] Additionally, the TPC of cultivated cardoon leaves was lower relatively to those published for artichoke,[10] cultivated cardoon and wild cardoon.[8,9] These differences in TPC can be due to the different factors explained above.

3.2 HT-UHPLC-UV-MS^n analysis of methanol/water/acetic acid (49.5:49.5:1) extracts of *C. cardunculus* L. var. *altilis* (DC)

Figure 1 highlights the HT-UHPLC-UV chromatograms of methanol/water/acetic acid extracts derived from stalks outer part and capitula florets of *C. cardunculus* L. var. *altilis* (DC), recorded at 330 nm. Phenolic compounds were identified by comparing the UV spectra and the MS^n fragmentation with those of standards or, when these were not available, with literature, as discussed below.

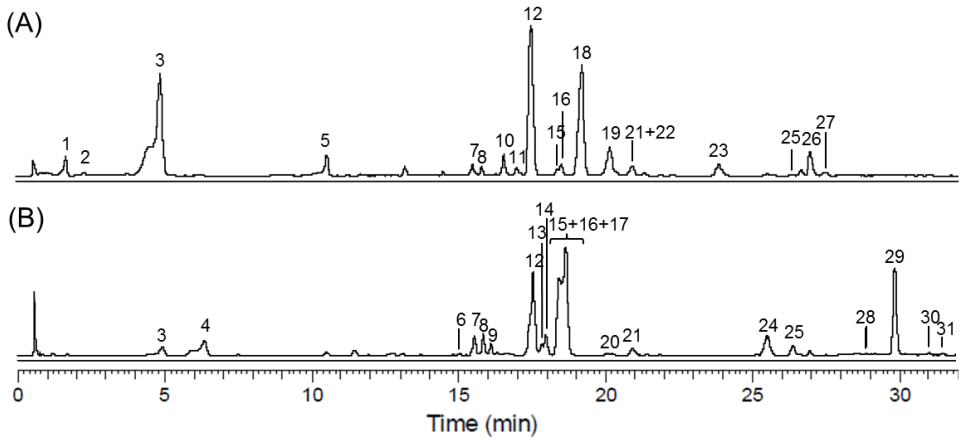


Figure 1: HT-UHPLC-UV chromatograms of methanol/water/acetic acid (49.5:49.5:1) extracts of different morphological parts of *C. cardunculus* L. var. *altilis* (DC), recorded at 330 nm: (A) stalks inner part and (B) capitula florets.

Twenty eight phenolic compounds were identified in the methanol/water/acetic acid extracts of *C. cardunculus* L. var. *altilis* (DC) (Table 3). Eriodictyol hexoside (**6**) was evidenced, for the first time, as a *C. cardunculus* L. component, with the exception of eriodictyol hexoside (**6**), scopolin (**4**) and dicaffeoyldisuccinoylquinic acid isomer (**26**), the remaining phenolic compounds noticed in this work were earlier described in stalks,[18,22,23] capitula, including receptacles and bracts,[17,22–27] leaves,[10,11,18,23,26,28–30] commercial supplements[30] and by-products of artichoke.[30–33]

Table 3: Retention time (RT) of the compounds detected by HT-UHPLC-UV-MS^n analysis in the metanol/water/acetic acid (49.5:49.5:1) extracts of *C. cardunculus* L. var. *altilis* (DC).

No.	RT (min)	Compound	Presence in *C. cardunculus* L.
1	1.70	1-*O*-Caffeoylquinic acid	WC,[16] Art,[10,18,22,24,25,28,31] CC[16]
2	2.34	3-*O*-Caffeoylquinic acid	WC,[15,18] Art,[18,24,25,28–31] CC[18]
3	4.90	5-*O*-Caffeoylquinic acid	WC,[15,16,18,26] Art,[10,18,22,24–26,28–33] CC[16,18]
4	6.33	Scopolin isomer	C[34]
5	10.52	1,3-Di-*O*-caffeoylquinic acid	WC,[15] Art,[10,11,24–26,28,30,31] CC[26]
6	14.97	Eriodictyol hexoside	RFCC
7	15.47	Luteolin glucuronide	WC,[16,18] Art,[17,22–25,30,31,33] CC[16,18]
8	15.76	Luteolin 7-*O*-glucoside	WC,[15,16,18,22] Art,[10,11,17,18,22–24,28,29,31,33] CC[16,18]
9	16.02	Luteolin rutinoside	WC,[16,18,22] Art,[10,11,17,18,22–25,33] CC[16]
10	16.52	1,4-Di-*O*-caffeoylquinic acid	Art[32]
11	16.93	Non-identified compound	
12	17.40	1,5-Di-*O*-caffeoylquinic acid	WC,[15,17,18] Art,[17,18,22,24,25,28–32] CC[18]
13	17.73	Naringenin 7-*O*-glucoside	Art[24,31,33]
14	17.88	Naringenin rutinoside	Art[10,27,31]
15	18.33	Apigenin glucuronide	WC,[16,18] Art,[17,18,22–25,30,31,33] CC[16–18]
16	18.44	Apigenin 7-*O*-glucoside	WC,[18] Art,[11,18,22,24,25,28,30,31] CC[18]
17	18.55	Apigenin 7-*O*-rutinoside	WC,[16–18] Art,[10,11,18,22,24,25,31,33] CC[16–18]
18	19.15	1,5-Di-*O*-caffeoylsuccinoylquinic acid isomer	WC,[16,18] Art,[18] CC[16,18]
19	20.11	4-Acyl-di-*O*-caffeoylsuccinoylquinic acid isomer	WC,[16,18] Art,[18] CC[16,18]
20	20.12	Chrysoeriol hexoside isomer	
21	20.79	Luteolin acetylhexoside	Art[30]
22	20.89	4-Acyl-di-*O*-caffeoylquinic acid isomer	Art[24,29–32]
23	23.79	Dicaffeoylsuccinoylquinic acid isomer	WC,[16,18] Art,[18] CC[16,18]
24	25.36	Luteolin	WC,[16–18] Art,[17,18,22,23,25,26,28,29,33] CC[16,18,26]
25	26.23	Apigenin acetylhexoside	Art[27]
26	26.86	Dicaffeoyldisuccinoylquinic acid isomer	WC[16]
27	27.43	Non-identified compound	
28	28.40	Naringenin	Art[24,33]
29	29.68	Apigenin	WC,[8,16–18] Art,[17,18,22,25,26,28,33] CC[16–18]
30	30.71	Chrysoeriol isomer	Art[33]
31	31.31	Non-identified compound	

Abbreviations: Art, artichoke; C, cardoon, without reference in literature regarding the variety; CC, cultivated cardoon; RFCC, reported for the first time in *C. cardunculus* L.; WC, wild cardoon.

Six phenolic compounds were here reported, for the first time, as cultivated cardoon components (Table 3), namely 1,4-di-*O*-caffeoylquinic acid (**10**), naringenin 7-*O*-glucoside (**13**), naringenin rutinoside (**14**), luteolin acetylhexoside (**21**), apigenin acetylhexoside (**25**) and naringenin (**28**). Isomers of 4-acyl-di-*O*-caffeoylquinic acid (**22**), dicaffeoyldisuccinoylquinic acid (**26**) and chrysoeriol (**30**) were also detected for the first time as cultivated cardoon components. An isomer of scopolin (**4**) was previously isolated from cardoon, but the variety was not indicated.[34]

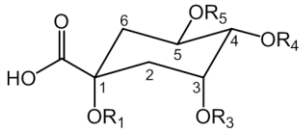
Although the remaining phenolic compounds were previously reported in cultivated and wild varieties of cardoon,[16–18] four of them were proven here, for the first time, to be cultivated cardoon stalks components, such as: 1-*O*-caffeoylquinic (**1**) and 1,3-di-*O*-caffeoylquinic acids (**5**), apigenin glucuronide (**15**) and apigenin 7-*O*-glucoside (**16**). Moreover, five phenolic compounds were referred, for the first time, in capitula receptacles and bracts of cultivated cardoon, namely 1-*O*-caffeoylquinic (**1**), 3-*O*-caffeoylquinic (**2**) and 1,5-di-*O*-caffeoylquinic acids (**12**), luteolin glucuronide (**7**) and luteolin 7-*O*-glucoside (**8**). Finally, 1,5-di-*O*-caffeoylquinic acid (**12**) was found for the first time in cultivated cardoon leaves.

3.2.1 Hydroxycinnamic acids and coumarins

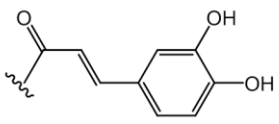
The hydroxycinnamic acids identified in the *C. cardunculus* L. var. *altilis* (DC) extracts were grouped into caffeoylquinic (**1**-**3**, **5**, **10**, **12** and **22**) and dicaffeoylsuccinoylquinic acids (**18**, **19**, **23** and **26**). A coumarin isomer (**4**) was also found in these extracts.

3.2.1.1 Caffeoylquinic acids

Compounds **1**-**3** were recognized as monocaffeoylquinic acids and compounds **5**, **10**, **12** and **22** as dicaffeoylquinic acids (Figure 2). Their identification relies on the UV spectra, the detection of $[M-H]^-$ and respective MS^n fragmentation (Table 4).



quinic acid



caffeoyl (caf)

No.	R_1	R_3	R_4	R_5
1	caf	H	H	H
2	H	caf	H	H
3	H	H	H	caf
5	caf	caf	H	H
10	caf	H	caf	H
12	caf	H	H	caf
22	H	caf	caf	H
	H	H	caf	caf

Figure 2: Structures of caffeoylquinic acids identified in the methanol/water/acetic acid extracts of *C. cardunculus* L. var. *altilis* (DC). Abbreviation: caf, caffeoyl.

Table 4: HT-UHPLC-UV-MS^n data of hydroxycinnamic acids and coumarins identified in the methanol/water/acetic acid extracts of *C. cardunculus* L. var. *altilis* (DC).

No.	Compound	UV λ_{max} (nm)	$[M-H]^-$ (*m/z*) [I]	ESI-MS^n product ions *m/z* (% base peak) [II]	Id. [III]
1	1-*O*-Caffeoylquinic acid	247, 298, 328	353	MS^2: **191** (100), 179 (5), 173 (1), 135 (1) MS^3: 127 (100)	[35,36]
2	3-*O*-Caffeoylquinic acid	242, 296, 324	353	MS^2: **191** (100), 179 (5), 135 (1) MS^3: 127 (100), 93 (49)	Co
3	5-*O*-Caffeoylquinic acid	244, 299, 324	353	MS^2: **191** (100), 179 (4), 135 (1) MS^3: 173 (50), 127 (100), 111 (29), 93 (46), 85 (77)	Co
4	Scopolin isomer	234, 250sh, 288, 339	399	MS^2: 353 (18), 191 (100), 176 (2)	[37]
5	1,3-Di-*O*-caffeoylquinic acid	237, 296, 320	515	MS^2: **353** (100), 335 (44), 191 (1), 179 (23) MS^3: 191 (100), 179 (46), 135 (5)	[38]
10	1,4-Di-*O*-caffeoylquinic acid	240, 296, 325	515	MS^2: **353** (100), 335 (18), 317 (42), 299 (53), 255 (11), 203 (54), 191 (3), 179 (2), 173 (7) MS^3: 191 (40), 179 (84), **173** (100), 135 (12) MS^4: 93 (100)	[38]
12	1,5-Di-*O*-caffeoylquinic acid	243, 298, 327	515	MS^2: **353** (100), 335 (5), 191 (13), 179 (1) MS^3: **191** (100), 179 (7), 173 (1), 135 (2) MS^4: 173 (66), 127 (100), 93 (59), 85 (56)	Co
18	1,5-Di-*O*-caffeoylsuccinoylquinic acid isomer	245, 298, 324	615	MS^2: 515 (12), **453** (100), 435 (2), 353 (54), 191 (2) MS^3: **353** (100), 191 (5) MS^4: 191 (100), 179 (10), 173 (5)	[39]
19	4-Acyl-di-*O*-caffeoylsuccinoylquinic acid isomer	239, 300, 327	615	MS^2: **515** (100), 453 (19), 435 (15), 353 (30), 335 (8), 317 (4), 255 (1) MS^3: **353** (100), 299 (7), 255 (6), 203 (12), 191 (5), 179 (2), 173 (5) MS^4: 191 (100), 179 (43), 173 (86)	[39]
22	4-Acyl-di-*O*-caffeoylquinic acid isomer	235, 297, 326	515	MS^2: **353** (100), 335 (5), 317 (5), 299 (11), 255 (6), 203 (17), 191 (2), 179 (4), 173 (2) MS^3: 191 (48), 179 (59), 173 (100), 135 (10)	[38]

No.	Compound	UV λ_{max} (nm)	$[M-H]^-$ (*m/z*) [I]	ESI-MS^n product ions *m/z* (% base peak) [II]	Id. [III]
23	Dicaffeoylsuccinoylquinic acid isomer	227, 245, 301, 325	615	MS^2: 515 (29), **453** (100), 435 (6), 353 (58), 335 (6), 191 (5) MS^3: 353 (100)	[16]
26	Dicaffeoyldisuccinoylquinic acid isomer	236, 300, 328	715	MS^2: 677 (100), **615** (4), 553 (42), 453 (36), **353** (16) MS^3 [715→615]: 515 (100) MS^3 [715→353]: 191 (100)	[16]

[I] Exception of compound 4, which was detected as a formate adduct. [II] *m/z* in bold was subjected to MS^n analysis. [III] Abbreviations: Co, co-injection of authentic standard; Id, identification.

Figure 3 demonstrates the MS^2 [353] and MS^3 [353→191] mass spectra and the product ions originated by the fragmentation of 5-*O*-caffeoylquinic acid (**3**), under the negative ESI. The MS^2 spectrum of the ion at *m/z* 353 (*I*) displayed the base peak at *m/z* 191 (*II*) ([quinic acid-H]$^-$) which resulted from the loss of cinnamate (-162 Da) (Table 4). A low intense peak was observed at *m/z* 179 ([caffeic acid-H]$^-$).[35] The MS^3 spectrum of the ion at *m/z* 191 revealed the characteristic product ions of the deprotonated quinic acid ion fragmentation, namely at *m/z* 173 (*III*) ([quinic acid-H-H_2O]$^-$), 127 (*IV*) ([quinic acid-H-H_2O-HCOOH]$^-$), 111 (*V*) and 93 ([phenoxide]$^-$) (Figure 3).[35,36] The identification of this compound was confirmed with the injection of the standard in the same chromatographic conditions.

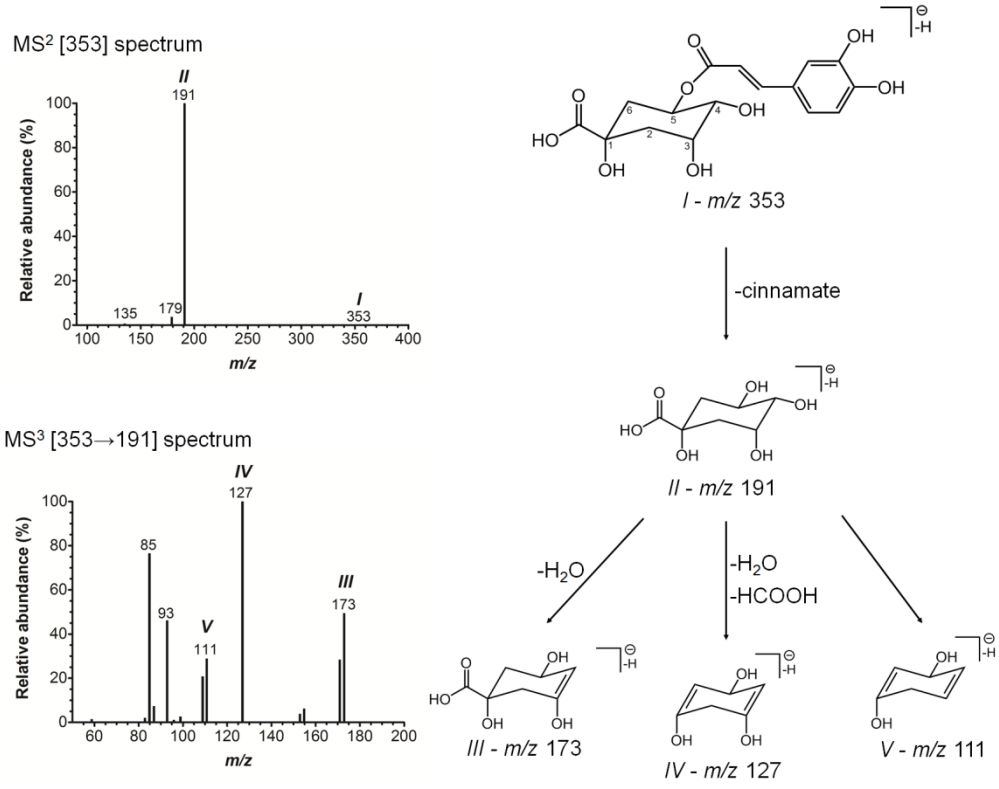


Figure 3: Negative ESI mass spectra and the main product ions arising from 5-*O*-caffeoylquinic acid (**3**) fragmentation.

Compound **2** was further assigned as 3-*O*-caffeoylquinic acid, based on the injection of the standard in the same chromatographic conditions. In addition to the UV spectrum and the MS^n data (Table 4), compound **1** was tentatively identified as 1-*O*-caffeoylquinic acid, since it was eluted before 3-*O*-caffeoylquinic acid, as earlier indicated.[31] The elution order of the monocaffeoylquinic acids indeed corroborated with the literature.[29] By using a C18 reversed-phase HPLC column, Adzet, *et al.*[29] had shown that monocaffeoylquinic acids with axial caffeoyl moieties (1- and 3-*O*-caffeoylquinic acids) firstly eluted from the HPLC column,

followed by monocaffeoylquinic acids with equatorial caffeoyl moieities (4- and 5-*O*-caffeoylquinic acids).

Compounds **5**, **10**, **12** and **22** (Figure 2) were considered as dicaffeoylquinic acids, through the UV spectra and the MS data. The negative ESI of these compounds gave origin to the $[M-H]^-$ at *m/z* 515 (Table 4). Additionally, the base peak of the respective MS^2 [515] spectra was noted at *m/z* 353, due to the loss of a cinnamate (-162 Da).[38]

Figure 4 reveals the MS^2 and the MS^3 spectra of 1,3-di-*O*-caffeoylquinic acid (**5**) (Figure 2), as well as the main fragmentation product ions. The MS^2 [515] spectrum exhibited the base peak at *m/z* 353 (*I*), due to the loss of a cinnamate, and an intense peak (>30%) at *m/z* 335 (*II*) resulting from successive losses of cinnamate and water (-18 Da). Furthermore, the MS^3 [515→353] spectrum exposed a base peak at *m/z* 191 (*III*) ([quinic acid-H]$^-$) and an intense peak (≈50%) at *m/z* 179 ([caffeic acid-H]$^-$).[38] Moreover, this compound was eluted much earlier than the other dicaffeoylquinic acids, as previously evidenced;[31,38] this may be related to the axial position of the two caffeoyl moieties.[29]

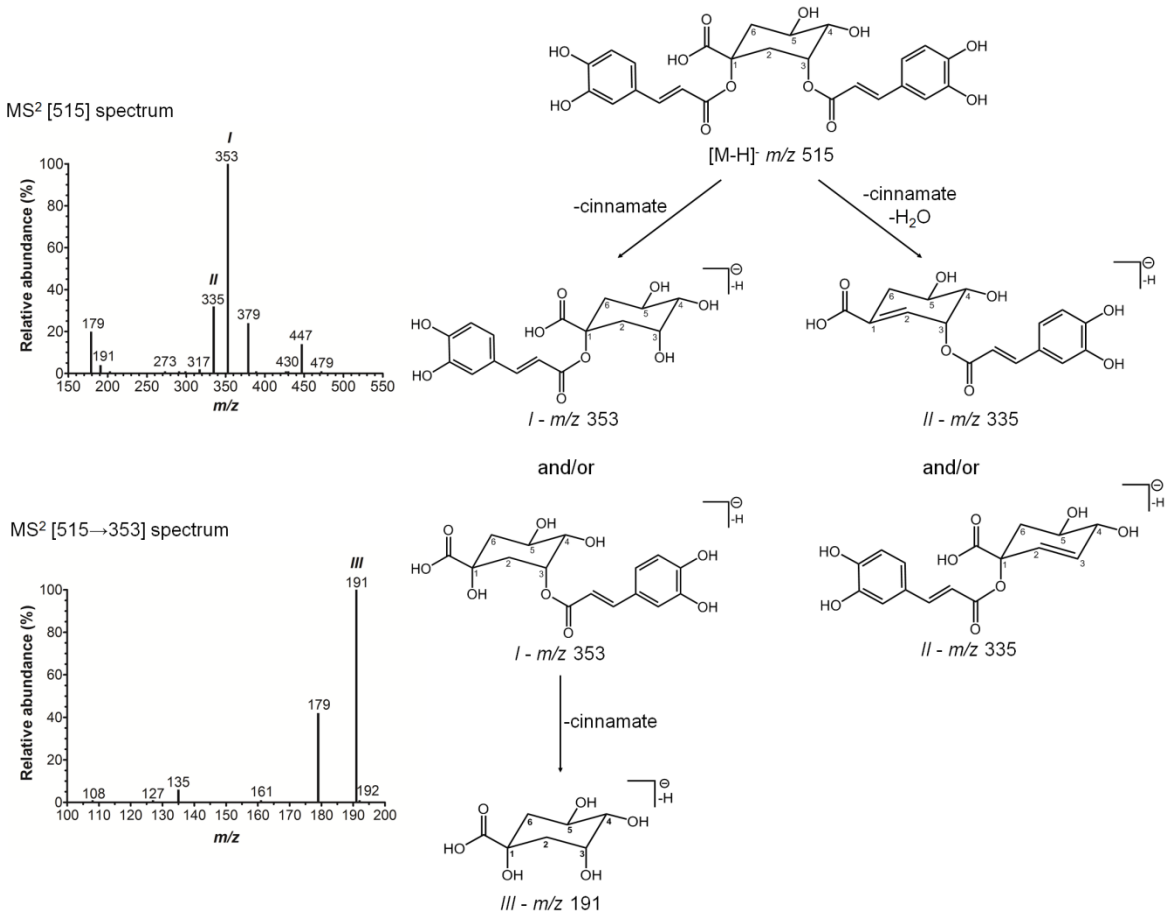


Figure 4: Negative ESI mass spectra and the main product ions arising from 1,3-di-*O*-caffeoylquinic acid (**5**) fragmentation.

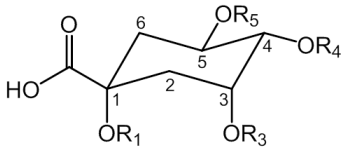
Compound **10** was tentatively assigned as 1,4-di-*O*-caffeoylquinic acid (Figure 2). The MS^2 [515] spectrum of this compound evidenced three characteristic product ions from 4-acyl dicaffeoylquinic acids, namely at *m*/*z* 317 ([M-H-H_2O-caffeoyl]$^-$), 299 ([M-H-2H_2O-caffeoyl]$^-$) and 255 ([M-H-2H_2O-caffeoyl-CO_2]$^-$) (Table 4), due to the successive loss of water (-18 Da), caffeic acid (-180 Da) and carboxylic acid (-44 Da).[38] Furthermore, the base peak of the MS^3 [515→353] spectrum was noticed at *m/z* 173, due to the loss of water and cinnamate molecules.[38] Additionally, the MS^4 [515→353→173] spectrum exhibited the base peak at *m/z* 93. Those base peaks are characteristic of the MS^n fragmentation of 4-*O*-acyl dicaffeoylquinic acids.[38] In fact, the product ions at *m/z* 173 and *m/z* 93 were previously reported in literature as the base peaks of respectively MS^2 [353] and MS^3 [353→173] spectra of 4-*O*-caffeoylquinic acid.[35] Three structures were hypothesized for compound **10**, namely 1,4-; 3,4- or 4,5-di-*O*-caffeoylquinic acid. Nevertheless, it might not be the last one, since the MS^2 fragmentation of [M-H]$^-$ yielded a product ion at *m*/*z* 335, which was not detected before in the MS^2 spectrum of the 4,5-di-*O*-caffeoylquinic acid.[38] Since two intense peaks at *m/z* 299 and 203 (>50% base peak) were observed in the MS^2 [515]$^-$ spectrum (Table 4), it was suggested that compound **10** was 1,4-di-*O*-caffeoylquinic acid.[38]

Compound **12** was identified as 1,5-di-*O*-caffeoylquinic acid (Figure 2), since the MS^2 [515] and the MS^3 [515→353] spectra revealed low intense peaks at *m/z* 335 (<10%) and *m/z* 179 (<10%), respectively.[38] In addition to the mass spectroscopic data, the identification of compound **12** with the injection of the standard in the same chromatographic conditions.

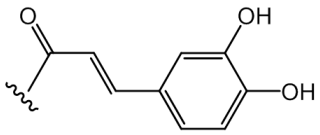
The MS^2 [515] spectrum of compound **22** also revealed three product ions, at *m*/*z* 335, 299 and 255 which are unique of 4-acyl dicaffeoylquinic acids MS^n fragmentation.[38] Moreover, the MS^3 [515→353] spectrum displayed a base peak at *m*/*z* 173. In this way, this compound might be either 3,4- or 4,5-di-*O*-caffeoylquinic acid. Compound **22** could be assigned as 3,4-di-*O*-caffeoylquinic acid, since the MS^2 spectrum demonstrated the product ion at *m/z* 335, as cited before.[38] However, it is not possible to elucidate the chemical structure of compound **22** as 3,4-di-*O*-caffeoylquinic acid, since a product ion at *m/z* 317 was noted in the MS^2 spectrum, which was not referenced in previous data.[31,38]

3.2.1.2 Dicaffeoylsuccinoylquinic acids

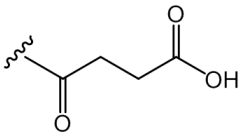
Compounds **18**, **19** (Figure 5) and **23** were considered as dicaffeoylsuccinoylquinic acids, based on the UV spectra, as well as on the detection of [M-H]$^-$ ion at *m/z* 615 and the MS^n fragmentation under negative ionization (Table 4).



quinic acid



caffeoyl (caf)



succinoyl (suc)

No.	R_1	R_3	R_4	R_5
18	caf	suc	H	caf
	caf	H	suc	caf
19	caf	suc	caf	H
	caf	H	caf	suc
	suc	caf	caf	H
	H	caf	caf	suc
	suc	H	caf	caf
	H	suc	caf	caf

Figure 5: Structures of dicaffeoylsuccinoylquinic acids identified in the methanol/water/acetic acid extracts of *C. cardunculus* L. var. *altilis* (DC). Abbreviations: caf, caffeoyl; suc, succinoyl.

The MS^2 [615] spectrum of compound **18** exhibited a base peak at *m/z* 453 due to the loss of a cinnamate (Table 4). Moreover, a weak peak at *m/z* 515 was evidenced in the MS^2 [615] spectrum, caused by the loss of succinoyl moiety (-100 Da). Additionally, an intense peak at *m/z* 353 was originated by the successive loss of the succinoyl moiety and a cinnamate. Furthermore, the MS^3 [615→453] spectrum displayed a base peak at *m/z* 353, due to the loss of succinoyl moiety.[16] The MS^4 spectrum of the ion at *m/z* 353 exposed a base peak at *m/z* 191 ([quinic acid]$^-$) and a weak peak at *m/z* 179, which is similar to the MS^3 spectrum [515→353] of 1,5-di-*O*-caffeoylquinic acid (**12**), as described before.[38] In this way, we proposed that compound **18** might be 1,5-di-*O*-caffeoyl-3-*O*-succinoylquinic acid or 1,5-di-*O*-caffeoyl-4-*O*-succinoylquinic acid[39] (Figure 5). Only based on the MS fragmentation, it was not possible to determine the position of succinoyl moiety in the structure.

Regarding compound **19**, the MS^2 spectrum of the $[M-H]^-$ ion at *m/z* 615 demonstrated a base peak at *m/z* 515, as well as an intense peak at *m/z* 353 and a weak peak at *m/z* 453 (Table 4). Additionally, the base peak at *m/z* 353 was noticed in the MS^3 spectrum of the ion at *m/z* 515, as well as two weak peaks at *m/z* 299 and 255 which are characteristic of the negative ESI fragmentation pathway of 4-acyl-dicaffeoylquinic acids.[38] Thus, compound **19** can be one of the following compounds: 1,4-di-*O*-caffeoyl-3-*O*-succinoylquinic, 1,4-di-*O*-caffeoyl-5-*O*-succinoylquinic, 3,4-di-*O*-caffeoyl-1-*O*-succinoylquinic, 3,4-di-*O*-caffeoyl-5-*O*-succinoylquinic, 4,5-di-*O*-caffeoyl-1-*O*-succinoylquinic or 4,5-di-*O*-caffeoyl-3-*O*-succinoylquinic

acids[39] (Figure 5). Nonetheless, it was not possible to determine the linkage positions of caffeoyl and succinoyl moieties in the quinic acid, only based on the MS data.

Finally, compound **23** was also noticied as a dicaffeoylsuccinoylquinic acid isomer, owing to the detection of the $[M-H]^-$ ion at *m/z* 615, as well as of the characteristic product ions of MS^2 [615] fragmentation, namely at *m/z* 515, 453, 353 and 191 (Table 4).[16,39] Moreover, the MS^3 spectrum of the ion at *m/z* 453 revealed a base peak at *m/z* 353. Nevertheless, it was not possible to identify unambiguously the substitution pattern of this compound with the MS data.

Compound **26** was considered as a dicaffeoyldisuccinoylquinic acid isomer, based on the $[M-H]^-$ at *m/z* 715 and on the respective MS^2 product ions, namely at *m/z* 615 ($[M-H\text{-succinoyl}]^-$), 553 ($[M-H\text{-cinnamate}]^-$), 453 ($[M-H\text{-cinnamate-succinoyl}]^-$) and 353 ($[M-H\text{-cinnamate-2succinoyl}]^-$) (Table 4).[16] Furthermore, the MS^3 fragmentation of the ion at *m/z* 615 yielded a product ion at *m/z* 515, due to the loss of a succinoyl moiety, while the MS^3 fragmentation of the ion at *m/z* 353 gave a product ion at *m/z* 191 ($[\text{quinic acid}]^-$). However, it was not possible to distinguish the substitution pattern of caffeoyl and succinoyl moieties in the quinic acid through the MS data. So far, only one dicaffeoylsuccinoylquinic acid was detected in Asteraceae plants (*Arctium lappa* L.), named as 1,5-di-*O*-caffeoyl-3,4-di-*O*-succinoylquinic acid.[39]

3.2.1.3 Coumarins

Compound **4** was assigned as a scopolin isomer, which means that it can be either scopolin or isoscopolin (Figure 6) whose structures only differ in the methoxyl position in the aromatic ring (C6 and C7, respectively).

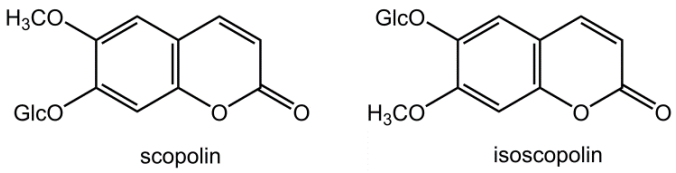


Figure 6: Structures of scopolin isomers, namely scopolin and isoscopolin. One of these compounds may be assigned to the identification of compound (**4**), detected in the methanol/water/acetic acid (49.5:49.5:1) extracts of *C. cardunculus* L. var. *altilis* (DC). Abbreviation: Glc, glucosyl.

Compound **4** proved to have similar UV spectrum to the standard of scopoletin. It was detected as formate adduct ($[M+HCOO]^-$) at *m/z* 399. The MS^2 [399] spectrum and the main fragmentation product ions of scopolin are given in Figure 7. The MS^2 $[M+HCOO]^-$ spectrum displayed a weak peak at *m/z* 353 ($[M-H]^-$) and a base peak at *m/z* 191, due to the loss of a glucosyl unit (-162 Da) from the $[M-H]^-$ ion. Nonetheless, it was not possible to identify unambiguously the position of the glucosyl moiety in the structure.

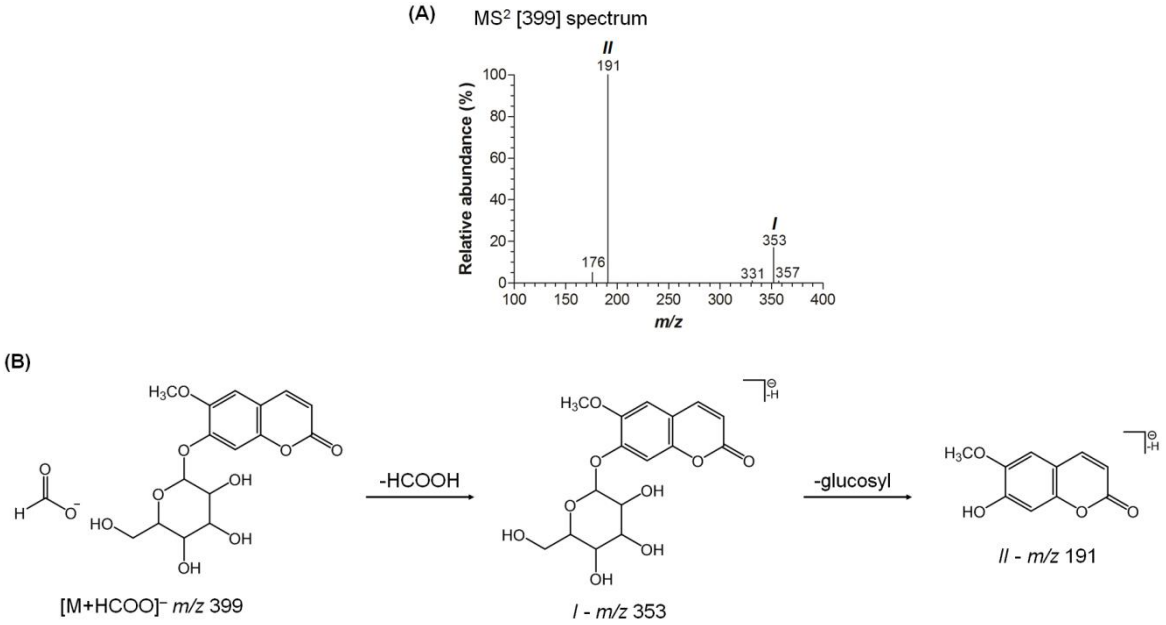


Figure 7: (A) Negative ESI MS^2 [399] spectrum and (B) the main fragmentation product ions of scopolin.

3.2.2 Flavonoids

Sixteen flavonoids were found in the methanol/water/acetic acid extracts of *C. cardunculus* L. var. *altilis* (DC), namely four flavanones (**6**, **13**, **14** and **28**) and twelve flavones (**7**-**9**, **15**-**17**, **20**, **21**, **24**, **25**, **29** and **30**), based on the UV and mass spectroscopic data (Table 5), as depicted bellow.

Table 5: HT-UHPLC-UV-MS^n data of the flavonoids identified in the methanol/water/acetic acid extracts of *C. cardunculus* L. var. *altilis* (DC).

No.	Compound	UV λ_{max} (nm)	$[M-H]^-$ (*m/z*)	ESI-MS^n product ions *m/z* (% base peak) [(I)]	Identification
6	Eriodictyol hexoside	234, 283, 328	449	MS^2: 431 (25), 413 (5), 404 (5), 381 (21), **287** (100), 175 (4) MS^3: 259 (22), 201 (19), 151 (100), 135 (17)	[40]
7	Luteolin glucuronide	236, 253, 266, 343	461	MS^2: 415 (2), 393 (3), 381 (6), 357 (5), 355 (4), 327 (6), **285** (100) MS^3: 268 (100), 257 (94), 173 (82)	[31]
8	Luteolin 7-*O*-glucoside	237, 253, 267, 346	447	MS^2: 379 (7), 357 (1), 327 (1), 311 (4), **285** (100) MS^3: 267 (62), 241 (92), 239 (39), 213 (62), 211 (22), 199 (100), 175 (86)	Co [(II)]
9	Luteolin rutinoside	235, 252, 267, 345	593	MS^2: 525 (1), **285** (100), 213 (1), 199 (1), 175 (1) MS^3: 267 (11), 243 (41), 241 (37), 217 (69), 199 (100), 175 (27), 151 (18)	[41]
13	Naringenin 7-*O*-glucoside	233, 282, 326	433	MS^2: 379 (1), 365 (2), 348 (1), **271** (100), 268 (1) MS^3: 177 (77), 151 (100), 125 (26)	Co [(II)]
14	Naringenin rutinoside	234, 282, 329	579	MS^2: **271** (100), 177 (1) MS^3: 227 (5), 177 (36), 151 (100)	[41]
15	Apigenin glucuronide	238, 266, 334	445	MS^2: 377 (14), **269** (100), 175 (7) MS^3: 225 (100)	[31]
16	Apigenin 7-*O*-glucoside	237, 267, 335	431	MS^2: 431 (100), 362 (1), 311 (1), **269** (27) MS^3: 269 (30), 227 (25), 225 (100), 201 (72), 183 (13), 149 (29)	Co [(II)]
17	Apigenin 7-*O*-rutinoside	239, 267, 335	577	MS^2: 440 (1), **269** (100), 169 (1) MS^3: 225 (100), 201 (40), 181 (37), 151 (57)	Co [(II)]
20	Chrysoeriol hexoside isomer	235, 251sh, 266, 342	461	MS^2: 446 (100), 415 (2), 393 (2), 341 (5), 313 (14), **299** (87), 298 (14), 297 (5), 284 (28), 283 (7), 269 (1), 255 (1) MS^3: 285 (1), **284** (100), 256 (1) MS^4: 257 (33), **256** (100)	[42]
21	Luteolin acetylhexoside	236, 252, 266, 346	489	MS^2: 447 (1), **285** (100), 284 (3), 241 (1) MS^3: 257 (40), 241 (67), 217 (100), 213 (48), 175 (56), 151 (55)	[43]
24	Luteolin	236, 251, 265, 347	285	MS^2: 285 (2), 267 (13), 257 (17), 243 (60), 241 (100), 217 (74), 213 (18), 199 (79), 197 (23), 175 (81), 151 (23), 133 (5)	[44]

No.	Compound	UV λ_{max} (nm)	$[M-H]^-$ (*m/z*)	ESI-MS^n product ions *m/z* (% base peak) [(I)]	Identification
25	Apigenin acetylhexoside	234, 266, 333	473	MS^2: 413 (4), 405 (4), 335 (1), 311 (3), 283 (2), **269** (100), 268 (56), 238 (2), 237 (1) MS^3: 225 (100), 181 (67)	[45]
28	Naringenin	234, 288, 327	271	MS^2: 253 (2), 227 (3), 177 (27), 151 (100), 119 (3), 107 (6)	[44]
29	Apigenin	237, 267, 335	269	MS^2: 269 (9), 241 (4), 227 (17), 225 (100), 201 (29), 183 (16), 181 (15), 159 (6), 151 (21), 149 (33), 117 (3), 107 (2)	[44]
30	Chrysoeriol isomer	234, 265, 287sh, 338	299	MS^2: 285 (2) **284** (100), 255 (<1), 243 (<1), 133 (<1) MS^3: 256 (100), 255 (1), 228 (1), 125 (1)	[46]

[(I)] *m/z* in bold was subjected to MS^n analysis. [(II)] Co, co-injection of authentic standard.

3.2.2.1 Flavanones

Four flavanones were reported in the methanol/water/acetic acid extracts of cultivated cardoon (Figure 8), namely eriodictyol hexoside (**6**), naringenin 7-*O*-glucoside (**13**), naringenin rutinoside (**14**) and naringenin (**28**).

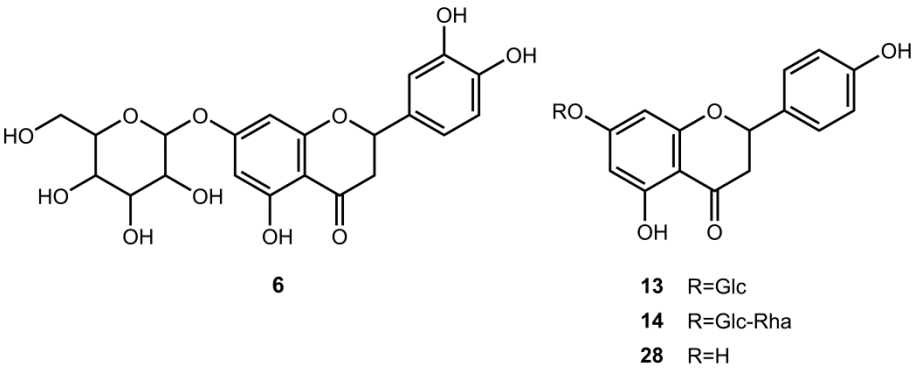


Figure 8: Structures of flavanones identified in the methanol/water/acetic acid extracts of *C. cardunculus* L. var. *altilis* (DC). The glycosyl substituent is attached to the common position of the aglycone. Abbreviations: Glc, glucosyl and Rha, rhamnosyl.

Compound **6** (Figure 8) was proposed to correspond to eriodictyol hexoside, since it was observed the $[M-H]^-$ ion at *m/z* 449 and its MS^2 spectrum exhibited a base peak at *m/z* 287 (Table 5), resulting from the loss of one hexosyl unit (-162 Da).[40] Furthermore, the MS^3 spectrum of the ion at *m/z* 287 displayed the characteristic product ions of eriodictyol, namely at *m/z* 259 ($[aglycone-CO]^-$), 201 ($[aglycone-C_2H_2O-CO_2]^-$), 151 ($[^{1,3}A]^-$) and 135 ($[^{1,3}B]^-$)[44] (the nomenclature follows the one that was explained before[47]).

Compounds **13** and **14** were considered as respectively naringenin 7-*O*-glucoside and naringenin rutinoside (Figure 8), based on the UV spectra and on the characteristic MS^3 product ions of naringenin at *m/z* 271 (Table 5).

Figure 9 indicates the MS^2 and MS^3 spectra, as well as the main product ions derived from the negative ESI of naringenin 7-*O*-glucoside (**13**), under negative ionization. This compound evidenced the $[M-H]^-$ ion at *m/z* 433 and the base peak of the MS^2 [433] spectrum at *m/z* 271 (*I*), due to the loss of glucosyl moiety. Additionally, the MS^3 fragmentation of the ion at *m/z* 271 yielded two product ions at *m/z* 177 (*II*) and *m/z* 151 (*III*), which is concordant with the one of naringenin, as discussed thereafter. The identification of this compound was thus confirmed with the injection of the standard in the same chromatographic conditions.

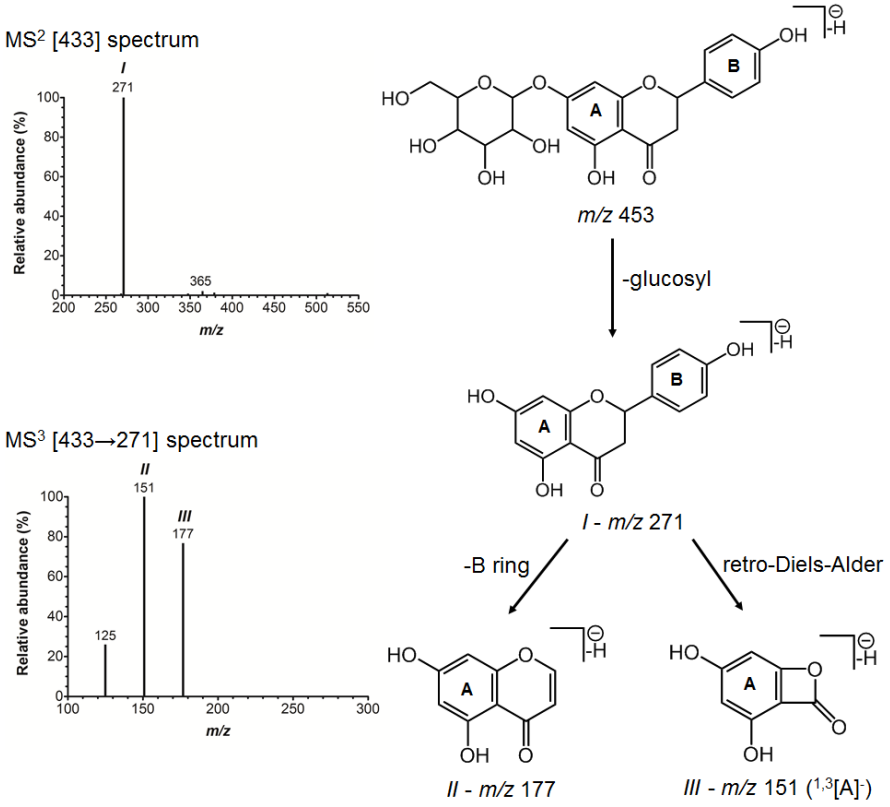


Figure 9: Negative ESI spectra and the main product ions arising from naringenin 7-*O*-glucoside (**13**) fragmentation.

The negative ESI of naringenin rutinoside (**14**) gave rise to the $[M-H]^-$ ion at *m/z* 579 (Table 5). The MS^2 [579] spectrum showed the base peak at *m/z* 271, resulting from successive losses of rhamnosyl (-146 Da) and hexosyl (-162 Da) moieties.[41] Moreover, the MS^3 fragmentation of the ion at *m/z* 271 originated characteristic product ions of naringenin, namely at *m/z* 227 ($[aglycone-H-CO_2]^-$), 177 ([aglycone-H-ring B]$^-$) and 151 ($[^{1,3}A]^-$); the last product ion was formed through the retro Diels-Alder fragmentation pathway.[44]

Compound **28** was assigned as naringenin (Figure 8), since the $[M-H]^-$ ion was found at *m/z* 271 (Table 5). In addition to the product ions referred before, the MS^2 [271] spectrum exposed other product ions at *m/z* 119 ($[^{1,3}B]^-$) and 107 ($[^{1,3}A\text{-}CO_2]^-$), as explained in literature.[44]

3.2.2.2 Flavones

Five luteolin derivatives (**7**-**9**, **21** and **24**), five apigenin derivatives (**15**-**17**, **25** and **29**) and two isomers of chrysoeriol derivatives (**20** and **30**) (Figure 10) were found in the methanol/water/acetic acid extracts of cultivated cardoon, based on the UV spectra and on the MS^n fragmentation data (Table 5).

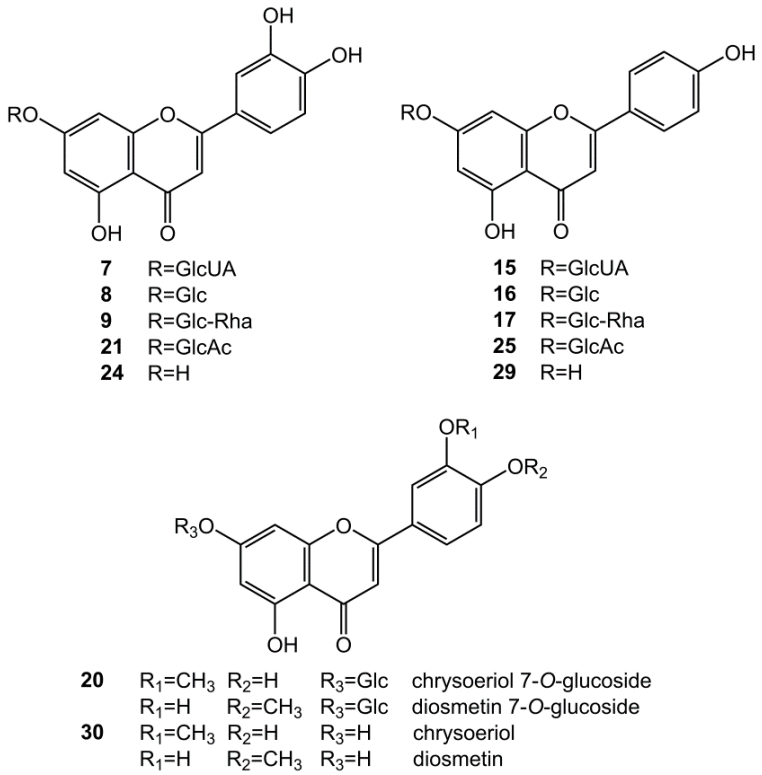


Figure 10: Structures of flavones identified in the methanol/water/acetic acid extracts of *C. cardunculus* L. var. *altilis* (DC). The glycosyl substituent is attached to the common position of the aglycone. Abbreviations: GlcAc, acetylglucosyl; Glc, glucosyl; GlcUA, glucuronyl; Rha, rhamnosyl.

In addition to the UV spectra, compounds **7** and **21** were indicated as respectively luteolin glucuronide and luteolin acetylhexoside, regarding the detection of the corresponding $[M-H]^-$ ions at *m/z* 461[31] and 489 and the respective MS^n fragmentation.[43] The MS^2 [461] spectrum of luteolin glucuronide (**7**) exhibited a base peak at *m/z* 285, arising from the loss of the glucuronyl moiety (-176 Da), while the MS^2 [489] spectrum of luteolin acetylhexoside (**21**) revealed a weak peak at *m/z* 447 and a base peak at *m/z* 285, due to successive losses of acetyl (-42 Da) and hexosyl (-162 Da) moieties. Moreover, the MS^3 spectrum of the ion at *m/z* 285 of both compounds **7** and **21** exhibited a characteristic product ion of luteolin negative ESI fragmentation, namely at *m/z* 257 ($[aglycone-H-CO]^-$).[44]

The identity of compound **8** was elucidated as luteolin 7-*O*-glucoside, based on the UV spectra, the detection of $[M-H]^-$ ion at *m/z* 447 and the MS^n fragmentation. Figure 11 displays the MS^2 and MS^3 spectra of luteolin 7-*O*-glucoside, as well as the main fragmentation product ions, under the negative ESI. The base peak of the MS^2 [447] spectrum was observed at *m/z* 285 (*I*), arising from the loss of the glucosyl moiety. Three product ions were noted at *m/z* 267 ($[aglycone-H-H_2O]^-$), 241 (*II*) ($[aglycone-H-CO_2]^-$) and 213 (*III*) ($[aglycone-H-CO_2-CO]^-$) (Figure 11). Additionally, several other characteristic product ions of luteolin were detected in the MS^3 spectrum of the ion at *m/z* 285, namely at: (i) *m/z* 199 (*IV*), due to the C-ring fragmentation

and cyclization involving the B-ring, leading to the loss of the C_2H_2O unit (-42 Da), followed by the loss of CO_2 group and the formation of the contracted A-ring; and (ii) *m/z* 175 (*V*) resulting from the loss of C_3O_2 unit (-68 Da) from A-ring fragmentation, followed by the loss of C_2H_2O unit due to the C-ring fragmentation and cyclization involving the B-ring (Figure 11).[44] The identification of this compound was confirmed with the injection of the standard in the same experimental conditions.

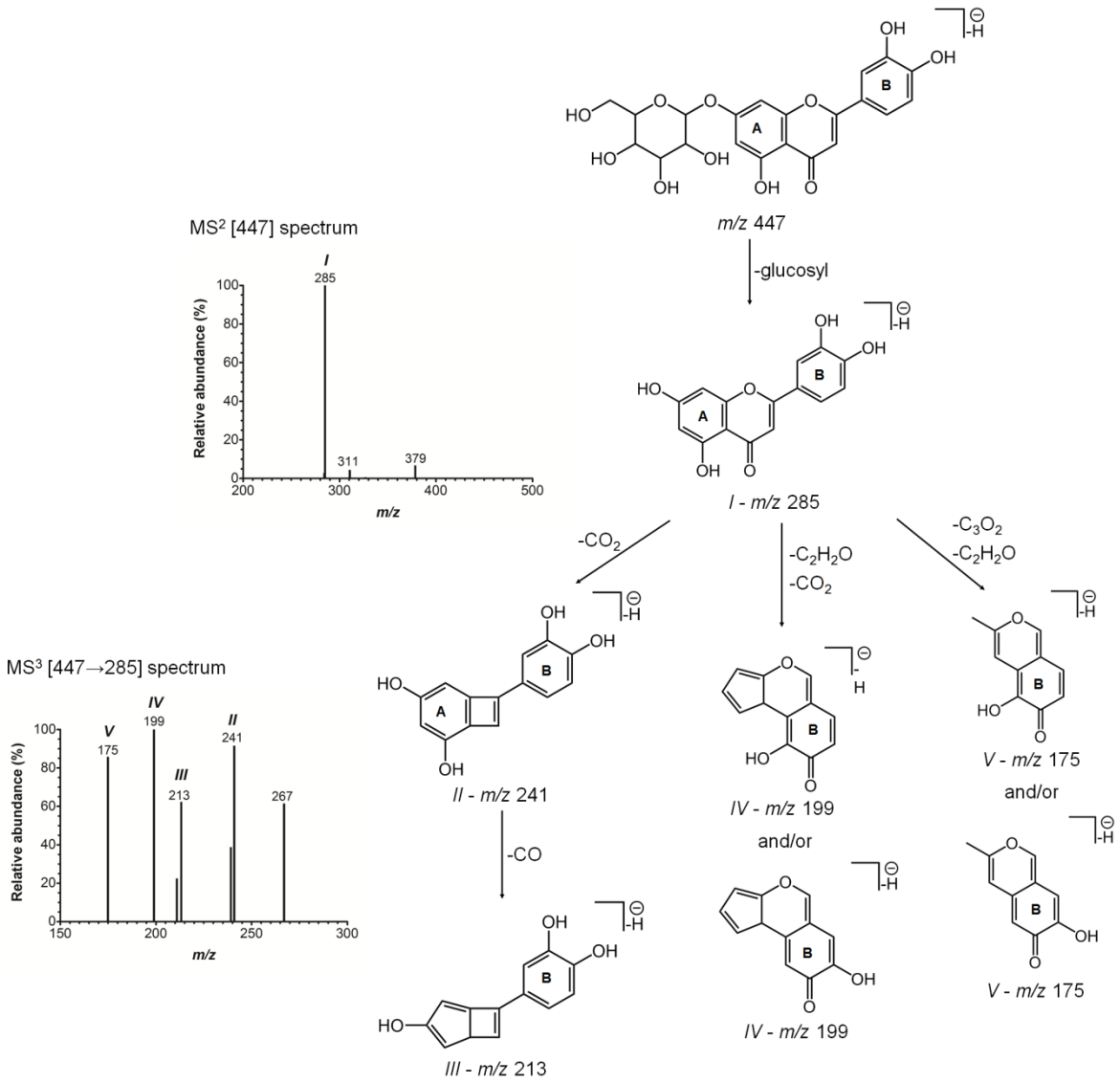


Figure 11: Negative ESI spectra and the main product ions resulting from luteolin 7-*O*-glucoside (**8**) fragmentation.

Compound **9** was tentatively assigned as luteolin rutinoside (Figure 10), due to the detection of the $[M-H]^-$ at *m/z* 593 and its MS^2 base peak at *m/z* 285 which resulted from successive losses of rhamnosyl (-146 Da) and glucosyl (-162 Da) moieties.[31] Furthermore, the

MS^3 fragmentation of the ion at *m/z* 285 yielded characteristic product ions of luteolin, namely at *m/z* 243 ([aglycone-H-C_2H_2O]$^-$), 217 ([aglycone-H-C_3O_2]$^-$)) and 151 ([^{1,3}A]$^-$).[44]

Compound **24** was assigned as luteolin (Figure 10), due to the UV spectrum, as well as the detection of the [M-H]$^-$ ion at *m/z* 285 (Table 5) and its MS^2 product ions, at *m/z* 267, 241, 213, 199, 197 ([aglycone-H-$2CO_2$]$^-$), 175 and 133 ([^{1,3}B]$^-$).[44]

Compounds **15** and **25** were ascribed as apigenin glucuronide and apigenin acetylhexoside (Figure 10), respectively, based on the detection of [M-H]$^-$ ions at *m/z* 445 (**15**) and *m/z* 473 (**25**). The MS^2 spectra of both compounds exposed a base peak at *m/z* 269, resulting from the loss of the glucuronyl moiety for compound **15**[31] and the acetylhexosyl moiety for compound **25**.[45] Moreover, the MS^3 spectrum of the ion at *m/z* 269 exhibited characteristic product ions of apigenin, namely at *m/z* 225 ([aglycone-H-CO_2]$^-$) and 181 ([aglycone-H-$2CO_2$]$^-$).[44]

Compounds **16** and **17** were identified respectively as apigenin 7-*O*-glucoside and apigenin 7-*O*-rutinoside (Figure 10), since their retention time, UV spectra, [M-H]$^-$ ions (*m/z* 431 and 577, respectively) and MS^n product ions (Table 5) were the same as the pure compounds injected in the same conditions.

The chemical structure of compound **29** was elucidated as apigenin (Figure 10), due to the UV spectrum and the detection of the [M-H]$^-$ ion at *m/z* 269 (Table 5). Furthermore, the MS^2 [269] spectrum highlighted characteristic product ions of apigenin, under negative ESI, namely at *m/z* 241 ([M-H-CO]$^-$), 227 ([aglycone-H-C_2H_2O]$^-$), 201 ([aglycone-H-C_3O_2]$^-$), 183 ([aglycone-H-C_2H_2O-CO_2]$^-$), 181 ([aglycone-H-$2CO_2$]$^-$), 151 ([^{1,3}A]$^-$), 159 ([M-H-C_3O_2-C_2H_2O]$^-$), 151 ([^{1,3}A]$^-$), 149 ([^{1,4}B+2H]$^-$), 117 ([^{1,3}B]$^-$) and 107 ([^{1,3}A-CO_2]$^-$).[44]

Compound **30** yielded the [M-H]$^-$ at *m/z* 299 and the base peak of MS^2 [299] spectrum was observed at *m/z* 284 (Table 5), due to the loss of a methyl group. Furthermore, a base peak at *m/z* 256 ([M-H-CH_3-CO]$^-$) and a weak peak at *m/z* 228 ([M-H-CH_3-2CO]$^-$) were detected in the MS^3 [299] spectrum. On the basis of the UV spectrum[48] and the MS^n fragmentation,[46] this compound **30** was tentatively noticed as a chrysoeriol isomer (Figure 10), which means that this compound may be chrysoeriol or diosmetin, whose structures only differ in the methoxyl position in the B-ring (C3´ and C4´, respectively).

Compound **20** had similar UV spectrum to compound **30**, and the respective [M-H]$^-$ ion was observed at *m/z* 461 (Table 5). The MS^2 spectrum of this ion showed a product ion at *m/z* 446 as the base peak, due to the loss of a methyl, and an intense peak at *m/z* 299, corresponding to the loss of a hexosyl moiety. Additionally, the MS^3 [446→299] spectrum displayed the base peak at *m/z* 284 (loss of a methyl), while the MS^4 [446→299→284] spectrum demonstrated the base peak at *m/z* 256 (loss of a carbonyl group), similar to MS^n fragmentation of compound **30**. The aglycone moiety of compound **20** can be a chrysoeriol isomer, and it was therefore tentatively assigned as a chrysoeriol hexoside isomer (Figure 10).

3.2.3 Unidentified compounds

The structure elucidation of compounds **11**, **27** and **31** could not be achieved only based on the MS^n data (Table 6).

Table 6: HT-UHPLC-UV-MS^n data of non-identified compounds in the methanol/water/acetic acid (49.5:49.5:1) extracts of *C. cardunculus* L. var. *altilis* (DC)

No.	UV λ_{max} (nm)	$[M-H]^-$ (*m/z*)	ESI-MS^n product ions *m/z* (% base peak) [(I)]
11	236, 300, 320, 337	483	MS^2: 415 (24), **263** (100), 253 (5)
27	236, 302, 329	793	MS^2: 725 (73), **631** (100) MS^3: 469 (100)
31	235	553	MS^2: 503 (2), 459 (23), 433 (4), 415 (8), 401 (2), **391** (100), 349 (2) MS^3: 347 (100), 319 (80), 304 (89), 151 (90)

[(I)] *m/z* in bold was subjected to MS^n analysis.

Compound **11** demonstrated the $[M-H]^-$ ion at *m/z* 483 and the MS^2 [483] spectrum had the base peak at *m/z* 263. However, the MS data did not allow the identification of this compound.

Compound **27** can be a caffeic acid derivative, since it presented similar UV spectrum to caffeoylquinic acid isomers. Moreover, the MS^2 spectrum of the $[M-H]^-$ ion at *m/z* 793 evidenced a base peak at *m/z* 731, resulting from the loss of 162 Da, which may be attributed to a cinnamate or an hexosyl unit. Furthermore, the ion at *m/z* 731, during the MS^3 fragmentation, also suffered the loss of 162 Da, which may be attributed to the loss of a second cinnamate or hexosyl unit. Nevertheless, the MS data were not sufficient to elucidate the structure of this compound.

Compound **31** exhibited the $[M-H]^-$ ion at *m/z* 553 and its MS^2 spectrum displayed the base peak at *m/z* 391 (loss of 162 Da), probably due to the loss of a hexosyl unit. Moreover, a low intense peak was observed at *m/z* 349 (loss of 204 Da) in the MS^2 spectrum of the ion at *m/z* 553, probably due to the loss of an acetylhexosyl unit. Furthermore, the MS^3 spectrum of the ion at *m/z* 391 exposed the base peak at *m/z* 347 (loss of 44 Da), probably due to the loss of one acetaldehydyl (CH_3COH) or carboxyl (CO_2) groups. Despite these data, it was not possible to indicate the chemical structure of this compound.

3.3 Quantitative analysis of phenolic compounds identified in *C. cardunculus* L. var. *altilis* (DC) methanol/water/acetic acid extracts

Phenolic compounds were quantified through the HT-UHPLC-UV standard curves of representative compounds from each family (Table 1). This study characterized, for the first time, the phenolic composition of stalks (outer and inner parts), capitula (receptacles, bracts

and florets) and leaves derived from *C. cardunculus* L. var. *altilis* (DC) (Figure 12 and Table 7).

The total contents of phenolic compounds in cultivated cardoon biomass varied between 2846 mg/kg dw in leaves and 12697 mg/kg dw in florets (Figure 12). Regarding the composition of methanol/water/acetic acid extracts, the phenolic concentrations ranged from 10.4 mg/g extract in leaves to 63.5 mg/g extract in stalks outer part.

The identified phenolic contents of both stalks parts and florets, when expressed either as mg/kg dw or mg/g extract, were higher than the corresponding total phenolic contents determined by the Folin Ciocalteu assay (Table 2) (from 17% in stalks outer part to 33% in florets). On the other hand, it was observed that the total identified phenolic contents of leaves were 54% lower relatively to those determined by the Folin-Ciocalteu assay. Despite the identified phenolic contents corresponded to the major phenolic compounds detected by HT-UHPLC-UV, the increment of total phenolic contents of leaves determined with the colorimetric assay (Table 2) may be related with a series of interfering compounds which also react with the Folin-Ciocalteu's reagent, and may be in those extracts, for instance sugars, ascorbic acid, organic acids, among others.[49]

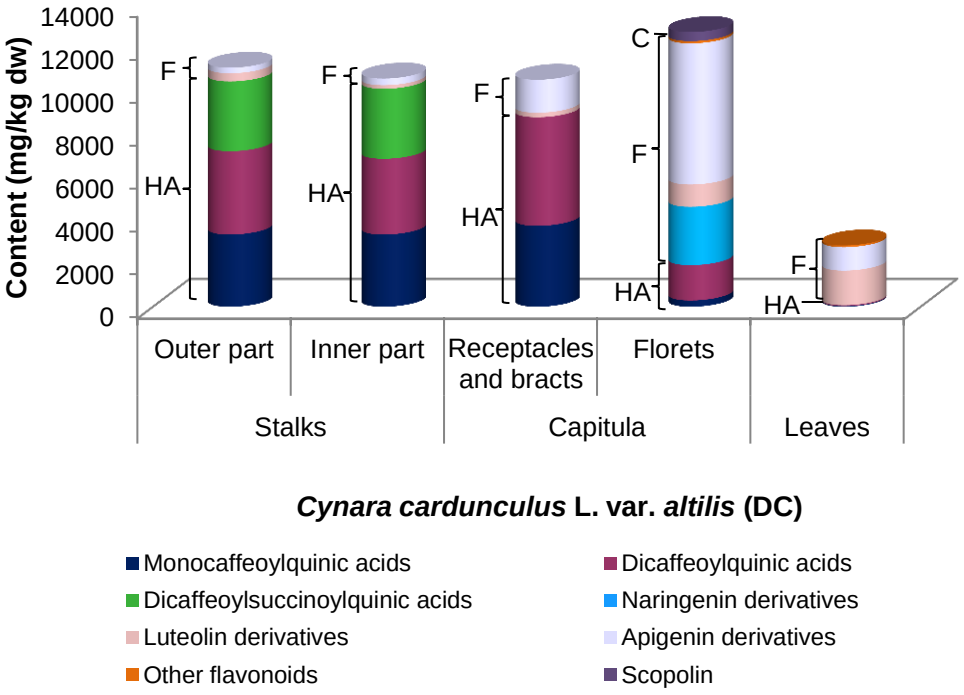


Figure 12: Phenolic composition of *C. cardunculus* L. var. *altilis* (DC) morphological parts. Abbreviations: C, coumarins; F, flavonoids and HA, hydroxycinnamic acids.

Table 7: HT-UHPLC-UV quantification of phenolic compounds identified in methanol/water/acetic acid (49.5:49.5:1) extracts of *C. cardunculus* L. var. *altilis* (DC). [I]

No.	Compound	Phenolic content (mg/kg dw)					Phenolic content (mg/g extract)				
		Stalks		Capitula		Leaves	Stalks		Capitula		Leaves
		Outer	Inner	Receptacles and bracts	Florets		Outer	Inner	Receptacles and bracts	Florets	
	Total hydroxycinnamic acids [V]	**10323** [a]	**9969** [a]	**8786** [b]	**1930** [c]	**71** [d]	**59.1** [a]	**49.5** [b]	**50.2** [b]	**5.7** [c]	**0.3** [c]
	Total monocaffeoylquinic acids	*3347* [b]	*3345* [b]	*3749* [a]	*291* [c]	*30* [c]	*19.1* [b]	*16.6* [c]	*21.4* [a]	*0.9* [d]	*0.1* [d]
1	1-*O*-Caffeoylquinic acid	112 [b]	216 [a]	56 [c]	–	–	0.6 [b]	1.1 [a]	0.3 [c]	–	–
2	3-*O*-Caffeoylquinic acid	159 [a]	44 [c]	94 [b]	–	–	0.9 [a]	0.2 [c]	0.5 [b]	–	–
3	5-*O*-Caffeoylquinic acid	3075 [b]	3085 [b]	3599 [a]	291 [c]	30 [c]	17.6 [b]	15.3 [c]	20.6 [a]	0.9 [d]	0.1 [d]
	Total dicaffeoylquinic acids [V]	*3742* [b]	*3381* [b]	*5036* [a]	*1640* [d]	*41* [e]	*21.4* [b]	*16.8* [c]	*28.8* [a]	*4.8* [d]	*0.1* [e]
5	1,3-Di-*O*-caffeoylquinic acid	211 [b]	316 [a]	181 [b]	–	–	1.2 [b]	1.6 [a]	1.0 [c]	–	–
10	1,4-Di-*O*-caffeoylquinic acid	258 [b]	190 [c]	466 [a]	–	–	1.5 [b]	0.9 [c]	2.7 [a]	–	–
12	1,5-Di-*O*-caffeoylquinic acid	3273 [b]	2876 [c]	4292 [a]	1640 [d]	41 [e]	18.8 [b]	14.3 [c]	24.5 [a]	4.8 [d]	0.1 [e]
22	4-Acyl-di-*O*-caffeoylquinic acid isomer	124 [a] (21+22)	135 [a] (21+22)	97 [b]	–	–	0.7 [a] (21+22)	0.7 [a] (21+22)	0.6 [a]	–	–
	Total dicaffeoylsuccinoylquinic acids	*3235* [a]	*3242* [a]	–	–	–	*18.5* [a]	*16.1* [a]	–	–	–
18	1,5-Di-*O*-caffeoylsuccinoylquinic acid isomer	2168 [a]	2151 [a]	–	–	–	12.4 [a]	10.7 [b]	–	–	–
19	4-Acyl-di-*O*-caffeoylsuccinoylquinic acid isomer	479 [a]	557 [a]	–	–	–	2.7 [a]	2.8 [a]	–	–	–
23	Dicaffeoylsuccinoylquinic acid isomer	262 [a]	215 [b]	–	–	–	1.5 [a]	1.1 [b]	–	–	–
26	Dicaffeoyldisuccinoylquinic acid isomer	326 [a]	318 [a]	–	–	–	1.9 [a]	1.6 [a]	–	–	–
	Total coumarins	**–**	**–**	**–**	**421**	**–**	**–**	**–**	**–**	**1.2**	**–**
4	Scopolin isomer	–	–	–	421	–	–	–	–	1.2	–

No.	Compound	Phenolic content (mg/kg dw)					Phenolic content (mg/g extract)				
		Stalks		Capitula		Leaves	Stalks		Capitula		Leaves
		Outer	Inner	Receptacles and bracts	Florets		Outer	Inner	Receptacles and bracts	Florets	
	Total flavonoids	**638** [d]	**478** [d]	**1740** [c]	**10345** [a]	**2775** [b]	**3.7** [c]	**2.4** [c]	**9.9** [b]	**30.6** [a]	**10.2** [b]
	Total flavanones	–	–	–	*2703*	–	–	–	–	*8.0*	–
6	Eriodictyol hexoside [(II)]	–	–	–	40	–	–	–	–	0.1	–
	Total naringenin derivatives	–	–	–	*2663*	–	–	–	–	*8.0*	–
13	Naringenin 7-*O*-glucoside	–	–	–	812	–	–	–	–	2.4	–
14	Naringenin rutinoside	–	–	–	1770	–	–	–	–	5.4	–
28	Naringenin	–	–	–	81	–	–	–	–	0.2	–
	Total flavones	*638* [d]	*478* [d]	*1740* [c]	*7642* [a]	*2775* [b]	*3.7* [c]	*2.4* [c]	*9.9* [b]	*22.7* [a]	*10.2* [b]
	Total luteolin derivatives	*379* [c]	*169* [d]	*211* [c,d]	*1035* [b]	*1607* [a]	*2.2* [c]	*0.8* [d]	*1.2* [d]	*3.1* [b]	*5.7* [a]
7	Luteolin glucuronide	171 [b,d]	101 [c]	146 [c,d]	208 [b]	388 [a]	1.0 [b]	0.5 [c]	0.8 [b]	0.6 [c]	1.4 [a]
8	Luteolin 7-*O*-glucoside	208 [b]	68 [c]	65 [c]	205 [b]	841 [a]	1.2 [b]	0.3 [c]	0.4 [c]	0.6 [c]	3.0 [a]
9	Luteolin rutinoside	–	–	–	109 [a]	31 [b]	–	–	–	0.3 [a]	0.1 [a]
21	Luteolin acetylhexoside [(IV)]	(21+22)	(21+22)	–	114 [b]	207 [a]	(21+22)	(21+22)	–	0.3 [a]	0.7 [b]
24	Luteolin	–	–	–	399 [a]	139 [b]	–	–	–	1.2 [a]	0.5 [b]
	Total apigenin derivatives	*259* [c]	*309* [c]	*1529* [b]	*6545* [a]	*1085* [b]	*1.5* [d]	*1.5* [d]	*8.7* [b]	*19.3* [a]	*3.9* [c]
15	Apigenin glucuronide	203 [d] (15+16)	281 [d] (15+16)	1423 [b]	4676 [a] (15+16+17)	804 [c] (15+16+17)	1.2 [d] (15+16)	1.4 [d] (15+16)	8.1 [b]	13.8 [a] (15+16+17)	2.9 [c] (15+16+17)
16	Apigenin 7-*O*-glucoside	(15+16)	(15+16)	–	(15+16+17)	(15+16+17)	(15+16)	(15+16)	–	(15+16+17)	(15+16+17)
17	Apigenin 7-*O*-rutinoside	–	–	–	(15+16+17)	(15+16+17)	–	–	–	(15+16+17)	(15+16+17)
25	Apigenin acetylhexoside	55 [c]	27 [d]	106 [b]	231 [a]	128 [b]	0.3 [c]	0.1 [d]	0.6 [b]	0.7 [a,b]	0.5 [b]
29	Apigenin	–	–	–	1638 [a]	154 [b]	–	–	–	4.8 [a]	0.5 [b]

No.	Compound	Phenolic content (mg/kg dw)					Phenolic content (mg/g extract)				
		Stalks		Capitula		Leaves	Stalks		Capitula		Leaves
		Outer	Inner	Receptacles and bracts	Florets		Outer	Inner	Receptacles and bracts	Florets	
	Total chrysoeriol derivatives	–	–	–	*63* [b]	*83* [a]	–	–	–	*0.3* [b]	*0.5* [a]
20	Chrysoeriol hexoside isomer [(III)]	–	–	–	30 [b]	83 [a]	–	–	–	0.2 [b]	0.5 [a]
30	Chrysoeriol isomer [(III)]	–	–	–	33	–	–	–	–	0.1	–
	Total identified phenolics	**11085** [b]	**10581** [b]	**10525** [b]	**12697** [a]	**2846** [c]	**63.5** [a]	**52.5** [b]	**60.2** [a]	**37.6** [c]	**10.4** [d]

[(I)] Results represent the means estimated from the analysis of two extracts analyzed in triplicate (standard deviation less than 5%). Means with different superscript letters within the same row, among the phenolic content expressed in mg/kg dw and among the phenolic content expressed in mg/g extract, are statistically different ($p < 0.05$). Numbers in parenthesis correspond to compounds whose chromatographic peaks were overlapped.

Standard curves used: [(II)] naringenin 7-*O*-glucoside and [(III)] luteolin 7-*O*-glucoside.

[(IV)] Standard curve of 1,5-di-*O*-caffeoylquinic acid was chosen, because the overlapped peaks (compounds **21** and **22**) was higher at 330 nm compared to 350 nm.

[(V)] In the case of stalks outer and inner parts, the total contents did not include the content of 4-acyl-di-*O*-caffeoylquinic acid isomer (**22**), due to the co-elution with luteolin acetylhexoside (**21**).

The phenolic contents in both parts of stalks (10581-11085 mg/kg dw) were enclosed within the published concentration range for floral stem of artichoke,[18,22,24] wild cardoon and cultivated cardoon.[18] Receptacles and bracts also contained considerably high amount of hydroxycinnamic acids (10525 mg/kg dw), being in the same order as those earlier reported for the same parts of artichoke by Pandino, *et al.*,[50] but lower than those shown by Negro, *et al.*[25] The analyzed leaves contained lower phenolic compounds concentrations, relatively to those formerly indicated for cultivated cardoon[16,18] and wild cardoon. Valentão[51] earlier investigated the phenolic composition of wild cardoon leaves, collected in North Portugal, at the florescence stage. The presented total identified phenolic content of cultivated cardoon leaves was much lower (up to 10.2-fold) comparatively with Valentão's results.[51] This could be associated not only with the edapho-climatic conditions, but also with the variety, age and life stage of plant, and extraction methodology.

3.3.1 Hydroxycinnamic acids and coumarins

Hydroxycinnamic acids were mainly retained in stalks outer and inner parts (Figure 12 and Table 7), accounting for 10323 and 9969 mg/kg dw, respectively. These compounds are commonly detected in stalks and capitula of *C. cardunculus* L.,[18,25,50] due to their integrity and structural roles in plant cell wall.[52] The hydroxycinnamic acids contents of stalks was in the same order as the content range previously referenced for floral stems of wild cardoon, artichoke,[18,22,24] and cultivated cardoon.[18] Stalks outer part extract revealed the highest hydroxycinnamic acids concentration (59.1 mg/g extract).

Receptacles and bracts demonstrated the highest dicaffeoylquinic acids content, accounting for 5036 mg/kg dw and 28.8 mg/g extract (Figure 12 and Table 7). These values were higher than those cited for receptacles and bracts of wild cardoon[17] and artichoke.[22,24,50] Monocaffeoylquinic acids were also found at considerable amounts, contributing for 3749 mg/kg dw and 21.4 mg/g extract in receptacles and bracts, which was within the concentration range cited for artichoke receptacles and bracts.[22,24,25,50] Dicaffeoylsuccinoylquinic acids were only noted in inner and outer parts of stalks, accounting for respectively 3242 and 3235 mg/kg dw (16.1 and 18.5 mg/g extract, respectively). Pandino, *et al.*[18] had proven that these compounds were present in floral stems of cultivated cardoon, whose concentration was 2.3-fold lower than the results obtained in this work.

The 1,5-di-*O*-caffeoylquinic acid (**12**) was the most common hydroxycinnamic acid identified in cultivated cardoon, representing 4292 mg/kg dw and 24.5 mg/g extract of receptacles and bracts. The content of this compound was much higher than that previously indicated for wild cardoon capitula (21 mg/kg dw).[17] Moreover, it was in the same order as the published concentration for receptacles and bracts of artichoke.[22,24,50] 5-*O*-caffeoylquinic acid (**3**) was the most abundant monocaffeoylquinic acid identified in cultivated cardoon, specially

in receptacles and bracts (3599 mg/kg dw and 20.6 mg/g extract). This was in the same order as that of artichoke receptacles and bracts.[24,50] This compound was not previously detected in capitula of wild cardoon and cultivated cardoon.[17] 1,5-di-*O*-caffeoylsuccinoylquinic acid isomer (**18**) was the major dicaffeoylsuccinoylquinic acid of cultivated cardoon, accounting for 2168 mg/kg dw (12.4 mg/g extract) and 2151 mg/kg dw (10.7 mg/g extract) in outer and inner parts of stalks, respectively. These concentrations were in the same order than those isomers found in cultivated cardoon floral stem.[18]

Coumarins (**4**) were only noted in the florets (421 mg/kg dw and 1.2 mg/g extract) (Table 7). This phenolic subclass was for the first time quantified in *C. cardunculus* L. varieties by LC-UV analysis.

3.3.2 Flavonoids

Capitula florets presented the highest amount of flavonoids (10345 mg/kg dw and 30.6 mg/g extract) (Table 7 and Figure 12), which may be linked with their role in plant reproduction.[53] The concentration of this phenolic subclass was in the same order of artichoke capitula,[17,25] and higher than those published regarding capitula of cultivated cardoon and wild cardoon.[17] Furthermore, flavonoids were also in leaves (2775 mg/kg dw and 10.2 mg/g extract), probably due to their important protective function against the ultraviolet radiation.[18] Leaves flavonoids concentration was lower than those referenced for artichoke and wild cardoon.[16,18] Nonetheless, it was included in the flavonoids concentration range, relatively to cultivated cardoon leaves.[16,18,51]

Flavones were the most abundant flavonoids (Table 7 and Figure 12), particularly in florets (7642 mg/kg dw and 22.7 mg/g extract), mainly represented by apigenin derivatives (6545 mg/kg dw and 19.3 mg/g extract). The apigenin derivatives concentration was in the same concentration order as those that were earlier noticed for capitula of artichoke,[17,31] wild cardoon and cultivated cardoon.[17] In fact, apigenin glucuronide (**15**), apigenin 7-*O*-glucoside (**16**) and apigenin 7-*O*-rutinoside (**17**) were the most abundant flavonoids in cultivated cardoon florets (4676 mg/kg dw and 13.8 mg/g extract). Moreover, leaves extracts exhibited the highest content of total luteolin derivatives (1607 mg/kg dw and 5.7 mg/g extract), being comparable with those earlier studied from wild cardoon leaves[18,51] and cultivated cardoon,[16,18] but lower than those from artichoke leaves.[16,18] Flavanones were only found in florets (2703 g/kg dw and 8.0 mg/g extract). This concentration was much higher than that indicated by Schutz, *et al.*[31] for artichoke capitula.

4. Conclusions

This work highlighted a detailed chemical characterization of phenolic compounds in stalks, capitula and leaves of *C. cardunculus* L. var. *altilis* (DC) from the south of Portugal, by HT-UHPLC-DAD-MS^n analysis. Among the 28 phenolic compounds identified in cultivated cardoon, eriodictyol hexoside was referred for the first time in *C. cardunculus* L., and six as cultivated cardoon components, namely 1,4-di-*O*-caffeoylquinic acid, naringenin 7-*O*-glucoside, naringenin rutinoside, naringenin, luteolin acetylhexoside and apigenin acetylhexoside. Stalks contained the highest hydroxycinnamic acids abundances (≈9.9-10.3 g/kg dw), while capitula florets demonstrated the highest flavonoids content (≈10.3 g/kg dw). Mono and dicaffeoylquinic acids were specially retained in receptacles and bracts (≈3.7 and ≈4.9 g/kg dw, respectively). Furthermore, florets and leaves revealed the highest contents of apigenin (≈6.5 g/kg dw) and luteolin (≈1.6 g/kg dw) derivatives, respectively.

In sum, the present work suggests that cultivated cardoon is promising in what concerns to phenolic composition, being comparable to other Asteraceae plants, like endemic *Helichrysum* species of Madeira Archipelago (Macaronesia).[54] The pre-extraction of phenolic compounds from cultivated cardoon can be integrated in a future industrial value chain, comprehending energy, pulp fibers production and nutraceutics, in order to contribute for the economic development of Mediterranean countries.

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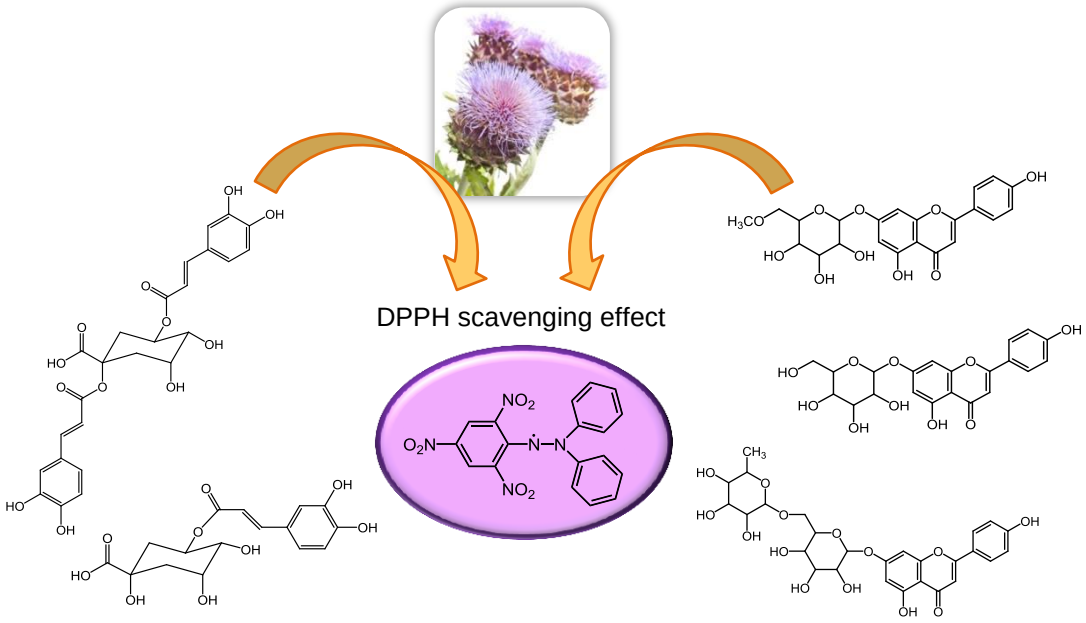
Part C

Evaluation of biological activity of *Cynara cardunculus* L. var. *altilis* extracts

Chapter V

Antioxidant activity of *Cynara cardunculus* L. var. *altilis* (DC) phenolic-rich extracts



Adapted from:

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Abstract

Given the presence of phenolic compounds, the antioxidant activity of methanol/water/acetic acid (49.5:49.5:1) extracts, derived from stalks, capitula and leaves of *Cynara cardunculus* L. var. *altilis* (DC) was approached through the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. For comparison reasons, ascorbic acid and 3,5-di-*tert*-4-butylhydroxytoluene (BHT) were used as, respectively, natural and synthetic reference antioxidants. Extracts of stalks outer part (IC_{50} = 34.35 µg/mL), and receptacles and bracts (IC_{50} = 35.25 µg/mL) exhibited the strongest DPPH scavenging effects. Nevertheless, cultivated cardoon extracts were less active to scavenge DPPH free radicals, comparatively to ascorbic acid (IC_{50} = 2.29 µg/mL) and BHT (IC_{50} = 16.02 µg/mL). The DPPH scavenging effect was linearly correlated with the total identified phenolic contents, assessed by HPLC-UV (r = -0.899), in particular with the total monocaffeoylquinic acids content (r = -0.996). Novel antioxidant formulations, containing cultivated cardoon phenolic-rich fractions, may potentially be considered for food applications.

1. Introduction

Cynara cardunculus L. (Asteraceae) comprises three varieties, namely wild cardoon (var. *sylvestris* (Lamk) Fiori), globe artichoke (var. *scolymus* (L.) Fiori) and cultivated cardoon (var. *altilis* (DC)).[1] This species is well-adapted to the Mediterranean habitat conditions, namely clay soils and dry and hot summers.[2] Cultivated cardoon has been produced in Spain, Italy, France and south of Portugal, given its potential to be transformed into biocombustibles.[3,4]

Since the Roman times, *C. cardunculus* L. leaves infusions have been consumed, due to their hepatoprotective[5] and anticholestatic properties.[6] These biological actions have been ascribed to phenolic compounds, specially to hydroxycinnamic acids (e.g., 5-*O*-caffeoylquinic and 1,3-di-*O*-caffeoylquinic acids)[5] and flavones (e.g., luteolin and luteolin 7-*O*-glucoside).[6] Moreover, *C. cardunculus* L. extracts, containing phenolic compounds, have also exhibited antimicrobial,[7–9] anti-inflammatory,[10] and antitumor[11,12] effects.

Artichoke phenolic-containing extracts have also evidenced antioxidant activity, by preventing oxidation of Cu^{2+}-mediated low density lipoprotein,[13] as well as by scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals[14] and reactive oxygen species (ROS).[15] Methanol extracts and infusions of wild cardoon leaves have also demonstrated scavenging effect on DPPH,[7] superoxide radical anion[7,16] and hydroxyl radical,[16] mainly due to the presence of phenolic compounds. It has also evidenced the ferric reducing antioxidant power of methanol aqueous extract derived from cultivated cardoon floral stems.[17] Our group[12] previously observed the DPPH scavenging effect of methanol extracts, derived from several morphological parts of wild and cultivated cardoon, but a detailed chemical composition of these extracts was not determined, particularly in what regards phenolic compounds.

After the chemical characterization of cultivated cardoon lipophilic fraction,[18] our group[19] found that phenolic compounds were mainly in methanol/water/acetic acid (49.5:49.5:1) extracts, derived from stalks outer part (63.5 mg/g), and receptacles and bracts (60.2 mg/g). Furthermore, we noticed that stalks outer and florets extracts displayed the highest content of hydroxycinnamic acids (59.1 mg/g) and flavonoids (30.6 mg/g), respectively.[19] Considering our interest for seeking plant antioxidants, this study intends to examine the DPPH scavenging ability of methanol/water/acetic acid extracts, derived from several morphological parts of *C. cardunculus* L. var. *altilis* (DC), given the phenolic composition.

2. Materials and Methods

2.1 Chemicals

Methanol (p.a., ≥99.8% purity) and water were supplied by Fisher Scientific (Pittsburgh, Pennsylvania, USA). Glacial acetic acid (p.a., ≥99.7% purity) was purchased from Panreac (Castellar del Vallès, Spain). DPPH and 3,5-di-*tert*-4-butylhydroxytoluene (BHT) (≥99% purity) were obtained from Sigma Chemicals Co. (Madrid, Spain). Ascorbic acid (≥99.5% purity) was supplied from Fluka Chemie (Madrid, Spain).

2.2 Preparation of *C. cardunculus* L. var. *altilis* (DC) methanol/water/acetic acid extracts

C. cardunculus L. var. *altilis* (DC) was collected at the flowering stage, in June 2010, at the Experimental Center of the Agriculture School from Instituto Politécnico de Beja, south of Portugal. Plants were separated in stalks, capitula and leaves, and preserved at -20 ºC until their use. Before extraction, samples were freeze-dried. Then, stalks were separated in outer and inner parts and capitula in receptacles, bracts and florets.

Methanol/water/acetic acid (49.5:49.5:1) extracts were prepared, after the lipophilic components removal, through solid-liquid extraction, and quantified by high performance liquid chromatography-ultraviolet detection (HPLC-UV), as discussed before.[19] For comparison reasons, the total phenolic contents were also determined through the Folin-Ciocalteu assay.[19] The detailed chemical composition of cultivated cardoon extracts may be consulted in Chapter IV.

2.3 DPPH scavenging effect assay

The antioxidant activity of methanol/water/acetic acid extracts was evaluated through the DPPH free radical scavenging assay, according to a procedure explained before.[20] Ascorbic acid and BHT were used as reference antioxidants from natural and synthetic origin, respectively.

Stock solutions of dry extracts, ascorbic acid and BHT were previously prepared in methanol/water (1:1). Sample aliquots (1 mL) were mixed with 0.25 mL of 0.95 mM DPPH methanolic solution and 2.75 mL of methanol. The ranges of final concentrations were 7.5-400.0 µg/mL for methanol/water extracts, 2.3-25.0 µM for ascorbic acid and 9.1-363.1 µM for BHT. The mixtures were vortexed and left to stand in the dark for 30 min. The absorbance of DPPH free radical was measured at 517 nm against a blank (methanol), and compared to a control without sample, using a UV/Vis V-530 spectrophotometer (Jasco, Tokyo, Japan). DPPH scavenging effect was calculated as the percentage of DPPH discoloration, using the following equation: % DPPH free radical scavenging effect = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where

A_{DPPH} is the control absorbance and A_S is the sample absorbance. The inhibitory concentration providing 50% DPPH scavenging effect (IC_{50}) was determined from the graph of scavenging effect percentage in function of concentration logarithm (GraphPad Prism 5, LaJolla, California, USA). Two extracts of each cultivated cardoon morphological part were analyzed in triplicate.

The antioxidant activity index (AAI) was calculated based on the following equation:[21] AAI = initial concentration of DPPH in the reaction mixture (μg/mL)/IC_{50} (extract) (μg/mL), where the initial DPPH concentration was 23.41 μg/mL.

2.4 Statistical analysis

The DPPH scavenging effect IC_{50} values were analyzed using the MIXED procedure option of SAS (SAS Institute Inc., Cary, North Carolina, USA), considering morphological parts of cultivated cardoon and extracts as fixed and random effects, respectively. Where differences existed, the source of the differences at $p < 0.05$ of significance level was identified by all pairwise multiple comparison procedure. The Tukey's test was used for pairwise comparisons. The Pearson correlation coefficients between phenolic compounds concentrations and DPPH scavenging effect were determined by using the CORR procedure option of SAS.

3. Results and Discussion

Antioxidant activity of methanol/water/acetic acid (49.5:49.5:1) extracts, derived from several morphological parts of *C. cardunculus* L. var. *altilis* (DC), was examined through DPPH scavenging effect. For comparison reasons, the ability of reference antioxidants, namely ascorbic acid and BHT, to scavenge DPPH was also evaluated (Table 1).

Extracts of stalks outer part, as well as of receptacles and bracts represented the most effective cultivated cardoon extracts to scavenge DPPH free radicals ($p > 0.05$), with IC_{50} values of 34.35 and 35.25 μg/mL, respectively (Table 1). The IC_{50} value of stalks inner part extracts was 1.7-fold higher than the former extracts ($p < 0.05$). Finally, florets and leaves extracts presented the highest IC_{50} values (163.92 and 164.90 μg/mL, respectively) ($p > 0.05$).

Table 1: IC_{50} and AAI values of methanol/water/acetic acid (49.5:49.5:1) extracts of *C. cardunculus* L. var. *altilis* (DC) and reference compounds, regarding the DPPH scavenging assay.

Cultivated cardoon extract/reference compound		IC_{50} (μg/mL) [(1)]	IC_{50} (μM) [(1)]	AAI
Stalks	Outer part	34.35 ± 2.27 [c]	–	0.68
	Inner part	58.65 ± 4.99 [b]	–	0.40
Capitula	Receptacles and bracts	35.25 ± 2.59 [c]	–	0.66
	Florets	163.92 ± 17.26 [a]	–	0.14
Leaves		164.90 ± 8.66 [a]	–	0.14
Ascorbic acid		2.29 ± 0.11 [e]	12.99 ± 0.61 [b]	10.22
BHT		16.02 ± 3.59 [d]	72.69 ± 16.28 [a]	1.46

[(1)] Results correspond to the means ± standard deviation estimated from the analysis from two extracts analyzed in triplicate. Means with different superscript letters within the same column are statistically different ($p < 0.05$).

Nevertheless, cultivated cardoon extracts displayed higher DPPH scavenging effect IC_{50} values, compared to those of antioxidant standards (Table 1), namely ascorbic acid (IC_{50} = 2.29 μg/mL) and BHT (IC_{50} = 16.02 μg/mL). Synthetic antioxidants, like butylated hydroxyanisole and BHT, are vastly used for preventing food lipid oxidation, due to their high performance, low cost and wide availability. However, there is a great search for plants phenolic compounds, as an alternative to potentially toxic and carcinogenic synthetic antioxidants.[22] Despite their lower DPPH scavenging effect compared to BHT, future research may be performed, in an effort to discover the potential of cultivated cardoon phenolic-enriched extracts to be included in novel food antioxidant formulations.

The antioxidant activity of artichoke,[14,23,24] wild cardoon[7,8,12] and cultivated cardoon[12] extracts have been assessed through the DPPH scavenging effect assay, but several methods have been performed, differently in terms of: (i) initial concentration of DPPH solution; (ii) reaction time; and (iii) way of presenting data. Therefore, the antioxidant activity index (AAI) was determined according to the equation suggested by Scherer and Godoy[21] (Table 1), in order to compare the DPPH scavenging effect of the tested cultivated cardoon extracts with the published results (Table 2). The tested capitula receptacles and bracts extracts were stronger (AAI = 0.66) than than several organic and hydroalcoholic extracts and fractions of wild cardoon capitula described in literature (AAI = 0.13[7] and AAI range of 0.23-0.31 [8]). Nonetheless, the analyzed capitula florets extract was as active (AAI = 0.14) as the methanol extract of wild cardoon capitula, earlier reported.[7] Cultivated cardoon leaves extract (AAI = 0.14) was much more active than infusions of artichoke leaves and dietary supplements (AAI = 0.01 and 0.002, respectively[24]), but it was less effective than the methanol extract of wild cardoon leaves, published in literature (AAI = 0.30).[7]

Table 2: AAI values regarding the DPPH scavenging effect of *C. cardunculus* L. extracts published in literature.

***C. cardunculus* L. extracts**		**AAI**	**Source**
Artichoke	Leaves infusion	0.01	[21]
	Dietary supplement infusion	0.002	[21]
Wild cardoon	Capitula methanol extract	0.13	[5]
	Capitula extracts	0.23-0.31	[6]
	Leaves methanol extract	0.30	[5]

The DPPH scavenging effect of the tested cultivated cardoon extracts were comparable with phenolic-containing extracts of other Asteraceae plants, tested at the same reaction time.[25,26] For example, the methanol/water/acetic acid extracts, derived from stalks outer part, and receptacles and bracts, were stronger relatively to *Centaurea gigantea* methanol extract (AAI = 0.56), containing chlorogenic acid, among other phenolic compounds.[25] Valentova, *et al.*[26] had noted that two ethyl acetate fractions, derived from *Smallanthus sonchifolius* leaves, exhibited DPPH scavenging effect (AAI = 0.55-0.83), containing 1.7-9.9 mg/g extract in terms of chlorogenic acid content.

Table 3 reveals the Pearson correlation coefficients between the total contents of phenolic compounds and the DPPH scavenging effect.

Table 3: Pearson correlation coefficients between phenolic compounds contents and DPPH scavenging effect of cultivated cardoon methanol/water/acetic acid (49.5:49.5:1) extracts.

Total contents	**Pearson correlation coefficient**
Phenolic compounds (Folin-Ciocalteu assay)	-0.929*
Identified phenolic compounds (HPLC-UV)	-0.899*
Hydroxycinnamic acids	-0.990**
Monocaffeoylquinic acids	-0.996**
Dicaffeoylquinic acids	-0.953*
Flavonoids	0.696
Apigenin derivatives	0.527
Luteolin derivatives	0.808

*Correlation is significant at $p < 0.05$.

** Correlation is significant at $p < 0.01$.

The DPPH scavenging effect of cultivated cardoon methanol/water/acetic acid extracts was linearly correlated with the phenolic compounds contents (Table 3), determined either by the Folin Ciocalteu assay (r = -0.929) or by the HPLC-UV quantification (r = -0.899). Furthermore, the DPPH scavenging effect of cultivated cardoon extracts was linearly

correlated with the total hydroxycinnamic acids content (r = -0.990), the total monocaffeoylquinic acids content (r = -0.996), and the total dicaffeoylquinic acids content (r = -0.953) (Table 3). These findings may be related with the resonance stabilization of *o*- or *p*-quinones originated by caffeoyl moieties.[27] Regarding flavonoids, the IC_{50} values of cultivated cardoon extracts increased with the total contents of flavonoids, luteolin and apigenin derivatives. In this manner, the DPPH scavenging effect of cultivated cardoon extracts was not correlated with the flavonoids, apigenin and luteolin derivatives contents. Wang, *et al.*[14] had showed that flavonoids, namely naringenin 7-*O*-rutinoside, luteolin 7-*O*-rutinoside and apigenin 7-*O*-glucoside, were less active to scavenge DPPH free radicals, compared to 1,3-di-*O*-caffeoylquinic acid.

Bioactivity-guided fractionation should be performed to clarify if phenolic compounds, or particularly hydroxycinnamic acids, were the main responsible for the DPPH scavenging effect of cultivated cardoon methanol/water/acetic acid extracts.

The preparation of cultivated cardoon enriched fractions, either in phenolic compounds or in hydroxycinnamic acids, may potentiate their antioxidant activity. For example, our group[28] recently indicated that the nanofiltration technology was useful to increment the contents of phenolic compounds (1.8-fold) and 5-*O*-caffeoylquinic acid (1.6-fold), in cultivated cardoon methanol/water leaves extracts. Brás, *et al.*[28] also indicated an 1.8-fold increment in the DPPH scavenging effect of cultivated cardoon phenolic-concentrated extracts, being correlated with the 5-*O*-caffeoylquinic acid content. Moreover, Kukic, *et al.*[8] reported a 7.3-fold enhancement in the DPPH scavenging effect of ethyl acetate fraction, after a 4.1-fold increase in the total phenolic contents, from the wild cardoon bracts 96% ethanol extract. Additionally, it was previously shown that 5-*O*-caffeoylquinic acid was 3.1-fold more active in neutralizing the DPPH free radicals, compared to the *C. gigantea* methanol extract, from which it was isolated.[25] In this manner, the preparation of cultivated cardoon enriched fractions in phenolic compounds, principally in hydroxycinnamic acids, derived from methanol/water/acetic acid extracts of stalks outer part and capitula receptacles and bracts, would probably enhance their DPPH scavenging effect.

Future work should also be carried out to investigate the antioxidant activity of the cultivated cardoon phenolic-rich extracts, as well as of their bioactive-enriched fractions, against ROS which are associated with the initiation, progression and promotion of cancer and cardiovascular diseases. Actually, Valentão, *et al.*[16] had noticed that wild cardoon leaves infusion was able to scavenge hydroxyl radicals. Besides the ROS scavenging effect,[15,16] the antioxidant activity of cultivated cardoon phenolic-enriched fractions can be further studied in immortalized human cell lines, through their influencing role upon ROS formation,[29] lipid peroxidation,[30] mitochondrial membrane potential,[11] and important antioxidant defense enzymes, namely superoxide dismutase, glutathione peroxidase and catalase.[31]

4. Conclusions

This study highlights the antioxidant activity of methanol/water/acetic acid (49.5:49.5:1) extracts derived from stalks, capitula and leaves of *C. cardunculus* L. var. *altilis* (DC), by using the DPPH free radical scavenging assay. Extracts of stalks outer part, and receptacles and bracts were the most active to scavenge DPPH free radicals, with IC_{50} values of 34.35 and 35.25 µg/mL, respectively ($p > 0.05$). The antioxidant capacity of cultivated cardoon extracts, by using the DPPH assay, demonstrated linear correlation with the total phenolic contents, determined either by the Folin-Ciocalteu assay (r = -0.929) or by the HPLC-UV quantification (r = -0.899). The DPPH scavenging effect of cultivated cardoon extracts were also linearly correlated with the total contents of monocaffeoylquinic acids (r = -0.996), hydroxycinnamic acids (r = -0.990) and dicaffeoylquinic acids (r = -0.953). Despite the weaker DPPH scavenging effect compared to ascorbic acid and BHT, the phenolic-rich fraction of cultivated cardoon may have potential to be investigated, in the scope of new food antioxidant formulations.

5. References

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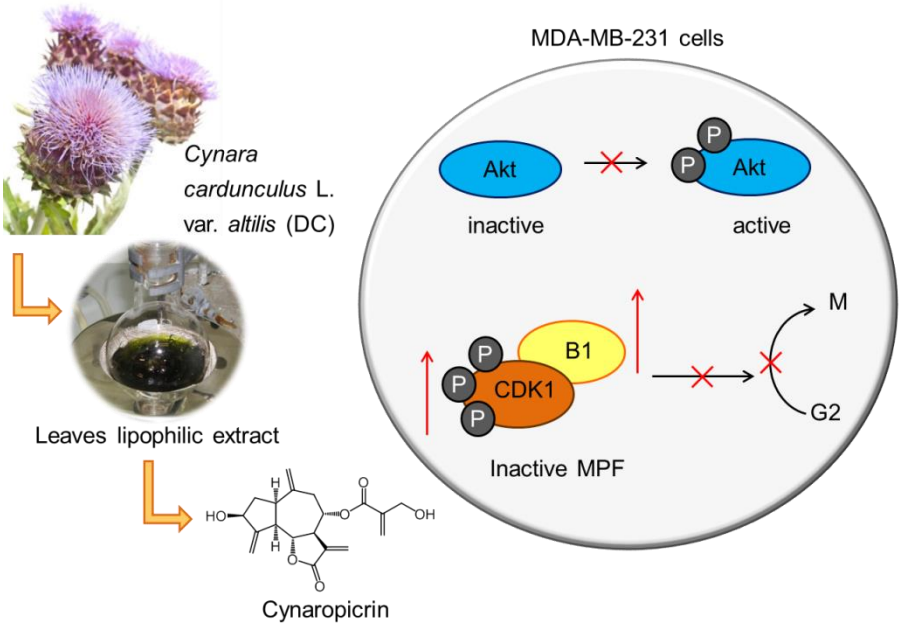
Chapter VI

Antiproliferative activity of *Cynara cardunculus* L. var. *altilis* (DC) extracts on human triple-negative breast cancer (TNBC) MDA-MB-231 cell line



Adapted from:

Ramos, P. A. B.; Guerra, A. R.; Guerreiro, O.; Santos, S. A. O.; Oliveira, H.; Freire, C. S. R.; Silvestre, A. J. D.; Duarte, M. F., Biochemical valorization of *Cynara cardunculus* L. var. *altilis* (DC) lipophilic extractives: antiproliferative activity of sesquiterpene lactones-rich extract on human triple-negative breast cancer MDA-MB-231 cell line, *Ind. Crops Prod.*, **submitted**.

Abstract

This study reports, for the first time, the antiproliferative activity of sequential lipophilic and phenolic-rich extracts from *Cynara cardunculus* L. var. *altilis* (DC), against the human triple-negative breast cancer MDA-MB-231 cell line. Leaves lipophilic extract was the most active cultivated cardoon extract (IC_{50} = 10.39 µg/mL), in inhibiting MDA-MB-231 cellular viability (48 h). Cynaropicrin also prevented MDA-MB-231 cell growth (IC_{50} = 17.86 µM), within the same time period. Moreover, leaves lipophilic extract and cynaropicrin were efficient in preventing colony formation, although leaves lipophilic extract effect was seen in a greater extension. Furthermore, leaves lipophilic extract and cynaropicrin blocked cell cycle at G2 phase, by upregulating $p21^{Waf1/Cip1}$ and, consequently, inducing accumulation of inactive phospho-Tyr15-CDK1 and cyclin B1. The leaves lipophilic extract effect upon MDA-MB-231 cell cycle, at G2 phase, was more evident compared to cynaropicrin. Suppressive activity of leaves lipophilic extract and cynaropicrin on MDA-MB-231 cell proliferation may also be related with decrease expression of phospho-Ser473-Akt. Present at considerably high content (455.2 mg/g extract), cynaropicrin may have greatly contributed for the antiproliferative effect of leaves lipophilic extract on MDA-MB-231 cells. These results showed the promising potential of cultivated cardoon leaves lipophilic extract and cynaropicrin to be researched, as a complementary approach to therapy of human triple-negative breast cancer.

1. Introduction

Breast cancer (BC) has been reported as the second most common form of cancer, and the most incident cancer among women, being the major cause of female cancer death.[1] European countries are among those with the highest incidence rate of breast cancer in women (Figure 1), ranging from 38.7 (Republic of Moldova) to 111.9 (Belgium) per 100,000 persons. Portugal also exhibits a considerable incidence rate of breast cancer among women (67.6 per 100,000 persons). Western Africa countries present the highest mortality rate (20 per 100,000 persons).[1]

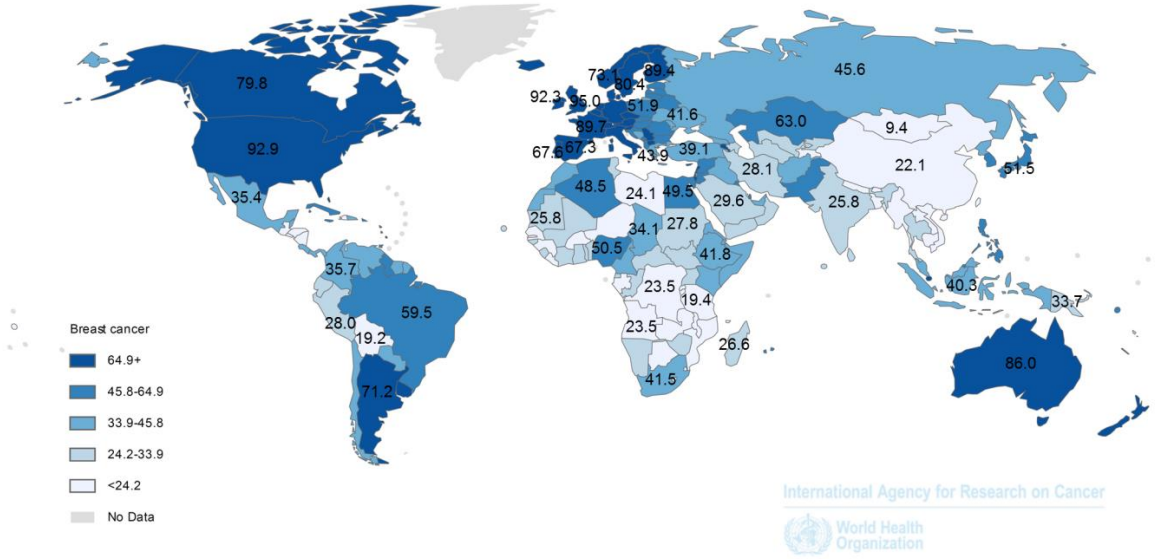


Figure 1: Estimated breast cancer worldwide incidence among women in 2012. The values correspond to estimated age-standardized rates per 100,000 persons (adapted from [1]).

Modern BC classification already includes the triple-negative subtype (TNBC) which is estrogen and progesterone receptors negative, and lack HER2 overexpression.[2] TNBC falls into basal-like BC subtype, based on gene profiling, but it also includes other molecular features. This subgroup shows different clinical behaviors, accounting for 10-20% of all invasive BC.[2] The main risk factors of TNBC in women include: (i) younger age; (ii) African origin; and (iii) BC susceptibility gene 1 mutation.[3] Currently, TNBC has not a targeted treatment; the most common chemotherapy approach is based on primary anthracycline and anthracycline/taxane derivatives therapies, as first line treatment, being in general not efficient, with high relapse risk, during the first three years after treatment,[4] and high incidence of visceral metastases, namely liver, central nervous system and lung.[3] The combination of cellular and molecular knowledge for new therapeutic strategies development, either as preventive or treatment, seems of high importance, especially in a scenario of so limited therapeutic options, with several side effects, and short survival rates. Several epidemiological

studies have indicated that the consumption of vegetables, fruits, fish and olive oil is inversely associated with BC risk.[5–7] The presence of secondary metabolites, namely ω-3 fatty acids, tetraterpenes (e.g., *β*-carotene and lycopene), phytosterols[5–7] and phenolic compounds,[8,9] may contribute for the reduction of BC incidence. Phytochemicals can be therefore considered for chemoprevention, as well as for new therapy strategies or as a complementary approach to BC chemotherapy, due to their promising reduced toxicity, desired range of efficacy and ability to influence simultaneously multiple pathways.[10]

Cynara cardunculus L. (Asteraceae) is a Mediterranean species which comprehends three varieties, namely wild cardoon (var. *sylvestris* (Lamk) Fiori), artichoke (var. *scolymus* (L.) Fiori) and cultivated cardoon (var. *altilis* (DC)). Since the Roman times, *C. cardunculus* L. leaves infusions have been traditionally consumed against hepatobiliary disorders, due to hypocholesterolemic,[11] choleretic and anticholestatic effects of phenolic compounds, such as caffeoylquinic acids and luteolin derivatives.[12] Moreover, artichoke lipophilic extracts evidenced anti-hyperlipidemic[13] and anti-inflammatory properties,[14] mainly due to the presence of sesquiterpene lactones[13] and pentacyclic triterpenes,[14] respectively. Some studies have reported the antiproliferative action of artichoke methanol extracts, by regulating apoptosis pathway, upon human hepatocarcinoma (Hep G2 cell line)[15] and TNBC cells (MDA-MB-231 cell line),[16] probably attributed to phenolic composition. Moreover, our group demonstrated the suppressive action of a methanol aqueous extract of cultivated cardoon leaves, on 48 h-cell growth of MDA-MB-231, but it was missing a detailed chemical composition.[17] Regarding *C. cardunculus* L. lipophilic extracts, their antitumoral potential has been less explored. At the current knowledge, only one study explained the *in vivo* inhibitory effect of artichoke capitula extract (composed by taraxasterol derivatives, among other pentacyclic triterpenes), on the 12-*O*-tetradecanoylphorbol-13-acetate tumor promotion of skin papilloma, in 7,12-dimethylbenz[*a*]anthracene-initiated mouse model.[14]

Recently, our group revealed that leaves and capitula florets of cultivated cardoon contained high abundances of sesquiterpene lactones and pentacyclic triterpenes respectively. Cynaropicrin (≈87.4 g/kg dw) and taraxasteryl acetate (≈8.9 g/kg dw) (Figure 2) represented the major sesquiterpene lactone and pentacyclic triterpene, respectively.[18] Few researchers have described that cynaropicrin prevented the cellular viability of several human tumor cell lines,[19,20] including the BC MCF-7 (estrogen receptor-positive) cell line.[19] Furthermore, the taraxasteryl acetate ingestion incremented the survival ratio of C3H/OuJ mammary tumor bearing mice to 66.6%, compared to control group, after 70 weeks. The taraxasterol treatment was even stronger than that with its acetate derivative, increasing the survival ratio to 80%.[21] Regarding the phenolic fraction, our group also published a study describing the high contents of hydroxycinnamic acids and flavonoids in stalks outer part and capitula florets, respectively. The 1,5-di-*O*-caffeoylquinic acid (Figure 2) was one of the main

hydroxycinnamic acid found in cultivated cardoon (≈4.2 g/kg dw).[22] Mileo, *et al.* (2012) reported the antiproliferative activity of 5-*O*-caffeoylquinic acid on TNBC MDA-MB-231 cell line, by increasing the percentage of apoptotic cells in a dose-dependent manner (200-1200 μM), for 24 h.[16]

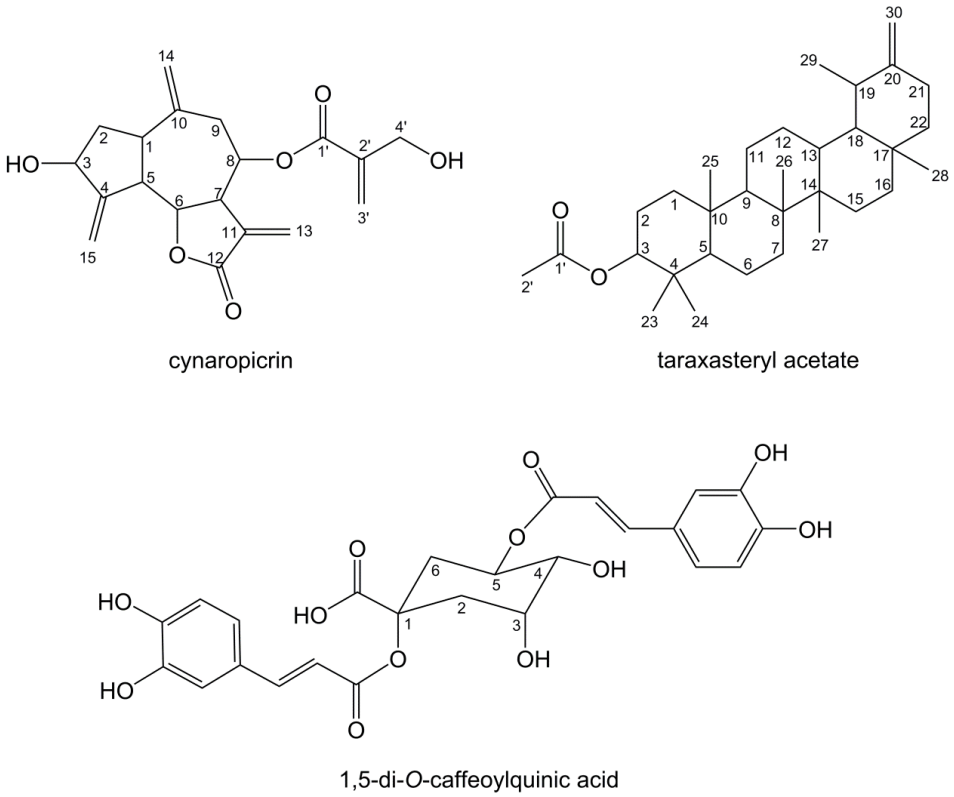


Figure 2: Structures of cynaropicrin, taraxasteryl acetate and 1,5-di-*O*-caffeoylquinic acid.

Following our research of new target bioactive-based TNBC treatment, this work aims to study the cell growth inhibitory action of lipophilic leaves and capitula florets, as well as of phenolic-rich extracts of stalks outer part and capitula florets, derived from cultivated cardoon on a TNBC cell line model (MDA-MB-231). The inhibitory effect of pure cynaropicrin, taraxasteryl acetate and 1,5-di-*O*-caffeoylquinic acid (Figure 2) on MDA-MB-231 cell growth was also assessed. The most effective antiproliferative extract and the related pure compound were further evaluated in terms of: (i) colony formation in an anchorage-independent growth assay; (ii) apoptosis assessment through caspase-3 activity; (iii) cell cycle analysis; (iv) protein expression of key cell cycle checkpoints; and (v) evaluation of Akt molecular cell signaling.

2. Materials and Methods

2.1 Chemicals

Dichloromethane (p.a. ≥99% purity) was supplied by Fischer Scientific (Pittsburgh, Pennsylvania, USA). *p*-Iodonitrotetrazolium violet, bovine serum albumin (BSA) (≥96% purity) and RNase were obtained from Sigma Chemicals Co. (Madrid, Spain). Cynaropicrin (≥97.5% purity) was purchased from Extrasynthese (Genay Cedex, France). Taraxasteryl acetate (≥99.2% purity) was provided by Avachem Scientific (San Antonio, Texas, USA). 1,5-di-*O*-caffeoylquinic acid (≥98% purity) was supplied by Biopurify (Chengdu Biopurify Phytochemicals Ltd., Chengdu, China). Dulbecco's modified Eagle's medium (DMEM) 4.5 g/L glucose and L-glutamine, fetal bovine serum (FBS) and trypsin (5 g/L)/EDTA (2 g/L) were obtained from Lonza (Verviers, Belgium). Penicillin (10000 units/mL)-streptomycin (10 mg/mL) mixture was provided by BioWest (Nuaillé, France). Dimethyl sulfoxide (DMSO) cell culture grade was supplied from Applichem (Gatersleben, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and propidium iodide was purchased from Calbiochem (San Diego, California, USA). Caspase-3/CPP32 colorimetric protease assay was provided by Invitrogen (Camarillo, California, USA). Anti-mouse horseradish peroxidase-conjugated secondary, cyclin-dependent kinase (CDK) inhibitory $p21^{Waf1/Cip1}$ (hereafter referred as p21), cyclin B1 and phospho(Ser473)-Akt antibodies (hereafter referred as p-Ser473-Akt) were supplied by Cell Signaling Technology (Danvers, Massachusetts, USA). Donkey anti-goat horseradish peroxidase-conjugated secondary, Akt1 and *β*-actin antibodies were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA), and phospho(Tyr15)-CDK1 (hereafter referred as p-Tyr15-CDK1) antibody was purchased from BD Biosciences (San Jose, California, USA). ECL reagents were provided by GE Healthcare Life Sciences (Buckinghamshire, U.K.).

2.2 Preparation of *C. cardunculus* L. var. *altilis* (DC) extracts

C. cardunculus L. var. *altilis* (DC) (cultivated cardoon) was collected at the flowering stage, in June 2010, at the Experimental Center of the School of Agriculture from Instituto Politécnico de Beja, Baixo Alentejo region, south Portugal. Stalks, capitula and leaves were separated and preserved at -20 ºC until analysis.

Lipophilic extracts, derived from cultivated cardoon leaves and capitula florets, were prepared and analyzed by gas chromatography-mass spectrometry, as indicated elsewhere.[18] The phenolic-rich extracts were prepared, after the removal of lipophilic components, from the stalks outer part and capitula florets, and thereafter analyzed by liquid chromatography-mass spectrometry, as earlier explained.[22]

Lipophilic extracts of capitula florets and leaves contained respectively: 320.6 and 38.5 mg/g extract of pentacyclic triterpenes; 8.8 and 3.4 mg/g extract of sterols; and 14.4 and 2.4 mg/g extract of fatty acids. Sesquiterpene lactones were only present in leaves lipophilic extract, accounting for 484.9 mg/g extract, mainly represented by cynaropicrin (455.2 mg/g extract). Phenolic-rich extracts of stalks outer part and capitula florets contained respectively: 53.9 and 0.7 mg/g extract of hydroxycinnamic acids, and 3.6 and 22.5 mg/g extract of flavonoids. The detailed chemical composition of lipophilic and phenolic-rich extracts of cultivated cardoon may be consulted in Appendix.

2.3 Cell culture

The human TNBC MDA-MB-231 cell line was purchased from American Type Cell Culture (ATCC, Manassas, Virginia, USA). Cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin–streptomycin mixture, at 37 ºC in a 5% CO_2 humidified atmosphere (C150, Binder GmbH, Tuttlingen, Germany). Before confluence, cells were washed with phosphate buffered saline (PBS), collected following trypsinization with trypsin (0.5 g/L)/EDTA (0.2 g/L) solution and suspended in fresh growth medium before platting.

2.4 Cell viability

Stock solutions of cultivated cardoon lipophilic extracts, as well as of pure cynaropicrin and taraxasteryl acetate were prepared in DMSO. Phenolic-rich extracts and pure 1,5-di-*O*-caffeoylquinic acid were prepared in 24% ethanol solution. Cells were seeded in 96-well plates at 2×10^5 cells/mL density, and incubated for 24 h, at 37 ºC. Then, cells were treated with capitula florets and leaves lipophilic extracts (1-500 µg/mL), stalks outer part and capitula florets phenolic-rich extracts (2000-4500 µg/mL), cynaropicrin (0.1-150.0 µM), taraxasteryl acetate (0.1-5.0 µM) and 1,5-di-*O*-caffeoylquinic acid (1000-2500 µM) for 48 h. The respective vehicle solvent controls received DMSO and ethanol (<1% (v/v)). Cell viability was determined through MTT assay. This method lies on the reduction of tetrazolium salt MTT, by dehydrogenases in active mitochondria, to formazan derivative which is monitored through ultraviolet-visible spectroscopy.[23] Cells were thus incubated with 20 µL, per well, of MTT stock solution (final concentration 0.5 mg/mL) in PBS, followed by 4 h of incubation, at 37 ºC. The medium was then discarded, and formazan crystals were solubilized in 100 µL of DMSO/ethanol (1:1) solution. The absorbance was read against a blank (ethanol/DMSO (1:1)) at 570 nm, by using Multiskan^{TM} FC microplate UV/Vis photometer (Thermo Scientific, Waltham, Massachusetts, USA). Results were expressed as the percentage of cell viability relative to that of the respective solvent control. The IC_{50}, defined as the sample concentration necessary to cause 50% inhibition of cell viability, was calculated by plotting the percentage of

cell viability in function of sample concentration logarithm. Triplicates were performed in three independent experiments for each treatment.

2.5 Soft agar colony formation assay

MDA-MB-231 cells (9200 cells per well) were seeded in 0.336% agar on top of 0.5% pre-solidified agar, in a 12-well plate. Each treatment was performed in triplicate. After 24 h of incubation at 37 ºC, cells were treated with the IC_{50} values of leaves lipophilic extract (10.39 µg/mL) and cynaropicrin (17.86 µM). Vehicle solvent control cells received DMSO (0.09% (v/v)). Every other day, agar layers were supplemented according to the above mentioned concentrations. After 14 consecutive days, cells were overnight incubated with 0.1% *p*-iodonitrotetrazolium violet, at 37 ºC. Colonies were thereafter observed by using an inverted microscope (Motic, Xiamen, P. R. China), at the 40 x magnification, and four fields of each well were photographed using Moticam 2500 camera (Motic, Xiamen, P. R. China). The images were processed using Motic Images Plus 2.0 program (Motic, Xiamen, P. R. China).

2.6 Assessment of caspase-3 activity

Caspase-3 activity was assessed according to manufacturer's protocol (Caspase-3/CPP32 colorimetric protease assay, Invitrogen, Camarillo, California, USA). DEVD-*p*NA, composed by the synthetic tetrapeptide substrate Asp-Glue-Val-Asp (the upstream amino acid sequence of the caspase-3 cleavage site in poly(adenosine diphosphate-ribose) polymerase); and tailed with *p*-nitroanilide (*p*-NA) chromophore. MDA-MB-231 cells were seeded at $5x10^5$cells/mL, and after 24 h-incubation, cells were treated with IC_{50} values of leaves lipophilic extract (10.39 µg/mL) and cynaropicrin (17.86 µM). Vehicle solvent control cells were exposed to DMSO (0.09% (v/v)). After 48 h, cells were collected and centrifuged at 769 x *g* for 5 min. The cell pellet (5 x 10^6 cells) was resuspended in 50 µL of chilled cell lysis buffer and incubated on ice for 10 min. After centrifugation at 10,000 x *g* for 1 min at 4 ºC, supernatant (cytosol) was collected to examine caspase-3 activity. Total protein concentration was assayed according to Lowry method[24] and BSA was used as protein standard. Cytosol samples (150 µg protein) were mixed with 50 µL of 2 x reaction buffer (10 mM dithiothreitol), and 5 µL of 4 mM DEVD-*p*NA. After 2 h-incubation, at 37 ºC in the dark, the absorbance of *p*-NA was read at 405 nm against a blank (without sample), by using Multiskan™ FC microplate UV/Vis photometer (Thermo Scientific, Waltham, Massachusetts, USA). Three independent experiments were carried out.

2.7 Cell cycle analysis

MDA-MB-231 cells were cultured in six-well plates at a density of 4 x 10^5 cells/mL for 24 h at 37 ºC. Then, cells were exposed to the IC_{50} values of leaves lipophilic extract (10.39 µg/mL) and cynaropicrin (17.86 µM). Vehicle solvent control cells received DMSO (0.09% (v/v)). After 48 h-incubation, cells were collected, PBS washed and fixed with 85% cold ethanol. Cell pellets were collected after centrifugation at 300 x *g* for 5 min at 4 °C, and resuspended in PBS. Then, cells were incubated with 50 µg/mL RNase and 50 µg/mL propidium iodide staining solution for 20 min at room temperature in dark. Propidium iodide-stained cells were analyzed in the Beckman-Coulter® EPICS-XL (Beckman-Coulter®, Brea, California, USA) flow cytometer equipped with an air-cooled argon-ion laser (15 mW, 488 nm). Results were obtained using the SYSTEM II software (version 3.0 Beckman-Coulter®, Brea, California, USA), in which at least 5000 nuclei per sample were acquired. Analysis of cell cycle distribution was performed by using the FlowJo software (Tree Star, Ashland, Oregan, USA). Four replicates were performed for each treatment.

2.8 Western blotting

MDA-MB-231 cells were plated at 5 x 10^5 cells/mL, and incubated for 24 h at 37 ºC. Medium was thereafter changed to medium containing IC_{50} values of leaves lipophilic extract (10.39 µg/mL) or cynaropicrin (17.86 µM). Vehicle solvent control cells were incubated with DMSO (0.09% (v/v)). After 48 h-incubation, cells were scraped, washed in cold PBS and centrifuged at 492 x *g* for 3 min, at 4 ºC. This procedure was repeated two more times. Cells were then lysed with RIPA buffer (1% NP-40 in 150 mM NaCl, 50 mM Tris-HCl (pH 8), 2 mM EDTA), containing 1 mM phenylmethylsulfonylfluoride, phosphatase inhibitors (20 mM NaF, 20 mM $Na_2V_3O_4$), and protease inhibitor cocktail (Roche, Mannheim, Germany), for 10 min, at 4 ºC. Cell lysates were centrifuged at 24104 x *g* for 10 min, at 4 ºC. Supernatants were collected, and total protein concentrations were quantified according to the Lowry method,[24] using BSA as the protein standard. Cell lysates (25-40 µg protein) were electrophoresed on sodium dodecyl sulfate 10% polyacrylamide gel, and then transferred onto poly(vinylidene difluoride) (PVDF) membranes (Amersham Biosciences, Buckinghamshire, UK). PVDF membranes were blocked with 5% (w/v) of nonfat dry milk at room temperature for 1 h, and incubated overnight at 4°C with a primary antibody against p21 (1:2000); cyclin B1 (1:1000); p-Tyr15-CDK1 (1:250); Akt1 (1:200); p-Ser473-Akt (1:1000); and *β*-actin (1:300). Bands were visualized by chemiluminescence using appropriate horseradish peroxidase-conjugated secondary antibodies, and developed with ECL reagents (Amersham Biosciences, Buckinghamshire, UK), according to manufacturer's instructions. Three independent experiments were performed for each treatment.

2.9 Statistical analysis

All parameters measured were analyzed using the PROC GLM option of SAS (SAS Institute Inc., Cary, North Carolina, USA). In the case of the statistical analysis of cell cycle data, the pairwise multiple comparisons were performed between treatments for each phase (G0/G1, S and G2). Where differences existed, the source of the differences at $p < 0.05$ of significance level was identified by all pairwise multiple comparison procedure, through the Duncan's test.

3. Results and Discussion

3.1 Inhibitory effects of cultivated cardoon lipophilic and phenolic-rich extracts on MDA-MB-231 cell growth

After the chemical characterization of cultivated cardoon (Chapters III and IV), two lipophilic and two phenolic-rich extracts were chosen to assess their MDA-MB-231 cell growth inhibition effects, during 48 h. In this manner, lipophilic extracts of capitula florets and leaves were evaluated, since they presented the highest content of pentacyclic triterpenes and sesquiterpene lactones, respectively (Chapter III). Moreover, phenolic-rich extracts of stalks outer part and capitula florets were also examined, as they displayed the highest concentration of hydroxycinnamic acids and flavonoids, respectively (Chapter IV). In order to evaluate the 48 h-inhibitory potential of cultivated cardoon extracts, on MDA-MB-231 cell viability, their IC_{50} values were determined through the MTT assay, as listed in Table 1. For comparison reasons, the inhibitory actions of pure compounds of cynaropicrin and 1,5-di-*O*-caffeoylquinic acid were also examinated, at the same experimental conditions (Table 1).

Table 1: IC_{50} values regarding the 48 h-cell growth inhibition of cultivated cardoon lipophilic and phenolic-rich extracts on the MDA-MB-231cell line, determined through MTT assay.

Cultivated cardoon extract/reference compound	IC_{50} upon MDA-MB-231 cell line	
	µg/mL	µM
Capitula florets lipophilic extract	315.22 ± 67.88 ^{c}	–
Leaves lipophilic extract	10.39 ± 0.41 ^{d}	–
Stalks outer part phenolic-rich extract	3341.20 ± 246.85 ^{a}	–
Cynaropicrin	6.19 ± 0.57 ^{d}	17.86 ± 1.65 ^{b}
1,5-Di-*O*-caffeoylquinic acid	899.50 ± 21.33 ^{b}	1741.69 ± 41.29 ^{a}

Results correspond to mean±standard deviation. Three independent experiments with triplicates were performed. Means with different superscript letters within the same column are statistically different ($p < 0.05$).

The IC_{50} value of leaves lipophilic extract against MDA-MB-231 cell proliferation, was 30-fold lower compared to that of capitula florets lipophilic extract (10.39 and 315.22 µg/mL, respectively) ($p < 0.0087$) (Table 1). The pure compound of cynaropicrin showed an IC_{50} value of 17.86 µM on MDA-MB-231 cell growth (Table 1). It was previously described that cynaropicrin suppressed the MCF-7 cell growth (human estrogen receptor-positive BC cell line), with an IC_{50} value of 3.18 µM.[19] Regarding the pure compound of taraxasteryl acetate, the IC_{50} value may be higher than 5.00 µM (2.34 µg/mL), but it was not determined due to solubility issues. It had earlier demonstrated that taraxasteryl acetate (42.67 µM) did not suppress the cellular viability of a BC cell line.[25]

The *α*-methylene-*γ*-lactone moiety present in sesquiterpene lactones is known to play a key role in cytotoxicity, as alkylating reactivity center.[26,27] The *α*,*β*-unsaturated ketone ($O=C-C=CH_2$), either in lactone, cyclopentenone ring or in an ester side chain, reacts with biological nucleophiles, namely thiol groups present in cysteine residues of proteins, through Michael addition reaction.[26,28] Cho, *et al.*[29] proposed that cynaropicrin may have reacted with thiol group of target protein(s), preventing therefore TNF-*α* production. Additionally, there are other structural moieties in sesquiterpene lactones that have been recognized to enhance their cytotoxicity *in vitro*, namely the presence of a side chain and its lipophilicity, as well as the molecular geometry and electronic features.[26] Bruno, *et al.*[30] noticed that the absence of unsaturated ester side chain, at C8 in germacranes, greatly decreased the cytotoxicity action, against several human tumor cells *in vitro*, including MDA-MB-231 cell line. Furthermore, Scotti, *et al.*[31] previously noted that the presence of a methylene group, at C10 in guaianolides, increased the inhibitory effect against human KB cells, nasopharynx tumor cell line. The unsaturated ester side chain at C8 and the methylene group at C10 are two structural features present in cynaropicrin (Figure 2), in addition to *α*-methylene-*γ*-lactone moiety, which may have contributed to the suppressive effect on MDA-MB-231 cell growth.

Cynaropicrin was identified as the major identified component of leaves lipophilic extract (455.2 mg/g extract). Therefore, this sesquiterpene lactone was, most probably, the main responsible for the inhibitory action of leaves lipophilic extract, upon MDA-MB-231 cell growth. In fact, leaves lipophilic extract and cynaropicrin IC_{50} values, when expressed as µg/mL, were not statistically different ($p > 0.05$). Nonetheless, it cannot be discarded the hypothesis that the suppressive effect of lipophilic leaves extract might also have resulted from synergistic or cumulative actions of cynaropicrin, together with other extract component(s), which needs to be further researched.

The IC_{50} value of stalks outer part phenolic-rich extract (3341.20 µg/mL) (Table 1) was much higher than those of lipophilic extracts from florets (11-fold) and leaves (322-fold). Moreover, the IC_{50} of florets phenolic-rich extract may be higher than 4500 µg/mL. MDA-MB-231 cells were thus more sensitive to lipophilic extracts than to phenolic-rich extracts. In what

concerns to the inhibitory effect of 1,5-di-*O*-caffeoylquinic acid, the respective IC_{50} value was 97.5-fold higher (1741.69 µM) (Table 1) compared to cynaropicrin ($p < 0.0001$), reflecting that MDA-MB-231 cells were much less resistant to cynaropicrin than to 1,5-di-*O*-caffeoylquinic acid.

The underlying biochemical mechanisms of cultivated cardoon leaves lipophilic extract, and its major compound cynaropicrin, on MDA-MB-231 cells were thereafter investigated.

3.2 Effect of cultivated cardoon leaves lipophilic extract and cynaropicrin on the formation of MDA-MB-231 cell colony

Soft agar has proven to be a powerful technique for screening active agents against the growth of several types of tumor cells, in a three-dimensional environment.[32] Following the strong effect upon cellular viability, in the two-dimensional monolayer assay, more insights were sought to know whether the leaves lipophilic extract and cynaropicrin, at the respective IC_{50} values, could also suppress the formation of MDA-MB-231 cell colonies, in a three-dimensional environment, using an anchorage-independent growth assay.

Treatments with 10.39 µg/mL leaves lipophilic extract and 17.86 µM cynaropicrin changed the size of MDA-MB-231 colonies, relatively to DMSO control (Figure 3A). Additionally, leaves lipophilic extract strongly prevented MDA-MB-231 cells to form colonies (Figure 3B), up to 19.8-fold regarding DMSO control cells ($p < 0.0002$). Similarly, MDA-MB-231 cells lost their ability to form colonies, after being exposed to 17.86 µM cynaropicrin (Figure 3B); however, this effect was much less marked (up to 2.4-fold regarding to DMSO control cells ($p < 0.0030$)). Once again, cynaropicrin probably contributed for the inhibitory effect of leaves lipophilic extract, upon the formation of MDA-MB-231 colonies, but other extract component(s) may also be involved.

At the current knowledge, this is the first time that inhibitory effects of cultivated cardoon leaves lipophilic extract and cynaropicrin, upon BC colony formation, are evidenced. Rao, *et al.*[33] previously demonstrated that antrocin, a sesquiterpene lactone isolated from the medicinal mushroom *Antrodia camphorata* (Polyporaceae), was able to prevent the formation of TNBC MDA-MB-231 colonies, after 10-14 days in an anchorage-independent growth assay, at dose-dependent manner (1-10 µM).

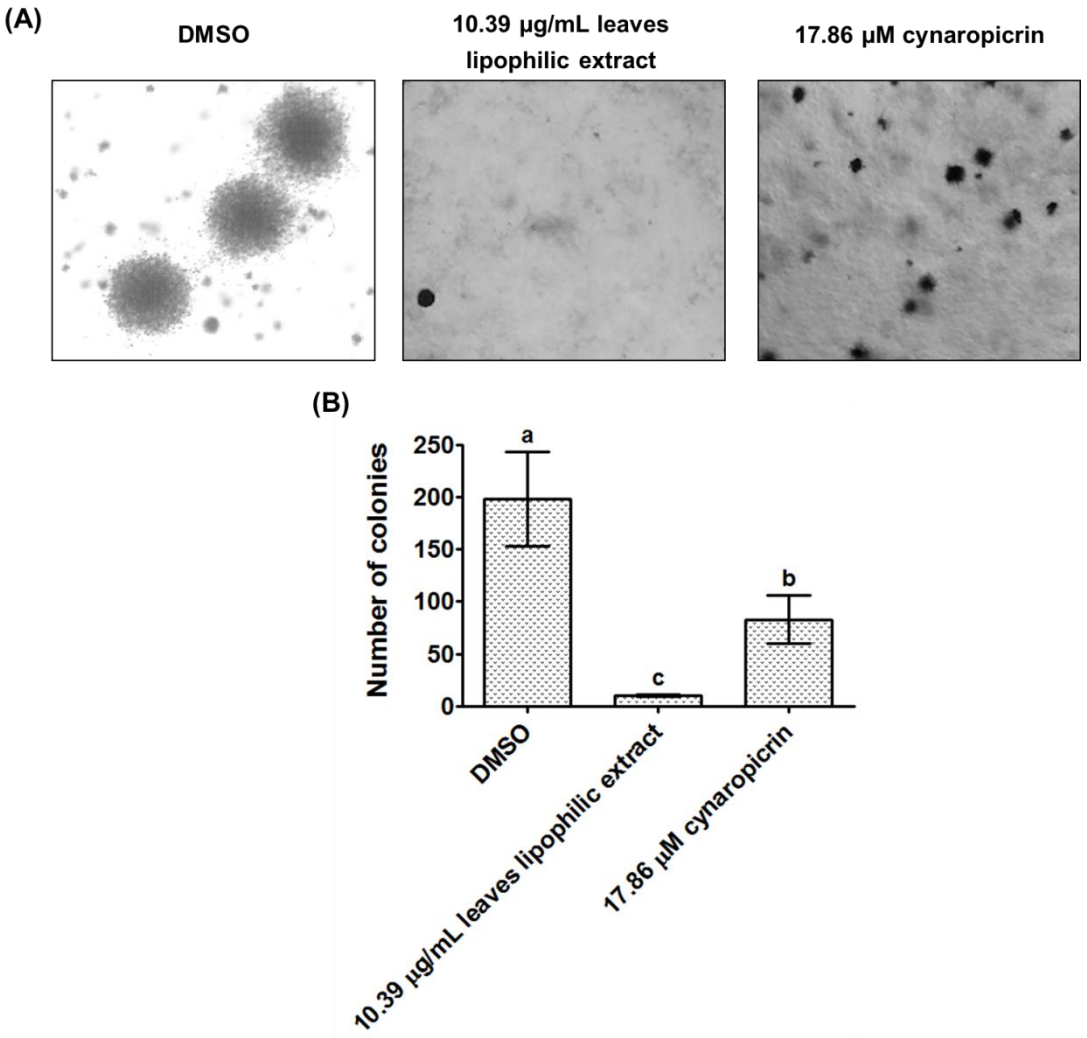


Figure 3: Effect of 10.39 µg/mL cultivated cardoon leaves lipophilic extract and 17.86 µM cynaropicrin upon formation of MDA-MB-231 colonies in soft agar, after 14 days: (A) representative photographs of colonies (40 x magnification); (B) number of colonies. Each column and bar represents respectively the mean and the standard deviation. Triplicates were carried out. Columns with different letters are statistically different ($p < 0.05$).

3.3 Measurement of caspase-3 activity in MDA-MB-231 cells, after treatment with cultivated cardoon leaves lipophilic extract and cynaropicrin

Regarding the antiproliferative effects of cultivated cardoon leaves lipophilic extract and cynaropicrin, the hypothesis of apoptosis was studied by assessing caspase-3 activity through a commercial colorimetric kit (Figure 4). Caspase-3 is considered as the most important caspase within the execution phase of apoptosis, because its active form specifically induces the Caspase Activated DNAse, causing chromosomal DNA degradation and chromatin condensation.[34]

After 48 h-incubation, leaves lipophilic extract increased the caspase-3 activity of MDA-MB-231 cells (1.3-fold *vs* DMSO control ($p < 0.0013$)) (Figure 4). On the other hand, MDA-MB-231 cells treated with 17.86 µM cynaropicrin did not significantly affect the caspase-3 activity, in relation to DMSO-treated control cells ($p > 0.05$). Contrarily, it was previously reported that cynaropicrin induced apoptosis in human gastric adenocarcinoma AGS cells, in dose (1-5 µM) and time (24 and 48 h) dependent ways.[35] Moreover, another study showed cynaropicrin (10 µM) as an apoptosis inducer, in human leukocyte U937 cells, being associated with reactive oxygen species generation and the proteolytic cleavage of protein kinase C.[36] More investigation should be carried out to know whether leaves lipophilic extract and cynaropicrin caused apoptosis in MDA-MB-231 cells.

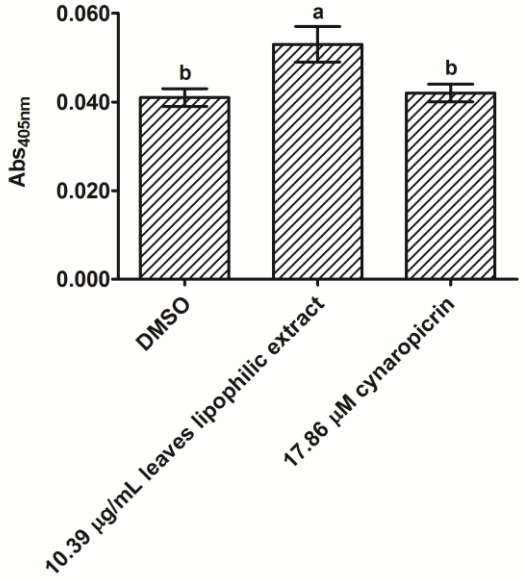


Figure 4: Caspase-3 activity assessment in the MDA-MB-231 cells, after 48 h-treatment with 10.39 µg/mL cultivated cardoon leaves lipophilic extract and 17.86 µM cynaropicrin. Each column and bar represents respectively the mean and the standard deviation. Three independent experiments were carried out. Columns with different letters are statistically different ($p < 0.05$).

3.4 Effect of cultivated cardoon leaves lipophilic extract and cynaropicrin on the MDA-MB-231 cell cycle

Flow cytometry was applied to gain further insights about the suppressive actions of cultivated cardoon leaves lipophilic extract and cynaropicrin, upon the MDA-MB-231 cells distribution through the different cell cycle phases (G0/G1, S and G2 phases) (Figure 5).

The 48 h-treatment with 10.39 µg/mL leaves lipophilic extract led to a significantly accumulation of 59.5% of MDA-MB-231 cells at G2 phase (Figure 5), representing a 4.7-fold cell percentage increase, regarding to DMSO control cells ($p < 0.0001$). Thereafter, there was

a corresponding decrease of cell percentage at G0/G1 and S phases (2.7- and 1.8-fold relatively to DMSO control cells, respectively). Leaves lipophilic extract thus induced cell cycle arrest at G2 phase. Similarly, cynaropicrin tested at 17.86 µM, for 48 h, also prevented MDA-MB-231 cells to enter into mitosis (37.8%), by blocking cell cycle progression at G2 phase (Figure 5), but in a less extension (3-fold increase of cell percentage compared to DMSO control cells ($p < 0.0001$)). Additionally, the cynaropicrin treatment reduced 2.1-fold the S phase cell percentage ($p < 0.0001$), but it did not change G0/G1 phase cell percentage, compared to DMSO control cells ($p > 0.05$). This finding corroborated with the G2 cell cycle arrest of human gastric AGS cells, caused by cynaropicrin at dose (1-5 µM) and time (24 and 48 h) dependent ways.[35] On the other hand, another study revealed that the incubation of human leukocyte U937 cells with cynaropicrin (10 µM), for 12 and 24 h, led to G1 phase cell accumulation.[36]

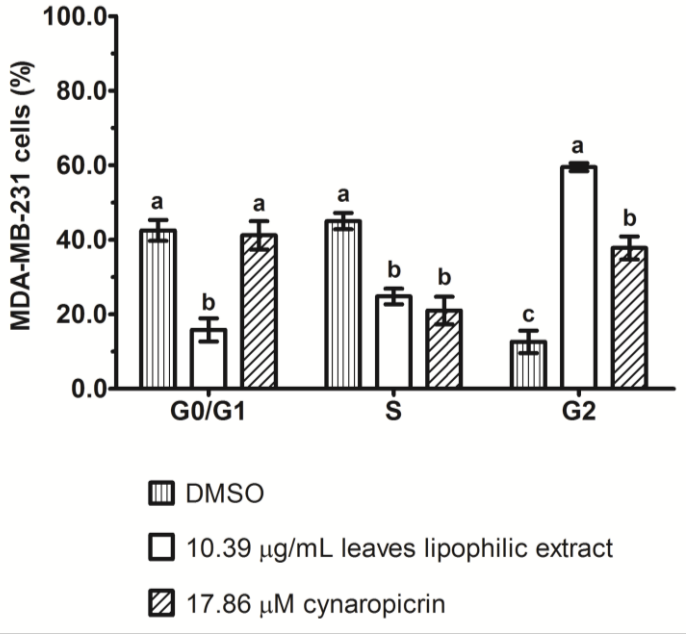


Figure 5: Cell cycle phase distribution of MDA-MB-231 cells, treated with 10.39 µg/mL cultivated cardoon leaves lipophilic extract and 17.86 µM cynaropicrin, after 48 h-incubation. DMSO was the solvent control. Each column and bar represents respectively the mean and the standard deviation. Four replicates were performed. Columns within each cell cycle phase presenting different letters are statistically different ($P < 0.05$).

3.5 Effect of cultivated cardoon leaves lipophilic extract and cynaropicrin on the p21$^{Waf1/Cip1}$, p-Tyr15-CDK1 and cyclin B1 protein expression in MDA-MB-231 cells

The protein expressions of three G2/M checkpoint regulators, namely p21, p-Tyr-15-CDK1 and cyclin B1, were examined (Figure 6), in order to understand the molecular mechanisms underlying the G2 cell cycle arrest in MDA-MB-231 cells, caused by cultivated cardoon leaves lipophilic extract and cynaropicrin.

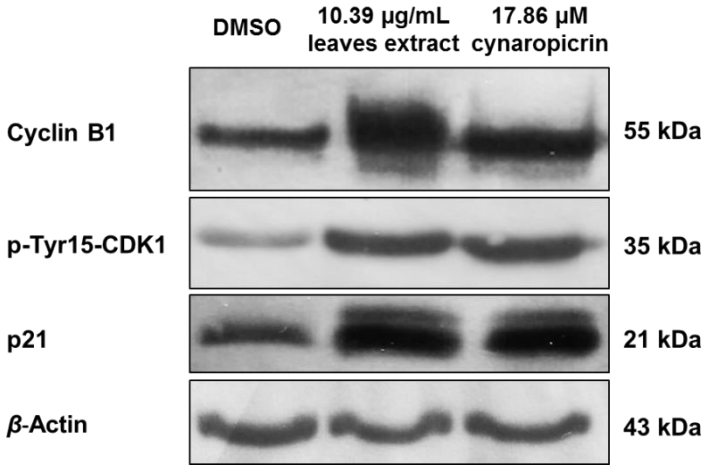


Figure 6: Western blot analysis of cyclin B1, phospho-Tyr 15-Cdk1, p21 and *β*-actin protein expressions in MDA-MB-231 cells, after 48 h-treatment with 10.39 µg/mL cultivated cardoon leaves lipophilic extract and 17.86 µM cynaropicrin. The blots shown are representative of three independent experiments.

Belonging to the class of kinase inhibitor proteins, p21 inhibits the kinase activity of CDK2 bonded to cyclin E and cyclin A during G1/S transition, as well as the CDK1-2/cyclin A and CDK1/cyclin B complexes activity required for G2/M cell cycle progression.[37,38] Moreover, it was proven that p21 mediates the nuclear retention of mitosis promoting factor (MPF), formed by CDK1/cyclin B1, thereby impeding its activation, through CDK1 dephosphorylation at Thr14 and Tyr15 by Cdc25 fosfatase.[39] Besides the periodic accumulation and degradation of cyclin B1, MPF activity is controlled by the phosphorylation and dephosphorylation of CDK1. The activation of MPF is truly decisive for the transition of mammalian cells from G2 to mitosis.

According to Figure 6, p21 protein expression increased in the 48 h-treated MDA-MB-231 cells with 10.39 µg/mL leaves lipophilic extract and 17.86 µM cynaropicrin, regarding to DMSO control cells. Furthermore, cyclin B1 and p-Tyr15-CDK1 protein expressions also increased in MDA-MB-231 cells after both treatments, compared to DMSO control cells (Figure 6). In this manner, cultivated cardoon leaves lipophilic extract and cynaropicrin induced p21 expression in MDA-MB-231 cells, which may be associated with the

accumulation of inactive p-Tyr15-CDK1 and cyclin B1, and consequently with the G2 cell cycle arrest.

So far, the regulating effects of cultivated cardoon leaves lipophilic extract and cynaropicrin on G2/M cell cycle checkpoint, in human tumor cells, was herein described for the first time. A previous report noticed that p21, p-Tyr15-Thr14-CDK1 and cyclin B1 protein expressions increased in G2/M arrested SK-28 human melanoma cells, after treatment (up to 4 h) with two sesquiterpene lactones isolated from *Inula viscosa* (Asteraceae), namely tomentosin and inuviscolide (18-54 μM).[40] Future investigation should be performed to know more about the regulating role of leaves lipophilic extract and cynaropicrin, upon key players in G2/M checkpoint.

3.6 Effect of cultivated cardoon leaves lipophilic extract and cynaropicrin on the p-Ser473-Akt and Akt1 protein expression in MDA-MB-231 cells

Akt has an important role in the glucose metabolism, survival, cell proliferation and programmed cell death.[41,42] Active Akt indeed occurs frequently in human cancer cells.[42] In this study, Akt1 and active p-Ser-473-Akt protein expressions, in MDA-MB-231 cells, were thus evaluated after 48 h-incubation with cultivated cardoon lipophilic extract and cynaropicrin, at the respective IC_{50} values (Figure 7).

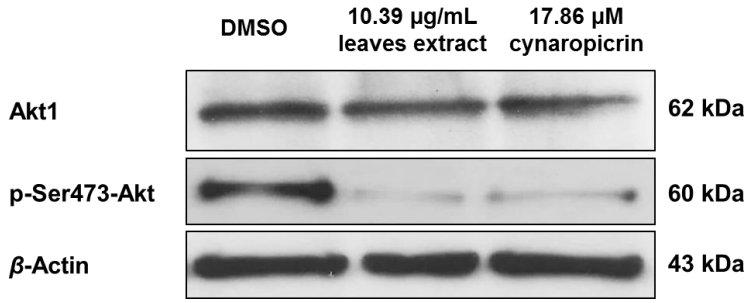


Figure 7: Western blot analysis of total Akt1, phospho-Ser-473-Akt and *β*-actin protein expressions in MDA-MB-231 cells after 48 h-treatment with 10.39 μg/mL cultivated cardoon leaves lipophilic extract and 17.86 μM cynaropicrin. The blots shown are representative of three independent experiments.

Notably, treatments with leaves lipophilic extract and cynaropicrin greatly decreased active p-Ser473-Akt protein expression in MDA-MB-231 cells, relatively to DMSO control (Figure 7). Nevertheless, total Akt1 protein expression in treated MDA-MB-231 cells remained unchanged (Figure 7). Therefore, decreased p-Ser473-Akt protein levels, caused by leaves lipophilic extract and cynaropicrin, were not influenced by variations in Akt1 protein expression. In this way, these data suggest that Akt signaling downregulation may be involved within the overall inhibitory effects of leaves lipophilic extract and cynaropicrin, upon TNBC

MDA-MB-231 cellular growth. At the present knowledge, few studies revealed the inhibitory actions of sesquiterpene lactones in the Akt signaling pathway. Rao, *et al.*[33] explained that antrocin (10 µM) caused downregulation of p-Ser473-Akt protein expression in MDA-MB-231 cells, in time dependent way (6-48 h), with no changes in total Akt protein level. These results indicate that it is necessary to gain more insights about the underlying mechanisms of leaves lipophilic extract and cynaropicrin, within the PI3K/Akt/mTOR pathway.

4. Conclusions

This work opens new perspectives for *C. cardunculus* L. var. *altilis* (DC) valorization, through the study of the antiproliferative effect of cultivated cardoon extracts and pure secondary metabolites, upon MDA-MB-231 cell line, in an effort to contribute for a potentially natural-based TNBC therapeutics. MDA-MB-231 cells were less resistant to cultivated cardoon lipophilic extracts, presenting sesquiterpene lactones and pentacyclic triterpenes, comparatively to phenolic-rich extracts. Cultivated cardoon leaves lipophilic extract and pure compound of cynaropicrin, representative of the main identified extract compound, inhibited strongly the TNBC MDA-MB-231 cell growth (IC_{50} = 10.39 µg/mL and 17.86 µM, respectively), for 48 h. Moreover, leaves lipophilic extract also suppressed the anchorage-independent growth of MDA-MB-231 cells, in soft agar, being even more active than cynaropicrin.

Although leaves lipophilic extract slightly increased the caspase-3 activity, more details are required to understand whether lipophilic components of leaves extract stimulated the MDA-MB-231 cell death, through apoptosis. Furthermore, leaves lipophilic extract caused G2 cell cycle arrest of MDA-MB-231 cells, in a more extension than cynaropicrin. The treatment of MDA-MB-231 cells with leaves lipophilic extract and cynaropicrin upregulated the CDK-inhibitor p21, consequently causing accumulation of p-Tyr15-CDK1 and cyclin B1. These findings may be associated with the G2 cell cycle arrest of MDA-MB-231 cells. Leaves lipophilic extract and cynaropicrin also affected the Akt upstream pathway, in MDA-MB-231 cells, by downregulating the active p-Ser473-Akt, without changing the total Akt1 expression.

Despite the fact that cynaropicrin being the most abundant identified compound in leaves lipophilic extract (455.2 mg/g extract), further research should be performed to understand if synergistic or cumulative effects of cynaropicrin with other extract component(s) were implicated in the inhibitory action, upon TNBC MDA-MB-231 cellular viability. Other task to be addressed is related with potentiating the inhibitory action of cytostatic drugs, upon MDA-MB-231 cell growth, through supplementation with cynaropicrin.

Taken all together, this study revealed that cultivated cardoon leaves lipophilic fraction and/or cynaropicrin may be promising agents for a complementary therapy of human TNBC.

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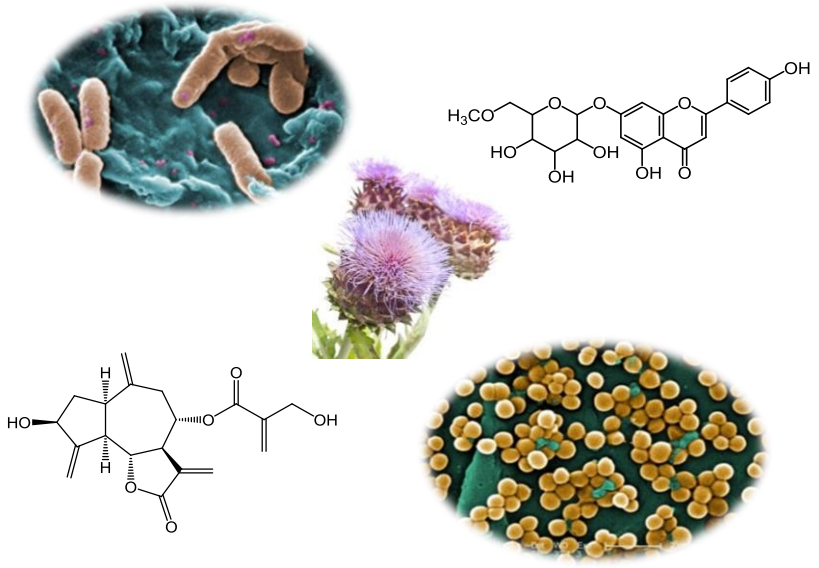
Chapter VII

Antibacterial activity of *Cynara cardunculus* L. var. *altilis* (DC) extracts on *Pseudomonas aeruginosa* PAO1 and methicillin-resistant *Staphylococcus aureus* (MRSA)



Ongoing work: Ramos, P. A. B.; Ferro, A. M. S.; Parreira, P.; Freire, C. S. R.; Silvestre, A. J. D.; Duarte, M. F. Antimicrobial activity of *Cynara cardunculus* L. extracts on multidrug-resistant bacteria.

Abstract

The present work highlighted, for the first time, the antibacterial activity of lipophilic and phenolic-rich extracts, derived from *Cynara cardunculus* L. var. *altilis* (DC), against two multidrug-resistant bacteria, known to be key players in healthcare-associated infections: the Gram-negative *Pseudomonas aeruginosa* and the Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA). Capitula florets lipophilic and phenolic-rich extracts did not have inhibitory effects against *P. aeruginosa* or MRSA. Leaves lipophilic extract also did not demonstrate antibacterial effect on *P. aeruginosa*, but cynaropicrin showed a slightly antimicrobial effect (MIC = 2048 µg/mL). Leaves lipophilic extract (MIC = 1024 µg/mL) and cynaropicrin (MIC = 256 µg/mL) prevented the growth of MRSA. Globally, these findings suggest that lipophilic extract of *C. cardunculus* L. var. *altilis* (DC) leaves and cynaropicrin have potential to be investigated as MRSA therapeutic agents. Cynaropicrin may be considered as a potential lead for the development of an antibacterial drug against *P. aeruginosa*.

1. Introduction

The multidrug-resistant (MDR) bacteria represent a severe threat to public health worldwide in the 21st century, with financial burden on health care systems and elevated risk of morbidity and mortality. This situation was favored from the constant selection pressure caused by the misuse of antibiotics, in both humans and food-producing animals, the increased disease transmission, and a decay in the development of new antibacterial drugs since the 1980s.[1–3] According to European Centre for Disease Prevention and Control, the MDR bacterial infections can cause 25000 deaths per year in Europe, in addition to the extra healthcare costs and productivity losses of at least € 1.5 billion.[4] Due to the less effective action of antibiotics, the MDR bacteria have emerged as the major health care-associated infections (HCAI), formerly known as "nosocomial" or "hospital" infections. The Gram-negative *Pseudomonas aeruginosa* and the Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) are two of the main MDR bacteria responsible for HCAI.[2,4]

Among the Gram-negative HCAI bacteria, *P. aeruginosa* is the second most frequent pathogen responsible for pneumonia, and the third and fourth most frequent pathogen that contributes for septicemia, urinary tract infections, and surgical wound infections. Immunocompromised individuals are the most susceptible to *P. aeruginosa* infection.[5] Being commonly disseminated in aquatic environments, *P. aeruginosa* can be transmitted by inadvertent contamination via water from household taps[6] and sinks within intensive care units.[5] *P. aeruginosa* has shown to selectively block several antimicrobial drugs, like fluoroquinolones, aminoglycosides and carbapenems, to penetrate its outer membrane. Regarding to the last European antimicrobial resistance surveillance, Portugal exhibited, in 2013, higher percentage of fluoroquinolones-resistant *P. aeruginosa* (23.9%) compared to the European Union mean (20.0%).[4]

S. aureus colonizes the skin of about 30% of healthy humans, which can afford several infections, namely in skin and in soft tissue, pneumonia, meningitis, endocarditis and toxic shock syndrome.[7] The MRSA appeared just after three years of the methicillin introduction, being considered as the major HCAI.[2] Based on the 2013 data of MRSA isolates in Europe, the percentages were generally lower in northern Europe and higher in southern and south-eastern countries (Figure 1). Portugal ranks at third position relatively to the percentage of MRSA isolates (46.8%) in Europe, with Romania (64.5%) and Malta (51.8%) at first and second positions, respectively.[4] MRSA causes a greater morbidity and mortality (up to 2-fold), compared to methicillin-susceptible *S. aureus*, which is mostly due to a delay in the administration of appropriate therapy and poor efficacy of the existing antibiotics.[8] Risk factors of MRSA infection comprise recent hospitalization or surgery, dialysis, residence in a long-term care facility, and the presence of percutaneous devices and catheters.[7]

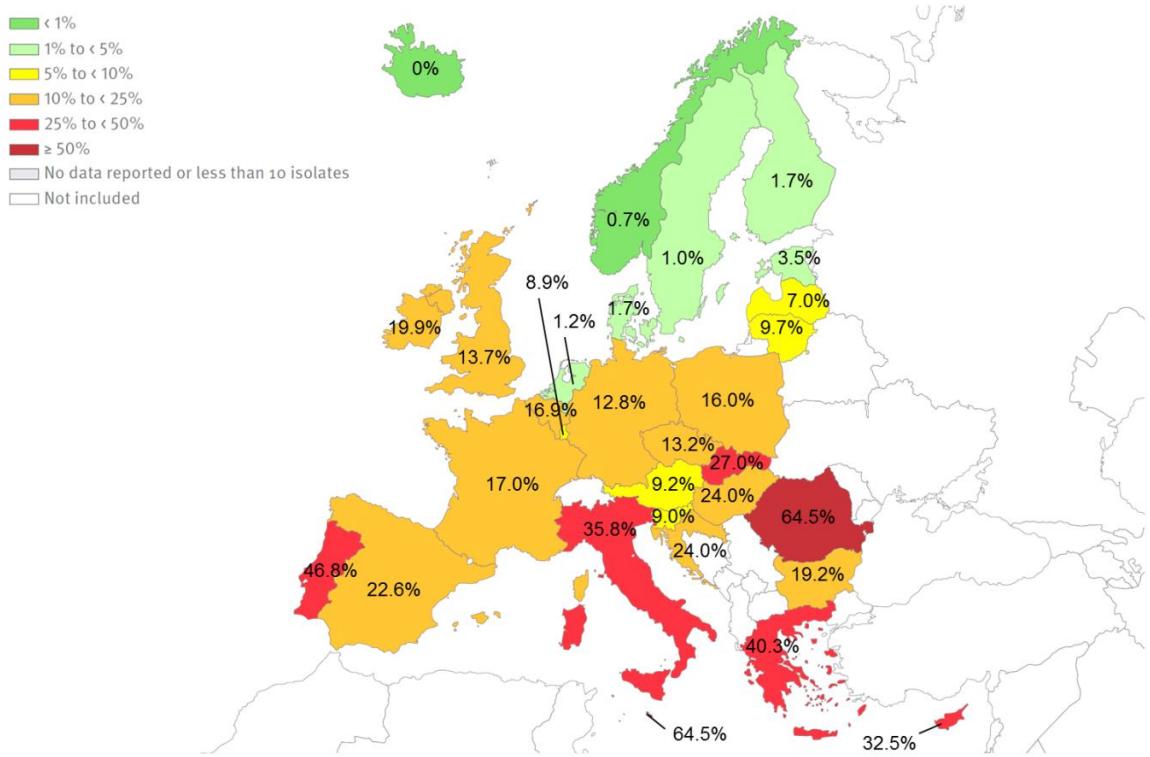


Figure 1: Percentage of MRSA invasive isolates in European Union/European Economic Area countries, in 2013 (adapted from [4]).

After a stagnation on the antibiotic discovery field during the last years,[1] natural products have gained renewed interest as sources of new or coadjutant therapeutic agents for combating the MDR bacterial infections, namely terpenes[9–11] and phenolic compounds.[12–14] Sesquiterpene lactones isolated from two Asteraceae plants, namely *Xanthium sibiricum Patrer Widd* and *Centratherum punctatum* Cass. ssp. *punctatum*, inhibited the growth of *P. aeruginosa*[10] and MRSA.[9] Taraxasterol, a pentacyclic triterpene isolated from *Chrysanthemum macrocarpum* (Asteraceae), demonstrated antibacterial effect on *P. aeruginosa* (MIC = 16 µg/mL).[15] Other pentacyclic triterpenes, like ursolic and oleanolic acids, could prevent MRSA growth (MICs of 8 and 16 µg/mL, respectively), but not the growth of *P. aeruginosa* PAO1 (MIC > 128 µg/mL),[11] a chloroamphenicol-resistant mutant of the original Australian PAO isolate.[16] In what concerns to phenolic compounds, ferulic acid[12] and apigenin 7-*O*-glucoside[17] inhibited the MRSA growth (MICs of 500 µg/mL and 216 µg/mL, respectively). Moreover, it was proven that apigenin and luteolin were able to inhibit the growth of *P. aeuruginosa* PAO1 strain.[18]

The Mediterranean species of *Cynara cardunculus* L. (Asteraceae) comprises wild cardoon (var. *sylvestris* (Lamk) Fiori), artichoke (var. *scolymus* (L.) Fiori) and cultivated cardoon (var. *altilis* (DC)). Besides the consumption of artichoke capitula as vegetable and the use of cardoon capitula for producing Iberian ewe's cheese, leaf infusions have been

traditionally used for therapeutic purposes, given to their hypocholesterolemic,[19] choleretic and anticholestatic properties.[20] These biological activities have been attributed to phenolic composition, namely caffeoylquinic acids and luteolin derivatives.[20] Furthermore, artichoke and cardoon methanol extracts have also displayed anti-hyperlipidemic[21] and anti-inflammatory properties,[22] mainly ascribed to sesquiterpene lactones[21] and pentacyclic triterpenes,[22] respectively.

Some reports have shown the antibacterial action of *C. cardunculus* L. extracts.[23–25] Artichoke leaves *n*-butanol fraction presented antibacterial effect on *P. aeruginosa* and *S. aureus*, being comparable to kanamycin sulfate and streptomycin. Luteolin 7-*O*-glucoside, isolated from artichoke *n*-butanol fraction, was the most active phenolic compound against bacteria growth.[23] Furthermore, wild cardoon leaves methanol extract also demonstrated to be active against the growth of *P. aeruginosa* and *S. aureus*, probably due to the presence of phenolic compounds.[24] Lipophilic compounds isolated from wild cardoon capitula, like *β*-sitosterol, have also exhibited antibacterial effect against for instance *E. coli* and *S. aureus*.[25]

Our group previously revealed that high proportions of pentacyclic triterpenes (≈27.5 g/kg dw) and sesquiterpene lactones (≈94.5 g/kg dw) were found in *C. cardunculus* L. var. *altilis* (DC) capitula florets and leaves, respectively.[26] Moreover, our group also denoted that flavonoids were mainly detected in capitula florets (≈13.3 g/kg dw).[27] At the current knowledge, the antibacterial activity of *C. cardunculus* L. var. *altilis* (DC) lipophilic and phenolic-rich extracts has not been already studied. The present work, in the scope of finding novel natural antibacterial substances, adresses to determine the minimum inhibitory concentration (MIC) of lipophilic and phenolic-rich extracts, derived from florets and leaves of cultivated cardoon, on *P. aeruginosa* and MRSA, the main bacteria of HCAI life-threatening infections.

2. Materials and Methods

2.1 Chemicals

Dichloromethane (p.a. ≥99% purity) was supplied by Fischer Scientific (Pittsburgh, Pennsylvania, USA). Cynaropicrin (≥97.5% purity) was purchased from Extrasynthese (Genay Cedex, France). Dimethyl sulfoxide (DMSO) cell culture grade was obtained from Applichem (Gatersleben, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased to Calbiochem (San Diego, California, USA). Glycerol, Mueller Hinton agar and broth, *Pseudomonas* (cetrimide) agar, mannitol salt agar and buffered peptone water were obtained from Liofilchem (Roseto degli Abruzzi, Italy). Brucella Broth, gentamicin and trimethoprim were provided by Sigma Chemicals Co. (Madrid, Spain).

2.2 Bacterial strains

P. aeruginosa PAO1 strain was donated by Professor Arsénio Fialho (Instituto Superior Técnico), being previously characterized.[28] MRSA was kindly donated and characterized by Professor Manuela Pintado (Escola Superior de Biotecnologia, Universidade Católica Portuguesa Porto (ESB/UCP)).[12] Bacterial strains were grown on Mueller Hinton Agar or Mueller Hinton Broth at 37 ºC. Selective media towards each bacterial strain was used to sustain the purity of the bacterial cultures. Bacteria were kept at -80 ºC, in Brucella Broth with 20% (v/v)) glycerol until further need.

The purity of *P. aeruginosa* and MRSA strains was evaluated by examination of the colony morphology in *Pseudomonas* (cetrimide) agar and mannitol salt agar plates, respectively, after overnight incubation at 37 ºC.

2.3 Preparation of *C. cardunculus* L. var. *altilis* (DC) extracts

C. cardunculus L. var. *altilis* (DC) (cultivated cardoon) was collected at the flowering stage, in June 2010, at the Experimental Center of the School of Agriculture from Instituto Politécnico de Beja, Baixo Alentejo region, south Portugal. Capitula and leaves were separated and preserved at -20 ºC until analysis was performed.

Lipophilic extracts, derived from cultivated cardoon capitula florets and leaves, were prepared and analyzed by gas chromatography-mass spectrometry, as referred elsewhere.[26] The phenolic-rich extract of capitula florets was prepared after the removal of lipophilic components, and their chemical composition was investigated by liquid chromatography-mass spectrometry, as earlier explained.[27]

Lipophilic extracts of capitula florets and leaves contained respectively: 320.6 and 38.5 mg/g extract of pentacyclic triterpenes; 8.8 and 3.4 mg/g extract of sterols; and 14.4 and 2.4 mg/g extract of fatty acids. Sesquiterpene lactones were only present in leaves lipophilic extract, accounting for 484.9 mg/g extract, mainly represented by cynaropicrin (455.2 mg/g extract). Phenolic-rich extracts of capitula florets contained 0.7 mg/g extract of hydroxycinnamic acids and 22.5 mg/g extract of flavonoids. The detailed chemical composition of lipophilic and phenolic-rich extracts of cultivated cardoon may be consulted in Appendix.

The antimicrobial effect of cultivated cardoon extracts and pure compound of cynaropicrin, representative of the major identified component of leaves lipophilic extract, was examined against Gram-negative *P. aeruginosa* and Gram-positive MRSA. For comparison reasons, gentamicin and trimethoprim were included as control antibiotics. Lipophilic extracts of florets and leaves, pure compound of cynaropicrin and trimethoprim were dissolved in DMSO. Florets phenolic-rich extract and gentamicin were prepared in buffered peptone water.

2.4 MIC determination

Bacteria were grown in Mueller Hinton plates, overnight at 37 ºC. Colonies were then dissolved in Mueller Hinton Broth, followed by dilution in the same medium broth to obtain an inoculum of 1 x 10^5 cfu/mL, as recommended by Clinical and Laboratory Standards Institute.[29] Culture suspensions were grown at 37 ºC, 200 rpm, until exponential phase. Afterwards, bacterial suspensions were then diluted to 1 x 10^5 cfu/mL and added to 96-well plates. Stock solutions were dissolved with the bacterial inoculum in two-fold serial dilutions, from 2048 µg/mL until 8 µg/mL. The solvent vehicle control for lipophilic extracts, cynaropicrin and trimethoprim received DMSO (4% (v/v)). The positive control was prepared with inoculum in Mueller Hinton Broth. Two negative controls were performed: one with Mueller Hinton Broth, and the other with the sample in Mueller Hinton Broth.

MIC values were determined after overnight incubation at 37 ºC, by using the MTT assay adapted from Eloff, *et al.*,[30] due to the color conferred by cultivated cardoon extracts to medium broth. MIC values were detected afte Briefly, 40 µL of MTT solution (200 µg/mL in peptone water) was added to each well, followed by incubation at 37 ºC for 45 min. MIC was considered as the minimum concentration of tested sample at which the yellow color of tetrazolium MTT salt did not change to the purple color of the formazan product. Three independent experiments were performed for each treatment, each in triplicate.

3. Results and Discussion

Lipophilic extracts of cultivated cardoon capitula florets and leaves demonstrated the highest contents of pentacyclic triterpenes and sesquiterpene lactones, respectively (Chapter III), whereas phenolic rich-extract of capitula florets displayed the highest flavonoids content (Chapter IV). In this manner, these extracts were selected to determine their MIC values on *P. aeruginosa* PAO1 and MRSA growth, through the MTT assay, as outlined in Table 1. Additionally, the MIC value of cynaropicrin pure compound, representative of the most abundant compound identified in leaves lipophilic extract, was also evaluated (Table 1). MIC values of two antibiotics, namely gentamicin and trimethoprim, were also assessed (Table 1).

Table 1: MIC values of lipophilic and phenolic-rich extracts of *C. cardunculus* L. var. *altilis* (DC) on *P. aeruginosa* PAO1 and MRSA growth, determined through MTT assay.

Cultivated cardoon extract/reference compound	MIC (µg/mL)	
	P. aeruginosa PAO1	MRSA
Capitula florets lipophilic extract	> 2048	> 2048
Leaves lipophilic extract	> 2048	1024
Capitula florets phenolic-rich extract	> 2048	> 2048
Cynaropicrin	2048	256
Gentamicin	8	128
Trimethoprim	nt	32

Abbreviation: nt, not tested.

Lipophilic extracts of cultivated cardoon capitula florets and leaves showed the highest Despite the considerably high concentrations of pentacyclic triterpenes and sesquiterpene lactones, lipophilic extracts of cultivated cardoon florets and leaves did not prevent the *P. aeruginosa* PAO1 growth (Table 1), at range 8-2048 µg/mL. The pure compound of cynaropicrin presented a slightly inhibitory effect on *P. aeruginosa* PAO1 growth (MIC = 2048 µg/mL). An earlier report revealed that cynaropicrin could inhibit irreversibly the enzymatic activity of MurA (uridine-N-acetyl-D-glucosamine (UDP-NAG) enolpyruvyl transferase), isolated from *P. aeruginosa,*[31] which is implicated within the biosynthesis of cell wall peptidoglycan. Phenolic-rich extract of florets did not show anti-*P. aeruginosa* PAO1 effect, at the tested concentrations, which may be related with the low concentration of flavonoids and other phenolic compounds.

Lipophilic and phenolic-rich extracts of cultivated cardoon florets did not inhibit MRSA growth, at range 8-2048 µg/mL (Table 1). Cultivated cardoon leaves lipophilic extract (45.5% of cynaropicrin) displayed anti-MRSA effect, with MIC of 1024 µg/mL. This antibacterial effect was comparable to the anti-*S. aureus* action of wild cardoon capitula fractions (MIC range 1000-1500 µg/mL).[25] It is worthy to notice that the MRSA, used in this work, was described to be resistant to oxacillin and to fluoroquinolones, like ciprofloxacin and levofloxacin.[12] Nonetheless, the anti-MRSA effect of lipophilic leaves extract was much weaker compared to gentamicin and trimethoprim (Table 1). The pure compound of cynaropicrin also inhibited MRSA growth (MIC = 256 µg/mL). This effect was stronger than the antibacterial action of cynaropicrin, shown by Schinor, *et al.*,[32] on two strains of *S. aureus*, namely a penicillinase producer (MIC = 500 µg/mL) and a non-penicillinase producer (MIC = 2500 µg/mL). In addition to the different *S. aureus* strain, Schinor, *et al.*[32] used the agar diffusion method. Further research will be needed to clarify if the anti-MRSA effect of leaves lipophilic extract resulted from inhibitory action of cynaropicrin, the most abundant compound identified in that extract.

These findings also reflected that *P. aeruginosa* PAO1 was more resistant towards the inhibitory actions of leaves lipophilic extract and cynaropicrin, compared to MRSA, probably due to the protective role of outer membrane.

In sum, these results suggested that lipophilic extract of *C. cardunculus* L. var. *altilis* (DC) leaves and cynaropicrin may have potential for the development of anti-MRSA therapeutics. Moreover, it was also evidenced that cynaropicrin could probably be part of an antibacterial formulation against *P. aeruginosa* PAO1.

4. Conclusions

The present work demonstrated the antibacterial activity of lipophilic and phenolic-rich extracts of *C. cardunculus* L. var. *altilis* (DC). Cultivated cardoon leaves lipophilic extract did not prevent the *P. aeruginosa* growth, but inhibited the MRSA growth (MIC = 1024 µg/mL). In fact, cynaropicrin presented a considerably anti-MRSA effect (MIC = 256 µg/mL). Furthermore, cynaropicrin was also able to prevent the *P. aeruginosa* PAO1 growth (MIC = 2048 µg/mL). Considering these findings, novel biocidal formulations may contain cultivated cardoon leaves lipophilic extract or cynaropicrin, in the scope of potentiating the inhibitory action of antibiotics, against two main MDR bacteria responsible for HCAI, namely *P. aeruginosa* and MRSA.

The pre-extraction of antibacterial compounds from cultivated cardoon can be a prospective task for its valorization and, simultaneously, to fulfill the urgency in finding novel therapeutic compounds against MDR bacteria.

5. References

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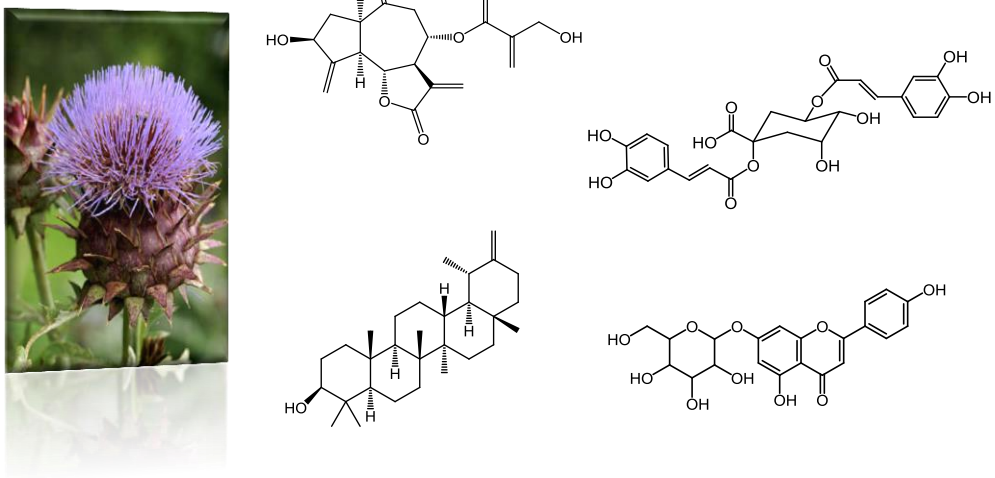
Part D

Chapter VIII

Concluding remarks and future perspectives



1. Concluding remarks

The cultivation of *Cynara cardunculus* L. var. *altilis* (DC) in the Mediterranean countries has gained increasing attention in the last decade. A vast search in literature (**Part A**) highlighted that this biomass can be converted, in a sustainable manner, into energy, pulp fibers, which perfectly fits with the biorefinary concept. The pre-extraction of terpenic and phenolic compounds would be an additional valorization pathway of cultivated cardoon, given the diversity of biological activities reported for these families. In fact, there has been a growing interest of pharmaceutical industry on natural bioactive compounds, in order to find new molecular entities, as well as to produce effective herbal products, which are more and more sought by the modern society. This thesis aimed to contribute to the increase of knowledge about the chemical composition of stalks, capitula and leaves of cultivated cardoon, planted in the south of Portugal (Baixo Alentejo region), regarding to lipophilic and phenolic compounds (**Part B**). Some relevant biological activities of cultivated cardoon extracts were also investigated, such as antioxidant, triple-negative breast cancer (TNBC) antiproliferative and anti-multidrug resistant (MDR) bacterial effects (**Part C**).

The lipophilic fraction of *C. cardunculus* L. var. *altilis* (DC) was analyzed by gas chromatography coupled with mass spectrometry (**Chapter III**). This technique allowed the identification of 65 lipophilic compounds, from which 33 and 41 were described in this thesis, for the first time, as *C. cardunculus* L. and cultivated cardoon components, respectively. In what concerns the terpenic composition, 1 sesquiterpene lactone and 4 pentacyclic triterpenes were indicated as cultivated cardoon components, for the first time: deacylcynaropicrin, and the acetates of *β*- and *α*-amyrin, lupenyl acetate and *ψ*-taraxasteryl acetate. Sesquiterpene lactones consisted in the most abundant lipophilic compunds found in leaves (≈94.5 g/kg dw), mainly represented by cynaropicrin (≈87.4 g/kg dw). Pentacyclic triterpenes were the most common lipophilic components of stalks and capitula, reaching at maximum ≈27.5 g/kg dw in florets. Taraxasteryl acetate was the most abundant triterpene in florets (≈8.9 g/kg dw), while taraxasterol was mainly retained in receptacles and bracts (≈5.3 g/kg dw). Leaves and florets lipophilic extracts were thus chosen to be studied in terms of biological activity, since these revealed the highest concentrations of sesquiterpene lactones (484.9 mg/g extract) and pentacyclic triterpenes (320.6 mg/g extract), respectively.

The phenolic composition of *C. cardunculus* L. var. *altilis* (DC) was thereafter studied by high performance liquid chromatography-ultraviolet detection-mass spectrometry (**Chapter IV**). The findings of this chapter indicated that 28 phenolic compounds were identified in stalks, capitula and leaves. Among these, eriodictyol hexoside was detected for the first time in *C. cardunculus* L., and 6 as cultivated cardoon components, namely 1,4-di-*O*-caffeoylquinic acid, naringenin 7-*O*-glucoside, naringenin rutinoside, naringenin, luteolin acetylhexoside and apigenin acetylhexoside. Florets exhibited the highest total concentration of the identified

phenolic compounds, accounting for ~12.7 g/kg dw. Stalks contained the highest the highest amounts of hydroxycinnamic acids, accounting for ≈10.3 g/kg dw and ≈9.9 g/kg dw in respectively outer and inner parts. Receptacles and bracts revealed the highest dicaffeoylquinic acids content (≈5.0 g/kg dw), mainly represented by 1,5-di-*O*-caffeoylquinic acid (≈4.2 g/kg dw). Flavonoids were the predominant phenolic compounds in capitula florets, accounting for ≈10.3 g/kg dw, mainly constituted by flavones (≈7.6 g/kg dw), such as apigenin glucuronide, apigenin 7-*O*-glucoside and apigenin 7-*O*-rutinoside (≈4.6 g/kg dw). Flavanones were only found in the florets (≈2.7 g/kg dw). Phenolic-rich extracts derived from stalks outer part and florets were selected to be assayed in terms of their biological effects, due to the highest contents in hydroxycinnamic acids and flavonoids, respectively.

The antioxidant activity of *C. cardunculus* L. var. *altilis* (DC) phenolic-rich extracts was tested through the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging effect (**Chapter V**). Phenolic-rich extracts of stalks outer part and receptacles and bracts were the most active in neutralizing DPPH free radicals (IC_{50} values of 34.35 and 35.25 µg/mL, respectively). Moreover, the DPPH scavenging effect of phenolic-rich extracts was linearly correlated specially with the total contents of monocaffeoylquinic acids (r = -0.996) and hydroxycinnamic acids (r = -0.990). Despite the weaker DPPH scavenging effects relatively to 3,5-di-*tert*-4-butylhydroxytoluene (BHT), cultivated cardoon phenolic-rich extracts could be possibly utilized in food industry, as an alternative to synthetic antioxidants.

Lipophilic and phenolic-rich extracts, derived from *C. cardunculus* L. var. *altilis* (DC), were screened in terms of the *in vitro* inhibitory action upon the cellular viability of TNBC MDA-MB-231 cell line (**Chapter VI**). Leaves and florets lipophilic extracts presented much more inhibitory action (IC_{50} values of 10.39 and 315.22 µg/mL, respectively), on the MDA-MB-231 cell growth, compared to the phenolic-rich extract of stalks outer part (IC_{50} = 3341.20 µg/mL) and florets (IC_{50} > 4500 µg/mL). MDA-MB-231 cells were also less resistant to cynaropicrin (IC_{50} = 17.86 µM) than to 1,5-di-*O*-caffeoylquinic acid (IC_{50} = 1741.69 µM). Considering the high concentration of cynaropicrin (455.2 mg/g extract), this sesquiterpene lactone may be mostly implicated in the suppressive effect of lipophilic leaves extract. Moreover, leaves lipophilic extract and cynaropicrin upregulated the $p21^{Waf1/Cip1}$ expression, which may have led to accumulation of phospho-Tyr15-CDK1 and cyclin B1. These findings may be involved in the G2 cell cycle arrest of MDA-MB-231 cells. Furthermore, leaves lipophilic extract and cynaropicrin largely downregulated the phospho-Ser473-Akt protein expression, without affecting Akt1 total protein level. The results of this chapter pointed out that leaves lipophilic extract or cynaropicrin may have potential to be explored in terms of therapy of TNBC, one of the most refractory human cancers.

The antimicrobial activities of lipophilic and phenolic-rich extracts, derived from cultivated cardoon, were evaluated, for the first time, against two MDR bacteria (**Chapter VII**),

main responsible for health care-associated infections (HCAI), namely Gram-negative *Pseudomonas aeruginosa* and Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA). Lipophilic and phenolic-rich extracts of florets, in the range 8-2048 µg/mL, were not efficient in preventing the growth of *P. aeruginosa* PAO1 and MRSA. Furthermore, lipophilic leaves extract did not exhibit antibacterial effect on *P. aeruginosa* PAO1 (MIC > 2048 µg/mL), contrarily to cynaropicrin (MIC = 2048 µg/mL). Nonetheless, lipophilic leaves extract was effective to suppress the MRSA growth (MIC = 1024 µg/mL). Cynaropicrin also inhibited the MRSA growth (MIC = 256 µg/mL). These data revealed the potential of cultivated cardoon leaves lipophilic extract and/or cynaropicrin to be investigated in the scope of MRSA therapeutics.

This thesis contributes for the valorization of *C. cardunculus* L. var. *altilis* (DC) biomass. Stalks, capitula and leaves present lipophilic and phenolic compounds with promising antioxidant, human TNBC (MDA-MB-231 cell line) antiproliferative and anti-MDR bacterial effects. The acquired scientific knowledge reflects that cultivated cardoon can be potentially exploited as a source of bioactive compounds, in concilliation with other added value perspectives, namely production of energy and pulp fibers, and Portuguese traditional cheeses manufacturing.

2. Future perspectives

The purpose of this thesis consisted of the chemical characterization of *C. cardunculus* L. var. *altilis* (DC) sequential extracts and the evaluation of some biological activities, namely antioxidant, human TNBC antiproliferative and anti-MDR bacterial effects. However, there are still some issues that need to be tackled before proceeding with the upgrade of bioactive compounds extraction from cultivated cardoon:

(i) to perform the bioactivity-guided fractionation of lipophilic and phenolic-containing extracts, in order to clarify the main responsible for the studied biological activities;

(ii) to prepare cultivated cardoon bioactive-enriched fractions, by using environmentally friendly techniques, namely supercritical fluid extraction and membrane filtration technology;

(iii) to assess the antioxidant action of cultivated cardoon extracts, containing phenolic compounds, in immortalized human cell lines, intending to understand their regulating role on scavenging and production of reactive oxygen species, as well as upon enzymes of cellular antioxidant system;

(iv) to investigate the hypothesis of interaction between cynaropicrin and other compound(s)/enriched-fractions of leaves lipophilic extract, regarding the antiproliferative action upon TNBC MDA-MB-231 cells;

(v) to study the possibility of extracts or enriched fractions in phenolic compounds to be used in novel food antioxidant formulations;

(vi) to evaluate the bioavailability of the most promising cultivated cardoon extracts/enriched fractions/pure compounds, through an *in vitro* simulated digestion model;

(vii) to develop novel biocidal formulations, containing cultivated cardoon leaves lipophilic extract or cynaropicrin, in an effort to contribute for a decrease of health care-associated infections, caused by MDR bacteria;

(viii) to explore the antitumoral potential of formulations, comprising cytostatic drugs and leaves lipophilic extract or cynaropicrin, to be applied in the development of a natural-based TNBC therapeutics.

Appendix

Tables 1 and 2 of Appendix list the chemical composition of respectively lipophilic and phenolic-rich extracts, derived from *Cynara cardunculus* L. var. *altilis* (DC).

Table 1: Chemical composition of lipophilic extracts derived from capitula florets and leaves of *C. cardunculus* L. var. *altilis* (DC).

Compound	Content (mg/g extract)	
	Capitula florets	Leaves
Aromatic compounds	**0.7 ± 0.1** [a]	**0.1 ± 0.0** [b]
Fatty acids	**14.4 ± 0.4** [a]	**2.5 ± 0.4** [b]
Saturated	*11.7 ± 0.3* [a]	*1.6 ± 0.1* [b]
Pentadecanoic acid	0.2 ± 0.1	–
Hexadecanoic acid	9.7 ± 0.1 [a]	1.0 ± 0.0 [b]
Octadecanoic acid	0.5 ± 0.1 [a]	0.1 ± 0.0 [b]
Eicosanoic acid	0.5 ± 0.1 [a]	0.2 ± 0.0 [b]
Docosanoic acid	0.6 ± 0.1	–
Tetracosanoic acid	0.2 ± 0.1 [b]	0.4 ± 0.0 [a]
Unsaturated	*2.7 ± 0.1* [a]	*0.8 ± 0.1* [b]
9Z,12Z-Octadeca-9,12-dienoic acid	2.0 ± 0.1 [a]	0.2 ± 0.0 [b]
9Z,12Z,15Z-Octadeca-9,12,15-trienoic acid	0.5 ± 0.0 [a]	0.3 ± 0.0 [a]
9Z-Octadec-9-enoic acid	0.2 ± 0.0 [a]	0.3 ± 0.1 [a]
Sesquiterpene lactones	–	**484.9 ± 15.8**
Grosheimin	–	20.7 ± 1.1
Deacylcynaropicrin	–	9.0 ± 0.2
Cynaropicrin	–	455.2 ± 14.7
Long chain aliphatic alcohols	–	**1.1 ± 0.0**
Tetracosanol	–	0.4 ± 0.0
Hexacosanol	–	0.7 ± 0.0
Sterols	**8.8 ± 4.4** [a]	**3.4 ± 0.2** [a]
Stigmaterol	2.2 ± 0.9 [a]	1.0 ± 0.0 [b]
β-Sitosterol	4.2 ± 2.2 [a]	2.4 ± 0.2 [a]
Δ^5-Avenasterol	2.4 ± 1.1	–
Pentacyclic triterpenes	**320.6 ± 80.7** [a]	**38.5 ± 0.3** [b]
β-Amyrin	33.1 ± 8.7 [a]	3.9 ± 0.3 [b]
α-Amyrin	17.9 ± 4.8 [a]	0.8 ± 0.0 [b]
Lupeol	10.6 ± 3.3 [a]	4.9 ± 0.2 [b]
β-Amyrin acetate	32.8 ± 8.4 [a]	1.7 ± 0.0 [b]
α-Amyrin acetate	13.9 ± 6.3 [a]	0.2 ± 0.1 [b]
Lupeoyl acetate	12.0 ± 3.7 [a]	1.5 ± 0.1 [b]
ψ-Taraxasterol	25.8 ± 3.4 [a]	5.7 ± 0.3 [b]
Taraxasterol	49.4 ± 13.0 [a]	12.1 ± 0.3 [b]
ψ-Taraxasteryl acetate	31.6 ± 14.9 [a]	2.0 ± 0.4 [b]
Taraxasteryl acetate	93.4 ± 14.1 [a]	5.7 ± 0.6 [b]
Others/non-identified	**12.9 ± 1.3** [b]	**326.6 ± 6.0** [a]
Total identified compounds	**357.4 ± 84.2** [b]	**856.9 ± 9.3** [a]

Results correspond to mean±standard deviation. Two aliquots of each extract were injected in duplicated. Means with different letters within the same row are statistically different (Tukey's test, $p < 0.05$).

Table 2: Chemical composition of phenolic-rich extracts from stalks outer part and capitula florets of *C. cardunculus* L. var. *altilis* (DC).

No.	Compound	Content (mg/g extract) [I]	
		Stalks outer part	Capitula florets
	Total hydroxycinnamic acids	**54.7 ± 2.4** [a]	**0.7 ± 0.1** [b]
1	1-*O*-Caffeoylquinic acid	1.1 ± 0.1	–
2	3-*O*-Caffeoylquinic acid	0.9 ± 0.1	–
3	5-*O*-Caffeoylquinic acid	18.2 ± 0.4 [a]	0.1 ± 0.0 [b]
5	1,3-Di-*O*-caffeoylquinic acid	1.2 ± 0.1	–
10	1,4-Di-*O*-caffeoylquinic acid	1.4 ± 0.1	–
12	1,5-Di-*O*-caffeoylquinic acid	15.8 ± 1.5 [a]	0.6 ± 0.1 [b]
22	4-Acyl-di-*O*-caffeoylquinic acid [II]	0.7 ± 0.0 (21+22)	–
18	1,5-Di-*O*-caffeoylsuccinoylquinic acid isomer	11.1 ± 0.5	–
19	4-Acyl-di-*O*-caffeoylsuccinoylquinic acid isomer	2.5 ± 0.0	–
23	Dicaffeoylsuccinoylquinic acid isomer	0.9 ± 0.1	–
26	Dicaffeoyldisuccinoylquinic acid isomer	1.7 ± 0.1	–
	Total coumarins	**–**	**1.5 ± 0.2**
4	Scopolin isomer	–	1.5 ± 0.2
	Total flavonoids	**3.6 ± 0.2** [b]	**22.5 ± 2.2** [a]
	Total flavanones	**–**	*8.5 ± 0.7*
6	Eriodictyol hexoside	–	nq [III]
13	Naringenin 7-*O*-glucoside	–	2.1 ± 0.4
14	Naringenin rutinoside	–	5.0 ± 0.3
28	Naringenin	–	1.4 ± 0.1
	Total flavones	*3.6 ± 0.2* [b]	*13.9 ± 1.5* [a]
7	Luteolin glucuronide	0.9 ± 0.0 [a]	0.3 ± 0.0 [b]
8	Luteolin 7-*O*-glucoside	1.2 ± 0.1 [a]	0.2 ± 0.0 [b]
9	Luteolin rutinoside	**–**	0.1 ± 0.0
21	Luteolin acetylhexoside	(21+22)	<0.1
24	Luteolin	**–**	0.6 ± 0.1
15	Apigenin glucuronide	1.1 ± 0.0 [b] (15+16)	8.4 ± 0.8 [a] (15+16+17)
16	Apigenin 7-*O*-glucoside	(15+16)	(15+16+17)
17	Apigenin 7-*O*-rutinoside	**–**	(15+16+17)
25	Apigenin acetylhexoside	0.4 ± 0.1 [a]	0.2 ± 0.0 [b]
29	Apigenin	**–**	4.1 ± 0.5
20	Chrysoeriol hexoside isomer	**–**	<0.1
30	Chrysoeriol isomer	**–**	<0.1
	Total identified phenolic compounds	**59.0 ± 2.4** [a]	**24.7 ± 2.4** [b]

[I] Results represent mean±standard deviation. Three samples of each extract were analyzed. Means with different letters within the same row are statistically different (Tukey's test, $p < 0.05$). Numbers in parenthesis correspond to compounds whose peaks were overlapped.

[II] Total hydroxycinnamic acid content did not include the content of 4-acyl-di-*O*-caffeoylquinic acid (**22**), due to the sum with luteolin acetylhexoside (**21**) (overlapped peaks).

[III] Abbreviation: nq, not quantified since it was below the limit of quantification.