University of Constantine 3 Salah BOUBNIDER

Faculty of Process Engineering

Department of Pharmaceutical Engineering

IMPACT OF HIGH-PRESSURE EXTRACTION TECHNIQUES ON THE QUALITY OF EXTRACTS OBTAINED FROM LOCAL PLANTS.

To apply for the Degree of:

Doctor in Process Engineering.

Option Pharmaceutical Engineering.

PhD Dissertation presented by:

ABDERREZAG Norelhouda

Academic year

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N°deSérie:

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THESIS

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Academic year

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Dedication

To Allah foremost.

To my dear parents Belkacem and Souad.

To my brother, my hero Ahmed.

To my sweet sister Hadjer.

To my soulmate Rafik.

To the soul of my grand-parents Mustapha and Abd-Elmadjid and my grand-mother Yakout.

To my queen Yamouna

To my sweet heart Nassima.

To my angel Hanane.

To my dear Wafa and her husband.

To all my family, one by one.

To all my friends, colleagues and neighbors.

To all of you, a thousand thanks.

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A. leucotrichus: Ammodaucus leucotrichus.

ABD-F: 7-fluorobenzoxadiazole-4-sulfonamide.

ABTS: 2,2-azino- bis (3-ethylbenzothiazoline-6-sulphonic acid).

ACh: Acetylcholine.

AChE: Acetylcholinesterase.

AD: Alzheimer's Disease.

ANOVA: Analysis of Variance.

ASE: Accelerated Solvent Extraction.

BBD: Box-Behnken Design.

BHT: Butylated Hydroxytoluene.

ChE: Cholinesterase.

CXE: Carbon Dioxide-Expanded Ethanol.

CXLs: Carbon Dioxide-Expanded Liquids.

DI: Degree of Inhibition (%).

DMSO: Dimethyl Sulphoxide.

DPPH: 2,2-diphenyl-1-picrylhydrazyl.

DW: Dried Weight (g).

EO: Essential Oil.

ESI: Electrospray Ionization.

FA: Fatty Acid.

FRAP: Ferric Ion Reducing Antioxidant Power.

GAE: Gallic Acid Equivalent.

GC: Gas Chromatography.

GC-MS: Gas Chromatography Mass Spectrometry.

GNPS: Global Natural Products Social Molecular Networking.

GRAS: Generally Recognized As Safe.

GXLE: Gas-Expanded Liquid Extraction.

GXLs: Gas-Expanded Liquids.

HMDB: Human Metabolome Database.

HPLC: High-Performance Liquid Chromatography.

IC_{50}: Inhibition Concentration by 50 % (ug/ml)

IS: Isosilybin.

LA: Linoleic Acid.

LC: Liquid Chromatography.

LOX: Lipoxygenase.

MAE: Assisted Microwave Extraction.

MAOs: Manoamine Oxidase.

MRO: Multiple Response Optimization.

MS: Mass Spectrometry.

NDDs: Neurodegenerative Diseases.

NMR: Nuclear Magnetic Resonance.

NO: Nitric Oxide.

PCA: Principal Component Analysis.

PCA: Principal Component Analysis.

PHWE: Pressurized Hot Water Extraction.

PLE: Pressurized Liquids Extraction.

Q-TOF: Quadrupole Time-Of-Flight.

RSM: Response Surface Methodology.

Rt: Retention Time (min).

S. Marianum: Silybum Marianum.

Sb $_{A+B}$: Silybin A + B.

Sc: Silychristin.

ScCO_2: Supercritical CO_2.

SD: Silydianin.

SD: Standard Deviation (%).

SFC: Supercritical Fluids.

SFE: Supercritical Fluids Extraction.

SLY: Silymarin.

SWE: Subcritical Water Extraction.

TCC: Total Carbohydrate Content (mg glucose/ g extract).

TEAC: Trolox Equivalent Antioxidant Capacity.

TFC: Total Flavonoids Content (mg quercetin/ g extract).

TIC: Total Ionic Current.

TPC: Total Phenolics Content (mg GAE/ g extract).

TX: Taxifolin.

U.S: United State.

Uae: Ultrasound-Assisted Extraction.

Abstract

In the present work, a multi-analytical approach was proposed to valorise two underused local Algerian plants from arid and non-arid zones, namely *Ammodaucus Leucotrichus* seeds and *Silybum marianum* seeds, and study their therapeutic performances. The first part was dedicated to the extraction of oils from the two plant using green processes based on the application of compressed fluids, such as pressurised liquid extraction (PLE), gas-expanded liquid extraction (GXLE) and supercritical fluid extraction (SFE), followed by the identification of the different constituents of the extracts by gas chromatography and liquid chromatography coupled with mass spectrometry. The second part of this thesis was reserved for the study of the neuroprotective activity of the different extracts, through acetylcholinesterase (AChE) and lipoxygenase (LOX) inhibition tests and the antioxidant activity, through free radical scavenging tests (DPPH and ABTS).

PLE using overheated liquid water at 180 °C proved to be a very effective extraction method for obtaining *Ammodaucus leucotrichus* extract with remarkable neuroprotective activity (IC50 (AChE) = 55, 6 µg/mL, IC50 (LOX)= 39.4 µg/mL and IC50 (DPPH)= 58.51 µg/mL). UHPLC-Q-TOF-MS/MS analysis allowed the preliminary identification of 94 compounds, mainly free and glycosylated phenols, lipids and organic acids. Furthermore, the extracts obtained by the ESF technique, optimized by the Box-Behnken design, showed a high carbohydrate content with low AChE inhibition. In addition, the extracts obtained by sequential PLE-SFE extractions possess relevant antioxidant activity compared to that of the extracts obtained by separate extraction processes. The results of the GC-MS analysis revealed the presence of 32 metabolites, 14 of which were reported for the first time in *Ammodaucus leucotrichus.* Concerning the seeds of *Silybum marianum*, the GXLE technique allowed the simultaneous extraction of the five favonolignans composing silymarin with predominance of silybin A + B (545.73 mg of silymarin/g of extract). Furthermore, the extract showed relevant antioxidant and anti-inflammatory potential with IC50 values equal to 8.80 µg/mL and 28.52 µg/mL, respectively, but a moderate AChE inhibition capacity (IC50 = 125.09 µg/mL).

Through this work, we have highlighted the promising potential of *Ammodaucus leucotrichus* and *Silybum marianum* extracts in the treatment of Alzheimer's and inflammatory diseases.

Key words: High pressure extraction, Silybum marianum, Ammodaucus leucotrichus, neuroprotective, antioxidant, anti-inflammatory.

Résumé

Dans le présent travail, une approche multi-analytique a été proposée pour valoriser deux plantes locales algériennes sous-utilisées provenant des zones arides et non arides, à savoir les graines d'*Ammodaucus Leucotrichus* et les graines de *Silybum marianum*, et étudier leurs performances thérapeutiques. La première partie a été consacrée à l'extraction des huiles des deux matrices végétales en utilisant des procédés verts basés sur l'application de fluides comprimés, tels que l'extraction liquide sous pression (PLE), l'extraction liquide par expansion de gaz (GXLE) et l'extraction par fluide supercritique (SFE), suivie de l'identification des différents constituants des extraits par chromatographie en phase gazeuse et chromatographie en phase liquide couplée à la spectrométrie de masse. La deuxième partie de cette thèse, a été réservée à l'étude de l'activité neuroprotectrice des différents extraits, par le biais des tests d'inhibition d'acétylcholinestérase (AChE) et de lipoxygénase (LOX) et l'activité antioxydante, par des essais de piégeage des radicaux libres (DPPH et ABTS).

La PLE utilisant de l'eau liquide surchauffée à 180 °C s'est avérée être une méthode d'extraction très efficace pour l'obtention de l'extrait d'*Ammodaucus leucotrichus* avec une activité neuroprotectrice remarquable (IC50 (AChE) = 55,6 µg/mL, IC50 (LOX)= 39,4 µg/mL et IC50 (DPPH)= 58,51 µg/mL). L'analyse par UHPLC-Q-TOF-MS/MS a permis l'identification préliminaire de 94 composés, principalement des phénols libres et glycosylés, des lipides et des acides organiques. De plus, les extraits obtenus par la technique de l'ESF, optimisée par le plan Box-Behnken, ont montré une teneur élevée en hydrates de carbone avec une faible inhibition de l'AChE. Par ailleurs, les extraits obtenus par extraction séquentielle (PLE-SFE) possèdent une activité antioxydante plus importante par rapport à celle des extraits obtenus par processus d'extraction séparés. Les résultats de l'analyse par GC-MS ont révélé la présence de 32 métabolites, dont 14 ont été identifiés pour la première fois chez *Ammodaucus leucotrichus*. En ce qui concerne les graines de *Silybum marianum*, la technique GXLE a permis l'extraction simultanée de cinq favonolignanes composant la silymarine avec prédominance de la silybine A + B (545,73 mg de silymarine/g d'extrait). L'extrait a montré un potentiel antioxydant et anti-inflammatoire remarquables avec des valeurs de IC50 égales à 8,80 µg/mL et 28,52 µg/mL, respectivement, mais une capacité d'inhibition d'AChE modérée (IC50 = 125,09 µg/mL).

A travers ce travail, nous avons mis en évidence le potentiel prometteur des extraits d'*Ammodaucus leucotrichus* et de *Silybum marianum* dans la prise en charge de la maladie d'Alzheimer et des maladies inflammatoires.

Mots clés : Extraction à haute pression, *Silybum marianum, Ammodaucus leucotrichus,* neuroprotectrice, antioxydante, anti-inflammatoire.

ملخص

في البحث الحالي، تم اقتراح نهج متعدد التحليلات لتقييم نوعين من النباتات الجزائرية المحلية البرية، من المناطق القاحلة وغير القاحلة، وهي بذور ام دريقة وحليب الشوك، بالإضافة لدراسة أدائها العلاجي.

خصص الجزء الأول لاستخلاص الزيوت من النبتيتين باستخدام العمليات الخضراء القائمة على تطبيق السوائل المضغوطة مثل استخلاص السائل المضغوط (PLE) واستخلاص السائل بالتمدد الغازي (GXLE) واستخلاص السوائل فوق الحرجة (SFE) ثم تم تحديد المكونات المختلفة للمستخلصات بواسطة كروماتوغرافيا الغازية وكروماتوغرافيا السائلة المرتبطتين بقياس الطيف الكتلي. تم تخصيص الجزء الثاني من هذه الرسالة لدراسة النشاط الوقائي العصبي للمستخلصات المختلفة، من خلال اختبارات تثبيط إنزيم الأسيتيل كولينستريز (AChE) والليبوكسيجيناز (LOX)ونشاط مضادات الأكسدة، من خلال اختبارات فحوصات الكسح المتطرفة DPPHوABTS .

أثبت استخدام PLE باستخدام الماء السائل المسخن بدرجة حرارة 180 درجة مئوية أنه طريقة استخلاص فعالة للغاية للحصول على مستخلص بذور ام دريقة ذو نشاط وقائي عصبي ملحوظ AChE (IC 50 =55.6 ميكروغرام/مل) ، LOX (IC 50 =39.4 ميكروغرام/مل) وDPPH (IC 50 =58.51 ميكروغرام/مل). سمح تحليل UHPLC-Q-TOF-MS/MS بالتحديد الأولي لـ 94 مركبًا، بشكل رئيسي الفينولات الحرة والغليكوزيلات والدهون والأحماض العضوية. اضافة على ذلك، أظهرت المستخلصات التي تم الحصول عليها باستخدام تقنية SFE ، والتي تم تحسينها بواسطة تصميم Box-Behnken، محتوى عاليًا من الكربوهيدرات مع تثبيط منخفض لـ AChE علاوة على ذلك، فإن المستخلصات التي تم الحصول عليها عن طريق عمليات الاستخلاص PLE-SFE المتسلسلة تمتلك نشاطًا مضادًا للأكسدة مقارنةً بالمستخلصات التي تم الحصول عليها عن طريق عمليات الاستخلاص المنفصلة. كشفت نتائج تحليل GC-MS عن وجود 32 مستقلبًا، تم الإعلان عن 14 منها لأول مرة في بذور ام دريقة. فيما يتعلق ببذور حليب الشوك ، سمحت تقنية GXLE بالاستخلاص المتزامن للفافونوليجنان الخمسة الموجودة في وقت واحد في السيليمارين مع غلبة السيليبين A + B بقيمة ملحوظة قدرها545.73 مجم من سيليمارين / جم من المستخلص. بالإضافة الى ذلك، أظهر المستخلص إمكانات مضادة للأكسدة ومضادة للالتهابات بقيم IC50 تساوي 8.80 ميكروغرام/مل و28.52 ميكروغرام/مل، على التوالي، ولكن قدرة تثبيط معتدلة لـ AChE (IC 50 = 125.09ميكروغرام/مل).

من خلال هذا العمل، قمنا بتسليط الضوء على الإمكانات الواعدة لمستخلصات بذور ام دريقة وحليب الشوك في علاج مرض الزهايمر والأمراض الالتهابية.

الكلمات المفتاحية: استخلاص الضغط العالي، ام دريقة، حليب الشوك ، محمي للأعصاب، مضاد للأكسدة، مضاد للالتهابات.

CHAPTER I : GENERAL INTRODUCTION

Chapter I: General Introduction

Biodiversity prospecting, or bio-prospecting for short, refers to the systematic and organized exploration for valuable products obtained from bio-resources that possess commercial potential [1]. Africa's high biodiversity presents significant potential for bio-prospecting. However, the arid desert area remains largely unexplored for this purpose, particularly in comparison to other continents. In addition, plants have adapted to the extreme conditions of the desert climate, leading to the synthesis of new secondary molecules with a wide range of interesting bioactivity [2]. Algeria, the largest country in Africa with approximately 2.38 million square kilometers, has a flora of 3,000 species from various botanical families, 15 % of which are endemic. Algeria, the largest country in Africa with approximately 2.38 million square kilometers, has a flora of 3,000 species from various botanical families, 15 % of which are endemic. Algeria, the largest country in Africa with approximately 2.38 million square kilometers, has a flora of 3,000 species from various botanical families, 15 % of which are endemic. However, this flora remains largely unexplored from a phytochemical or pharmacological perspective [3]. The diversity of flora in Algeria is closely related to the wide range of soil types and climates found throughout the country. The climate in the north is Mediterranean, while the Atlas Mountains dominate the central region and the Sahara Desert covers the south [4]. The use of medicinal plants from the national flora could make a significant contribution to the Algerian pharmaceutical industry, with a positive economic impact. In this study, we have chosen to focus on the plant species Ammodaucus leucotrichus from arid areas and Silybum marianum from non-arid areas, which have not been given much consideration despite their significant diversity.

The consumption patterns of modern society are constantly changing. Currently, there is a notable surge in the utilization of natural products produced through environmentally sustainable processes. This trend is being driven by increased public awareness of the health benefits and environmental considerations. Consequently, industrial production must adapt to changing consumption trends, which requires innovation in the search for products and processes that meet social, environmental, and economic criteria simultaneously. In the past two decades, there has been a growing interest in new extraction processes for bioactive compounds, both for analytical and industrial applications. Interest in the effectiveness of natural compounds against various diseases has increased due to the growing number of publications. Additionally, there is a demand for environmentally sustainable techniques. The target compounds in natural products are traditionally derived through exhaustive

extraction of the sample using solid-liquid extraction techniques. Recently, emerging technologies have been implemented using green solvents and eliminating energy-consuming methods and organic solvents. High-pressure extraction methods, such as supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE), are excellent alternatives to traditional methods. These methods are within the green technology concept, using solvents like CO2, ethanol, and water. The extracted materials are appealing because they are environmentally friendly and free of potentially harmful contaminants.

Natural products have diverse biological and pharmacological activities, making them valuable for scientists seeking effective compounds to treat health problems and diseases. They are characterized by low toxicity, complete biodegradability, and availability from renewable sources. Recent research has focused on identifying natural sources with beneficial effects on human health, including diabetes, cancer, and neurodegenerative diseases. Alzheimer's disease (AD) is a neurodegenerative disease that affects approximately 45 million people worldwide [5]. This number is expected to reach 135 million by 2050 [6]. AD is an age-related, progressive, and irreversible disease, and there is currently no effective cure due to its complex and multifactorial nature. Neurodegenerative diseases, such as Alzheimer's disease, can cause a decline in brain tissue and functionality, resulting in impaired psychomotor skills and cognitive processes, including speech, memory, and learning. The exact causes of this disease remain unclear, but studies suggest that a combination of genetic and environmental factors may contribute to its development [7]. The scientific community is actively searching for effective treatments for neurodegenerative diseases, including Alzheimer's disease. Novel therapeutic approaches involving the exploration of bioactive compounds from natural sources have been tested in preclinical and clinical settings to prevent AD [8]. Natural extracts are complex mixtures that can interact with multiple pathological targets, enabling the exertion of various neuroprotective effects. Therefore, these natural extracts are excellent candidates for AD therapy or prevention.

The manuscript presenting all this PhD thesis work is divided into four chapters as follows:

1. The first chapter presents a literature review that is divided into three main parts for clarity. The first part is devoted to the bibliography of green extraction techniques, specifically compressed fluid extraction methods such as pressurized liquid extraction

(PLE), supercritical fluid extraction (SFE), and gas-expanded liquid extraction (GXLE). It includes a detailed description of the principles, equipment, and influencing factors of each method. The second part provides a general description of the studied plants. This article discusses the botanical description, medicinal uses, chemical composition, and various biological activities of Ammodaucus *leucotrichus* and *Silybum marianum*. The final section provides a brief description of Alzheimer's disease and its possible therapeutic approach.

2. The second chapter describes in details the methodology used to carry out the experimental work, the handled materials and the operating protocols applied for the extraction of bioactive molecules, biological activities and chemical characterizations.

3. The final chapter summarises all the results obtained and their discussion. It is divided into two main sections. The first part presents the results obtained with *Ammadaucus leucotrichus* using different extraction techniques PLE and SFE. In addition, the chemical characterization by gas chromatography- mass spectrometry (GC-MS) and liquid chromatography – mass spectrometry (LC-MS) analyses and the in vitro biological activities of the obtained extracts, such as antioxidant, anti-inflammatory and anti-cholinesterase activity. The second part concerns the results obtained from *Silybum marianum* using GXLE and the biological potential (ABTS, LOX and AChE), in addition to the quantification of the silymarin mixture of the obtained extracts.

4. The manuscript concludes with a general summary of the main findings and contributions of this work, as well as the potential implications and future directions that it may open up.

CHAPTER II: BIBLIOGRAPHIC RESEARCH

CHAPTER II: BIBLIOGRAPHIC RESEARCH

In this chapter, we present a comprehensive literature review, divided into three sections for better clarity. The first section focuses on green extraction techniques, with an emphasis on compressed fluid extraction methods. It provides a detailed overview of the principles, equipment, and key factors influencing each technique. The second section covers the plants under study, including a botanical description, medicinal uses, chemical composition, and the various biological activities of selected plants. The final section introduces Alzheimer's disease and discusses potential therapeutic approaches for its treatment.

2.1.Green extraction techniques

The extraction process is essential for isolating high-value compounds from various solid matrices. The choice of solvent and technique greatly influences the selectivity and efficiency of the extraction, as well as the avoidance of unwanted substances. A green solvent should ideally possess the following characteristics: a) low human and environmental toxicity, b) easy biodegradability without harmful effects, c) natural occurrence, d) renewable origin, e) by-product status, f) low-vapor pressure, g) no need for evaporation after extraction. However, finding a solvent that meets all these criteria is difficult.

Solid-liquid extraction techniques can be classified into two categories: conventional and non-conventional methods. Conventional methods include Soxhlet, maceration, infusion, decoction, percolation, stirring-assisted, and stream distillation, while non-conventional or modern methods comprise pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), ultrasound-assisted extraction (UAE), enzyme-assisted extraction (EAE), microwave-assisted extraction (MAE), gas-expanded liquid extraction (GXLE), enhance solvent extraction (ESE) and pulsed-electric field extraction (PFE) [9][10]. Figure (1.1) illustrates the main conventional and non-conventional extraction techniques.

Green non-conventional extraction techniques are innovative methods that use less or no organic solvents, less energy, and less time to extract bioactive compounds from natural sources. These methods are aligned with the principles of green chemistry and sustainability, and they have many applications in various industries such as food, pharmaceutical,

cosmetic, and textile. These techniques have several advantages over conventional methods, such as:

- Reduced solvent usage: Green non-conventional extraction techniques use water, carbon dioxide, ionic liquids, or other benign solvents that are safer for human health and the environment than organic solvents. This reduces the risk of toxicity, flammability, and pollution, and also lowers the cost of solvent disposal and recovery.

- Shorter extraction times: Green non-conventional extraction techniques use high pressure, temperature, or electromagnetic waves to enhance the mass transfer and solubility of the target compounds. This results in faster extraction rates and higher yields than conventional methods that rely on slow diffusion and equilibrium processes.

- High throughput: Green non-conventional extraction techniques can process large amounts of raw materials in a short time, which increases the productivity and efficiency of the extraction process. Some techniques, such as microwave-assisted extraction and ultrasound extraction, can also be performed in batch or continuous modes, which allows for greater flexibility and scalability.

- Specificity: Green non-conventional extraction techniques can selectively extract the desired compounds from complex matrices by adjusting the operating parameters, such as pressure, temperature, frequency, and power. This improves the quality and purity of the extracts and reduces the need for further purification steps.

- Environmentally friendly conditions: Green non-conventional extraction techniques operate under different conditions that preserve the integrity and functionality of the extracted compounds. This avoids the degradation, oxidation, or loss of activity that may occur in conventional methods that use high temperatures or harsh solvents. Moreover, green non-conventional extraction techniques generate less waste and emissions than conventional methods, which minimizes the environmental impact of the extraction process.

But these techniques are not always advantageous and present some drawbacks. The main advantages and disadvantages associated with these novel extraction techniques are listed in Table (2.1).

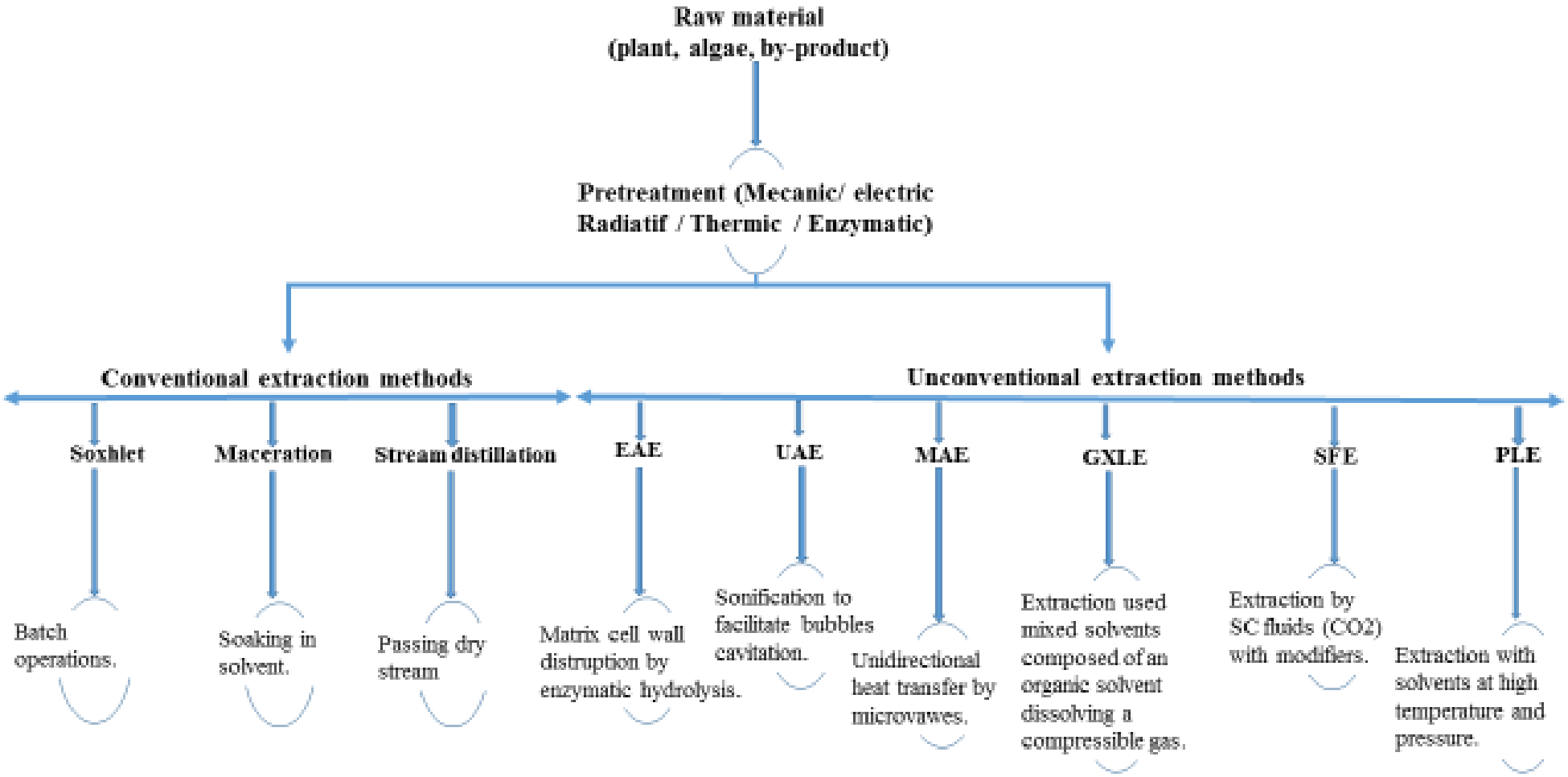

Figure 2.1: Convectional and un-convectional extraction techniques (Created by Author).

Table 2.1: Advantages and disadvantages of non-conventional green extraction techniques (Created by Author).

Green extraction techniques	**Advantages**	**Disadvantages**	**References**
Supercritical fluid extraction (SFE).	High extraction yields, short extraction time, automated system, ranging from laboratory scale (>1g), pilot scale (up to kg), to industrial scale (up to tonnes), no filtration required, possibility to reuse CO_2, no use of toxic solvents, possibility to tune the polarity of SC-CO_2, possibility to extract thermolabile compounds at low temperature, can be coupled with NMR or GC-MS for compound characterization online.	High equipment cost, complex system configuration of the system, elevated pressure required, risk of volatile compounds losses, and limited selectivity towards polar compounds due to low polarity of SC-CO_2.	[10][11][12]
Pressurized liquid extraction (PLE).	Short extraction time, higher repeatability, keeping the samples in a light- and oxygen-free environment, low amount of solvent required and enhanced extraction yield.	High equipment cost.	[13][14]
Gas-expanded liquid extraction (GXLE).	Low energy consumption, low enviremontal impact, limited waste solvent treatment, improved extraction yield.		
Microwaves assisted extraction (MAE).	Reduced extraction duration, minimal energy requirement, laboratory and industrial, enhanced extraction yield, scale and lower capital required for equipment.	Non-selective, heating and/or overheating of the sample may reduce extraction efficiency or cause thermal degradation, limited penetration depth of microwaves during scaling up, very poor efficiency for volatile compounds and separation and purification steps are required.	[15][16]
Ultrasound assisted extraction (UAE).	Low energy consumption, shorter extraction time, easy to handle, no need for complex equipment, less solvent, and improved extraction yield.	Non-selective, heat generated can damage thermolabile compounds, and low efficiency in extraction oil.	[16][17]

Pulsed electric field extraction (PFE).	Energy efficient, short extraction time, selective, non-thermal technology, low operation cost, easy scaling up, minimal energy consumption, low toxicity, waste-free process and continuous operability.	High cost, depend on medium composition, particularly the conductivity.	[18][19]
Cold plasma assisted extraction.	Improve extraction yield, absence of chemicals/solvents, non-thermal, rapid sterilization, economical, environmentally friendly, no production of toxic and hazardous waste compound, and capable of operating at atmospheric pressure and ambient temperature.	Limited applications, need for trained and experienced personnel, initial installation cost and need for special equipment.	[20][21]
High voltage electric discharge extraction (HVED).	Enhanced cell structure disruption efficiency, decreased solvent consumption, and non-thermal technique.	Generation of free radicals that may oxidize target extracted compounds, reduced selectivity, and achieving outcomes observed at the laboratory scale demands higher energy input for pilot-scale applications.	[22][23]
Enzyme assisted extraction (EAE).	Enhanced selectivity, increased yield, environmentally friendly.	Enzymes are expensive and require careful control of the PH and temperature of the medium to ensure optimal enzyme activity.	[24][25]

2.1.1. Green compressed fluid–based extraction techniques

High-pressure extraction techniques, such as: sub and supercritical fluid extraction (SFE), pressurized liquid extraction (PLE) (in which subcritical water extraction (SWE) is included), and gas-expanded liquids extraction (GXL), are progressively used for the extraction of different bioactive compounds from various natural sources, such as plants, algae, fungi, and bacteria [26]. These techniques have high efficiency and selectivity, and they are generally faster, flexible, and integrable with other processes as part of an intensification [27][28] or biorefinery strategy [29][30]. Moreover, high-pressure extraction techniques are characterized by the ability to use environmentally-friendly solvents, while reducing the total required volume to a minimum.

Figure 2.2: Example of green high-pressure solvents in order of increasing polarity (Created by Author).

2.1.1.1. Pressurized liquid extraction

a. General aspects

Pressurized-liquid extraction (PLE) is a rapid and efficient technique for the extraction of bioactive molecules from different matrices. It is based on the use of liquid solvents at elevated temperatures (below the critical point) and pressures high enough to keep the solvent in the liquid state, which enhances extraction efficiency in comparison to techniques performed at near room temperature and atmospheric pressure [14][31]. Pressurized-liquid extraction (PLE) has received different names such as: Accelerated Solvent Extraction® (ASE), Pressurized Fluid Extraction (PFE), Subcritical Solvent Extraction (SSE), Pressurized Hot Solvent Extraction (PHSE) and Super-Heated Liquid Extraction (SHLE). When the extracting solvent used is water, these techniques are referred to as Subcritical

Water Extraction (SWE), Pressurized Hot Water Extraction (PHWE) or Super-Heated Water Extraction (SHWE) [13][32].

Moreover, PLE is often considered as *a greener process* compared to traditional methods for the following reasons:

- Minimizing solvent usage;

- Improving selectivity by reducing the need for additional purification steps and leading to less chemical waste and resource consumption;

- Reducing energy consumption and lowering the overall environmental footprint of the extraction process by shortening extraction times;

- Offering the potential for automation;

- Covering a wide range of compounds polarities using generally recognized as safe (GRAS) solvents, such as water ($\varepsilon = 80$), ethanol ($\varepsilon = 24$) and limonene ($\varepsilon = 2.3$);

- Extracting thermo-labile and volatile compounds at elevated temperatures without degradation [5], [25], [26].

b. Required equipment

The basic equipment for the PLE system, whether commercial or a home-made, consists of: a solvent reservoir, a high-pressure pump (usually the one found in a liquid chromatography system) to precisely control the pressure P_{ext} (typically between 5-15 MPa) and the flow in the system, an oven to heat up the extraction cell T_{ext} and to control the P_{ext}, a pressure-control valve or restrictors, and a collecting vessel which may be equipped with a cooling bath to minimize the thermal degradation of the extract [32]. PLE can be performed in static or dynamic mode and the selection of one mode over the other depends on what is needed to be analysed [35]. Therefore, considering the extraction conditions applied in PLE, it becomes essential to use materials resistant to corrosion when constructing a PLE system, since some solvents such as water becomes highly corrosive in sub and supercritical conditions [14].

c. Parameters Affecting process

Several process parameters affect the PLE performance, including temperature, pressure, extraction time, solvent type, matrix characteristics [31]. Mustafa et al. [33] and Sun et al. [36] gave a comprehensive and detailed overview of the main and different factors affecting PLE process.

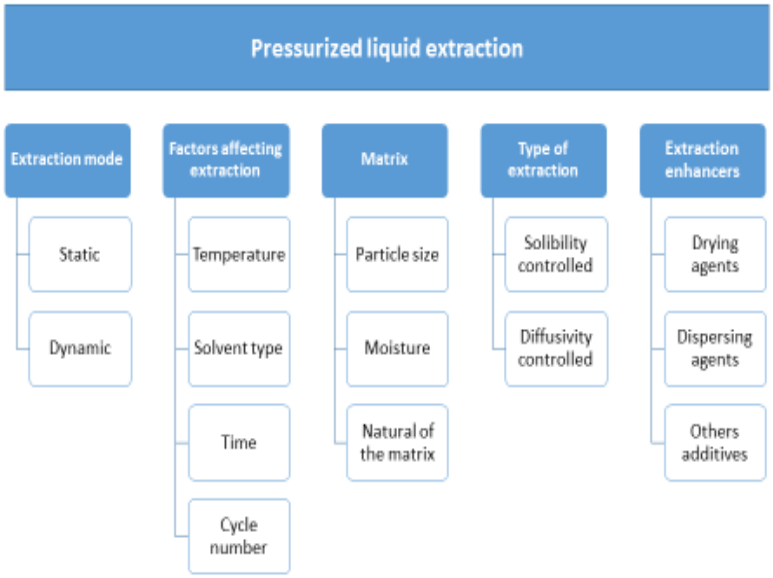

Figure 2.3: Factors affecting pressurized liquid extraction (PLE) and optimization parameters (adapted from [33]**).**

According to the literature, temperature and solvent type are generally the parameters with the greatest impact on the PLE technique [27], [29], [30], [31]. The selection of the solvent is primarily determined by the affinity between the solvent and the target solute to be recovered from the biomass. Since it is known that all solvents typically used in traditional extraction methods are applicable in PLE, opting for "GRAS" (Generally Recognised as Safe) solvents, such as D-limonene, ethyl acetate, ethanol, water, or even their combinations, under the PLE conditions, enhances the eco-friendly nature of the process and allows extracting target compounds with a wide range of polarities. The use of high temperatures increases diffusivity and viscosity in solvent and decreases the surface tension. Consequently, this allows better solvent penetration into the matrix, leading to faster mass

transfer [10]. Moreover, elevated temperature can alter the physicochemical characteristics of the solvent, including its dielectric constant (polarity). In particular, the dielectric constant of water is substantially reduced from ε = 78 at 25 °C to 27 at 250 °C, similarly to the dielectric constants of organic solvents like methanol (ε = 33) and ethanol (ε = 24) under ambient conditions, thus making it possible to extract polar, mid-polar and non-polar compounds.

In addition to temperature and solvent type, other parameters that affect PLE are pressure, extraction time, and solvent-to-solid ratio. Pressure is an important factor that influences the density, solubility, and viscosity of the solvent, as well as the cell volume and the extraction yield. Generally, higher pressures lead to higher extraction efficiencies, but they also increase the operational costs and safety risks [30], [32]. Therefore, an optimal pressure should be chosen according to the solvent and the matrix characteristics, normally as long as the solvent remains liquid, pressure has a small effect in extraction results. Extraction time is another parameter affect PLE, it determines the duration of the contact between the solvent and the solute. Longer extraction times may increase the yield, but they may also cause degradation, hydrolysis, or loss of activity of the bioactive compounds. Hence, a balance between extraction time and quality should be achieved. Solvent-to-solid ratio is the ratio of the volume of the solvent to the mass of the solid sample. Higher solvent-to-solid ratios may improve the extraction performance, but they may also increase the solvent consumption and the energy demand. Therefore, a minimum solvent-to-solid ratio that ensures sufficient solvent penetration and solute dissolution should be selected.

2.1.1.2. Supercritical fluid extraction

a. General aspects

Supercritical Fluid Extraction (SFE) is an efficient and emerging approach for recovering various types of substances. It avoids some of the drawbacks of conventional extraction techniques and is considered an environmentally friendly technology [41]. This technology is significant across various industries as it offers the potential to produce products that are free from toxic residues, without degradation of active components, and with notable purity. SFE employs a supercritical fluid, which is defined as any substance that is subjected to thermodynamic pressure and temperature conditions above its critical point. At this state, there is no distinction between liquid and gas phases, and the fluid phase

shares the characteristics of both a gas and a liquid. These simultaneous properties, high liquid-like density and low gas-like viscosity, lead to an effectively enhanced mass transfer between the target compound and the supercritical fluid (SF) [42][43][44].

Furthermore, Supercritical Fluid Extraction (SFE) offers several advantages over conventional extraction methods. These include reduced solvent consumption and waste generation, shorter extraction times, lower energy demand, higher selectivity and specificity, and preservation of thermo-labile and volatile compounds [4]. As a result, SFE has been widely used to extract various bioactive compounds from different natural sources, including plants, algae, fungi, and bacteria [45][46]. Some examples of the applications of SFE include the extraction of essential oils, flavours, and fragrances from spices, herbs, and flowers; the extraction of antioxidants, pigments, and vitamins from fruits, vegetables, and algae; the extraction of lipids, fatty acids, and sterols from seeds, nuts, and microorganisms; the extraction of alkaloids, terpenoids, and cannabinoids from medicinal plants; and the extraction of pharmaceuticals, nutraceuticals, and cosmeceuticals from various sources [45][46].

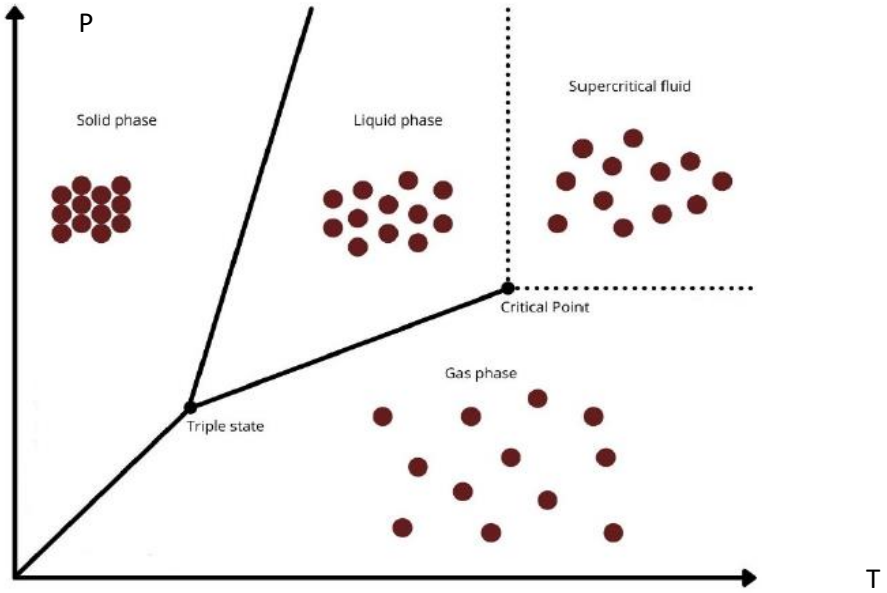

Figure 2.4: General phase diagram in terms of pressure and temperature [43].

Supercritical carbon dioxide (SC-CO_2) is the most commonly used solvent in Supercritical Fluid Extraction (SFE), which is a green and efficient technique for recovering various bioactive compounds from natural sources. SC-CO_2 has several advantages, including its non-toxic, non-corrosive, and non-flammable nature, as well as its low viscosity

and high diffusivity, which contribute to its high output quality. In addition, due to its low critical temperature and pressure values (31 °C and 7.38 MPa), CO_2 is suitable for extracting thermo-sensitive molecules [47]. Separating solute from the SC-CO_2 can be easily achieved through the depressurization of the SF. Moreover, SC-CO_2 can be recycled and reused, which helps minimize waste generation.

However, the use of SC-CO_2 as a solvent has a drawback due to its low polarity. This makes it suitable for lipids, fats, and non-polar substances, but less effective for most polyphenols and polar compounds [48]. To address this limitation, modifiers or co-solvents such as ethanol, methanol, and water, or combinations of co-solvents, can be introduced. This increases polarity, strengthens solvating power, and allows for the extraction of more polar compounds, thereby expanding the range of bioactive compounds that can be targeted [49][50]. Ethanol and methanol are the most commonly used organic co-solvents. Water and ethanol are commonly used as co-solvents in the SFE process due to their low cost and eco-friendliness. They have gained attention for their potential direct application in the food and pharmaceutical industries [51].

b. Required equipment

The design of a SFE system can vary in complexity depending on specific requirements. Essentially, there are two types of systems: analytical instruments and preparative systems, which can be either pilot-scale or industrial-scale. Analytical systems are used for sample preparation before chromatographic analysis, for instance, to obtain mg/g of extracts. Preparative systems are utilized to extract compounds on a pilot scale of grams or an industrial scale of kilograms [52][53].

A supercritical fluid extraction (SFE) system typically consists of a solvent reservoir, a pump or compressor, an extraction vessel, a separator, and a collection vessel. The principles of operation for a SFE system are as follows: pressurize and heat the solvent above its critical point, pass it through the extraction vessel containing the solid or liquid sample, where it dissolves the target compounds, depressurize and cool it in the separator, where the solute precipitates and the solvent is recycled, and finally collect the solute in the collection vessel. The parameters for operating a SFE system, including pressure, temperature, flow rate, and co-solvent, can be adjusted to optimize extraction efficiency, selectivity, and yield.

Additional resources are available elsewhere for more detailed information and schemes [54][55].

c. Parameters Affecting process

The efficiency of the SFE process is affected by several factors, including temperature, pressure, time, CO_2 flow rate, moisture content, particle size of the matrix, and type and percentage of co-solvent [45][46]. To optimize extraction yield and minimize operational costs, it is crucial to control these extraction parameters. However, most studies have only investigated the effects of temperature and pressure, which typically range from 40 to 70 °C. The lower limit for temperature is determined by the proximity to the critical temperature of CO_2, while the upper limit is influenced by its impact on the solvent density. It is challenging to predict the effect of temperature on extraction. Elevated temperatures decrease the density and solvating power of the supercritical fluid, which reduces extraction efficiency. Lowering temperatures reduces the vapor pressure of the solute and the volume of the solvent, thereby increasing the density and solvating power of the supercritical fluid (SF), which subsequently increases the extraction yield [38][52]. In terms of pressure, most studies investigate the range between 10 to 50 MPa. Increasing the pressure increases the density and solvation capacity of the supercritical fluid, making it easier to penetrate the sample matrix. However, excessive pressure is not always recommended as it may compact the extraction bed, reducing diffusivity and subsequently decreasing extraction efficiency [56].

2.1.1.3. Gas-expanded liquid extraction

a. General aspects

Among the studied techniques, Gas Expanded Liquid Extraction (GXLE) is the less known. It is an intermediate technique between PLE and SFE, in which a compressible gas (usually CO_2) is dissolved in a liquid organic solvent [57][58]. The effect of this combination, under certain pressure and temperature conditions, is to expand the solvent, resulting in reduced viscosity and increased extraction capacity. As mentioned above, CO_2 is often chosen as the compressible gas in most GXLE due to its environmental and economic advantages. The resulting process is called Carbon Dioxide Expanded Liquid (CXL). Therefore, GXLE has similar advantages as SFE, such as increased diffusivity, while

improving the extraction of high polar compounds. This improvement is achieved at mild operating pressures compared to SFE and with lower amounts of organic solvent compared to PLE [59]. This makes GXLE attractive for industrial applications and economically viable.

In previous work Jessop and Subramaniam [60] categorized GXL into three types based on the solubility of the gas in the liquid phase:

- One-phase GXL: gas is highly soluble in the liquid solvent, leading to significant variation in the physicochemical properties; in this sense, the most commonly used organic solvents are ethanol, methanol, and hexane (compounds with high affinity to CO_2).
- Two-phases GXL: the liquid phase does not totally dissolve in the gas, there are two distinct phases in the mixture (resembling tiny bubbles within the liquid). For instance, combinations of CO_2 and water or strong polar solvents where the physicochemical properties do not change significantly (except acidity).
- Mixed type: Only small quantities of gas can be dissolved in the organic solvent. The viscosity changes significantly, while the polarity or dielectric constant remain relatively unaffected. For example, polymers, oils, and ionic liquids.

This classification is not a fixed rule; indeed, the same mixture can be in all the three categories depending on pressure and more notably temperature.

GXLE can be effective in recovering bioactive molecules from various matrices. It has been used to extract cholesterol-lowering compounds from the olive pits using CO_2-expanded ethyl acetate [61], lipids from *B. braunii* using CO_2-expanded methanol [62], astaxanthin from *H. pluvialis* microalgae using CO_2-expanded ethanol (CXE) [63], β-carotene from *A. platensis* cyanobacteria using CXE [64], fucoxanthin from *P. tricornutum* microalgae was carried out using CO_2-expanded limonene [65] and phytosterol from *P. autumnale* cyanobacteria using CXE to investigate their potential neuroprotective properties [66].

b. Required equipment

Given the similarities with SFE noted above, the equipment required to work with GXLs can be the same as that required for SFE [43][67]). This equipment is shown in Figure

(3.7) (Chapter 3). However, the design requirements may be less demanding since the pressures typically applied in the GXLE are less than 10 MPa [38]. In addition, it is important to consider that a higher amount of solvent is used compared to SFE. The basic instrumentation consists of two pumps, one for the liquid solvent and the other for the gas (CO_2), a heated extraction cell, valves for flow/pressure control and separators [59]. In addition, additional equipment such as gauges, thermocouples, and a passive safety system (rupture disk or safety valve) are recommended, but not required, for laboratory scales.

c. Parameters effect on the process

Significant changes in physicochemical properties can influence extraction conditions when modifying the three primary parameters of GXLs: pressure, temperature, and solvent composition (molar ratio).

- **Diffusion coefficient**

It is a critical transport parameter that determinates the interactions between solvents and solutes. Regarding mass transfer characteristics, GXLs are in midway between supercritical fluids and liquids, because their viscosity and density is in a range intermediate between neat liquids and supercritical fluids. For instance, the diffusion coefficient of pure alcohols is lower than that of a GXL formed by CO_2 and alcohol, and this, in turn, is lower than that of a supercritical fluid composed of them. However, diffusion is also influenced by changing the pressure, the temperature, and the molar ratio [68].

- **Volume expansion**

It is the parameter that most distinctly characterizes GXLs – the expansion of the volume of a neat liquid solvent by introducing a gas phase and pressure. This results in a volume increase with pressure, not like in pure gases, pure liquids, or supercritical fluids. The change in volume depends on the solubility of the gas phase in the organic solvent. Abbot et al. illustrated that the addition of the same amount of CO_2 does not result in the same expansion across all solvents [69]. Aida et al. linked this expansion to the hydrogen bond network [70]. On the one side, at elevated pressures, the composition consists of a single liquid phase, where the volume increases with pressure and decreases with temperature. This phenomenon is attributed to the dissolution of CO_2 into the liquid phase, causing an expansion in volume. On the other side, it is essential to consider the impact of

high temperatures, as they ease the formation of a new phase from the gas. Consequently, higher temperatures lead to the formation of two-phase GXL [71].

- **Density**

It is a crucial parameter, since it is strongly related to mass transfer phenomena. It is affected by pressure, temperature, and composition of the solvent. In a single-phase GXL, increasing the pressure or decreasing the temperature leads to an increase in density. It is more complex in a two-phase GXL where the liquid density can increase if the gas phase expands (by adding CO_2), but increasing the temperature has a double effect: it expands the gas phase and decreases the density in the liquid phase. Conversely, small increases in pressure can lead to a decrease in the density of the mixture, attributed to an increase of solubility of the gas in the liquid. The impact of the molar fraction of gas varies depending on the liquid. For instance, in systems comprising CO_2 and alcohols, acetonitrile, or even cyclohexane, the density is higher at a low CO_2 molar fraction in the liquid and drop at high CO_2 pressures.

- **Polarity**

It can be modified mainly by adjusting the molar ratio of gas in the liquid phase. For example, in the case of CO_2, increasing its quantity in the liquid phase results in a GXL with lower polarity but higher acidity (particularly in water and alcohols). In one-phase GXL, the molar ratio of gas in the liquid can be increasing without adding additional gas to the mixture. This adjustment can be achieved, for instance, by reducing the temperature or increasing the pressure. The change in the polarity can be calculated numerically using Kamlet–Taft solvatochromic and electronic transition energy (ET) parameters [72] [73]. The main challenge with numerical calculation methods lies in their application primarily to model molecules rather than complex natural raw materials. Nevertheless, their results serve as highly accurate approximations, aiding in the selection of the suitable solvent mixture and determination of the optimal conditions of pressure and temperature based on the desired polarity of the target compounds in the extraction of natural products.

2.1.2. Green combined technologies

Sequential extraction of compounds from a matrix sample using solvents of different polarities provides diverse profiles of high-added value natural products. This approach minimises the amount of generated waste and maximises the products obtained from a single raw material. However, the complexity of the composition of natural raw materials means that a single extraction process may not be able to solubilise different classes of compound, especially since selective extraction methods are expected.

This approach is also referred to as a sequential multistage process [29], integrated operational processes [74], multi-unit operational processes [67], biorefinery [75] and fractionation [76]. The method involves subjecting the same raw material to distinct extraction techniques and/or employing the same extraction technique under varying conditions sequentially to obtain different fractions of extract. Importantly, this strategy enables to obtain lipophilic extract fractions, fractions comprising compounds of elevated polarity, and further to separate fractions containing strongly bonded compounds in the matrix.

Various high-pressure techniques can be used based on the characteristics of the desired products. When targeting the nonpolar compounds, SFE with CO_2 as the solvent is recommended for solid feeds, while Supercritical Fluid Fractionation SFF is suitable for liquid feeds. Conversely, if the goal is to recover compounds with polar characteristics, PLE using polar solvents and SFE with CO_2 and polar modifiers are the most appropriate methods.

The application of combined high-pressure extraction technologies for obtaining compounds from different matrices has been reported in Table (2.2).

2.1.3. Applications of novel green extraction technologies in the recovery of bioactive compounds

There are many examples in the literature of the use of high-pressure extraction techniques to recover high value molecules from various plant matrices (see Table (2.2)). Although the focus today is on the use of green solvents in environmentally friendly extraction processes, the majority of high value compounds have been extracted using ethanol, water or their mixtures as extraction solvents, mainly due to their greener nature and the diversity of solvents that could be used

Table 2.2: Recent application of sub and supercritical fluid extraction for high-value molecules from different natural matrix (period 2016-2023) (Created by Author).

Matrix natural	Compounds recovered	Extraction methods	Extraction conditions	References
Pressurized liquid extraction (PLE)				
***Rosmarinus oficinalis* L.**	Rosmarinic acid, carnosic acid and carnosol.	PLE	EtOH: H_2O 0, 20, 40, 60, 80, and 100%, 100 °C, 80 bar, Static time 5min and 3 cycles.	[77]
***Euglena cantabrica* microalga.**	Carotenoids, Chlorophylls and carbohydrates.	PLE	EtOH-H_2O mixture (0, 50, 100 %) 40, 110 and 180 °C, 10 MPa, 20 min.	[78]
***Convallaria majalis* L.**	Convallatoxin, k-Strophanthidin, Bufalin, Digoxin, Digitoxigenin, Odoroside A, Proscillaridin, and Withanolide A.	PLE	MeOH-H_2O mixture (0, 25, 50, 75 %), 40, 60, 80 and 100 °C, 2 cycles, 10.34 MPa, Static time 5 min.	[79]
Green coffee beans.	Chlorogenic acid and their derivatives.	PLE	Pure H2O, 3.45-17.24 MPa, 40-200 °C, 2-18 min.	[80]
Pomegrante peels.	Proteins and bioactive peptides.	PLE	0, 30, 40, 50, 60, 70, 80 and 100 % EtOH, 10.34 MPa, 120 °C, Static time 5 min, Extraction time 12 min and one cycle.	[81]
Edible insects. *Acheta domesticus* and *Tenebrio molitor*	Fatty acids	PLE	EtOH, EtOH:H2O, 120 °C, 100 bars and 15 min.	[82]
Cocoa Bean Shell.	Flavanols and alkaloids.	PLE	EtOH, 60, 75 and 90 °C, 10.35 MPa and static time 5-50 min.	[83]
***Echium plantagineum* seeds.**	Omega-3 fatty acids.	PLE	Ethyl acetate, hexane, EtOH, H_2O and EtOH: H_2O (90:10,	[84]

			80:20, 70:30 and 50:50 V/V), 60, 90, 120, 150 and 200 °C and static time 10 min.	
Thinned peaches.	Polyphenol-derived.	PLE	0, 50 and 100 % EtOH, 50, 115 and 180 °C, 10 MPa, static time 5 min and Extraction time 20 min.	[85]
Mauritia flexuosa shell.	Carotenoids and phenolics compounds.	PLE	35 -65 °C, 20-80 % EtOH in H_2O, 40 min and 10 MPa.	[86]
***Stevia rebaudiana* leaf.**	Glycosides, phenolics, and flavonoids.	PLE	40, 70 % EtOH in H_2O, 5, 10 MPa, 100, 125 and 150 °C and 10, 20 and 30 min.	[87]
Eugenia uniflora L. leaf.	Phenolic compounds.	PLE	10 MPa, 50 °C and 0, 50, 100 % EtOH in H_2O.	[88]
***Citrus sinensis* by-products.**	Terpenoids.	PLE	ETAC, 25, 62.5 and 100 ◦C, 10 MPa and time 10, 20 and 30 min.	[89]
Tamarillo (*Cyphomandra betacea*) Epicarp.	Phenolics and flavonoids.	PLE	20 min, 10.34 MPa, 0, 50, and 100 % EtOH in H_2O and 60, 120, and 180 °C.	[90]
***Mentha pulegium*, *Equisetum giganteum*.**	Flavonoids	PLE	50 % EtOH in H_2O (M. pulegium) EtOH (E. giganteum), 40 °C, 100 bar, 2 ml/min and 210 min.	[91]
Tuber aestivum and *Terfezia Claveryi* Fungis.	Carbohydrates, β-glucans, chitin, proteins, phenolic compounds, and sterols.	PLE	H_2O (carbohydrates and phenolic compounds), EtOH (sterols), 50, 115 and 180 °C, and 5, 17.5 and 30 min.	[92]
Supercritical fluid extraction (SFE)				
***Chlorella sorokiniana* Microalga.**	Carotenoids and Chlorophylls.	SFE	CO_2 + (0-10 % EtOH), 40-60 °C, 10-30 MPa, 180 min.	[93]
***Gracilaria mammillaris* seaweed.**	/	SFE	10, 20 and 30 MPa, 40, 50 and 60 °C, and 2, 5 and 8 % EtOH.	[94]
Haskap berry pulp.	Anthocyanin.	SFE	CO_2 + H_2O, 35 min, 35, 50 and 65 °C and 10, 27.5 and 45 MPa.	[95]
***Lupinus mutabilis*.**	Alkaloids	SFE	CO_2 +10 % EtOH, 50 °C, 27 MPa and 30 min.	[96]
***Idesia polycarpa*.**	Essential oil	SFE	50 L/h CO_2, 1 h, 40-60 °C, and 7-15 MPa.	[97]

Acacia dealbata.	Triterpenoids	SFE	CO_2 + 5 % EtOH or 5 % Ethyl acetate, 40-80 °C, 20-30 MPa and 360 min.	[98]
Curcuma longa.	Curcuminoid	SFE	CO_2, 65 °C, 35 MPa, and 20 min.	[99]
***Moringa oleifera* leaves.**	Flavonoids	SFE	10, 15 and 20 MPa, 50, 65 and 80 ◦C, 30, 60, 90, 120, 150 and 180 min, and the effect of the different % of EtOH and H_2O as co-solvents.	[44]
***Hippophae salicifolia* leaves.**	Polyphenols	SFE	40-60 °C, 18-32 MPa, 10-20 g/min (CO_2 flow rate), and 1-3 % co-solvent.	[100]
Seaweeds *Alaria esculenta,* *Laminaria digitata,* and *Ascophyllum nodosum* seaweeds.	β-carotene, α-tocopherol, β/γ-tocopherol, δ-tocopherol, fucoxanthin, and phloroglucinol.	SFE/ GXLE	30 MPa, 40-80 °C, 40 min, co-solvent: 0-15 % EtOH in H_2O and co-solvent in CO2: 5-90 %.	[48]
Spent coffee grounds.	Oil fraction, antioxidants	SFE	T = 39.85-59.85 °C, 50 MPa, $1.9*10^{-3}$ kg /min (CO_2 flow rate), co-solvents: isopropanol, EtOH and ethyl lactate.	[101]
Wild thyme by-product.	Polyphenols, essential oil, fatty acids.	SFE	SFE1: 10 MPa, 40 °C and SFE2: 35 MPa, 50 °C	[102]
***Japonica Luna* rice bran.**	Fatty acids.	SFE	CO_2, $1.8*10^{-3}$ kg/min, 50-80 °C and 30-40 MPa.	[103]
***Dunaliella salina* microalga.**	Carotenoids.	SFE	CO_2, 90 min, 4.5 ml/min, 25, 35, and 40 MPa, and 15, 30, and 45 °C.	[104]
Ginseng fruit (*Potentilla anserina* L)	Fatty acids	SFE	CO_2, 60-180 min, 30-40 MPa, and 30- 60 °C.	[105]
Sinami fruit (*Oenocarpus mapora*)	Polyphenols	SFE	CO_2, 35 MPa, and 60 °C.	[106]
Gas-expanded liquid extraction (GXLE)				
Acerola by-products.	Ascorbic acid, phenolic compounds and carotenoids.	GXLE	CO_2 + EtOH, 40 °C, 7 MPa, 90 min.	[107]

Olive seeds.	Phenolic compounds and phytosterols.	GXLE	CO_2 + Ethyl acetate, 40, 60, 80 °C, 8, 16.5, 25 MPa, 10 min	[61]
***Gardenia jasminoides* Ellis fruit pulp.**	Pigments (crocin-1 and crocin-2).	GXLE	0, 50, 80 and 100 % EtOH in H_2O, 8, 10 and 15 MPa, 5, 20 and 25 °C.	[108]
N.A	Curcumin	GXLE	10-50 % co-solvent, EtOH, 10-30 MPa, and 35-50 °C.	[109]
N.A	Quercetin	GXLE	70 °C, 10 MPa, and 30 % mol CO_2: EtOH mixture. 35 °C, 10 MPa, and 10 % mol CO_2: ethyl lactate.	[110]
N.A	Acetylsalicylic acid. Acetaminophen. Caffeine.	GXLE	50 °C, 8.5 MPa.	[111]
***Schizochytrium sp*. Microalgae.**	Fatty acids (Docosahexaenoic acid).	GXLE	40-70 °C, 6.9 MPa, CO_2 0.16 and EtOH 5 mL/min.	[112]
Garlic husk.	Garlic acid. 4-hydrobenzoic acid. Caffeic acid. p-coumaric acid. Trans-ferulic acid.	GXLE	50-200 °C, 180 min, CO_2 0.5-2 mL/min and EtOH 3 mL/min.	[113]
***Candida antarctica*.**	Lipase enzyme.	GXLE	20 °C, 6 MPa and Bio-based solvent (MeTHF) 10 %.	[114]
South African medicinal plants: *Cydonia oblonga* fruit, *Allium cepa* bulb, and quince fruit.	Quercetin.	GXLE	10-90 % co-solvent, 0-20 % H_2O in EtOH, 10-30 MPa, and 30-80 °C.	[115]
Apple fruit varieties.	Catechin, chlorogenic acid, guaiaverin, hirsutrin, hyperoside, phloridzin,	GXLE	10-70 % co-solvent, 5-20 % H_2O in EtOH, 10 min, 10-30 MPa, and 30-80 °C.	[116]

	epicatechin reynoutrine, and rutin.			
Comparison purpose				
Chia seeds.	Fatty acids. Triacylglycerols	PLE	Dichloromethane: methanol (2:1, v/v), EtOH, 40, 60 and 80 ºC, one cycle, 10.3 MPa and 10 min.	[117]
		SFE	25 and 45 MPa, 40 and 60 ºC, 40 g/min of CO_2 and 240 min.	
Comfrey root.		PLE	EtOH: H_2O mixtures 0-100 % 40- 200 °C, 10.34 MPa, and 20 min.	[118]
		SFE	CO_2+EtOH at 7 and 15 %, 150 and 300 bar, 40°C, and 120 min.	
***Vetiveria zizanioides* roots.**	Essential oil (Valerenol Valerenal β-cadinene).	SFE	CO_2, 50 °C, 20 MPa, and 90 min.	[119]
		GXLE	CO_2+EtOH, 50 ° C, 8.4 MPa, CO_2 0.22 mol, EtOH: 5 mL/min, and 150 min.	
Biorefinery approach				
***Viburnum opulus* pomace and berries.**	Tocopherols. Fatty acids.	Sequential SFE-PLE	SFE-CO_2: 35-55 MPa, 30-50 °C, 1.5-2.5 L min^{-1} and 60-120 min, by CCD. PLE: solvents (acetone, EtOH, H_2O), 15 min, 70 °C and 10.3 MPa.	[120]
Elephant grass (*Pennisetum purpureum*).	Phenolics. Sterols. Fatty acids.	Sequential SFE-PLE	SFE-CO_2: 35 MPa, 40 °C, 0.175 Kg S^{-1}. PLE: 50 % EtOH/H_2O, 3 cycle (15 min for each), 100 °C and 10.3 MPa.	[121]
Sardine waste.	Fish oil. PUFAs. Fish proteins.	Sequential SFE-SWE	SFE-CO_2: 25 MPa, 40 °C, 15 g/min and 140 min. SWE: 10 ml/min, 30 min, 90, 140, 190, 250 °C, and 10 MPa.	[122]
***Passiflora mollissima* seeds.**	Fatty acids. Phenolics.	Sequential PLE-PLE	PLE: solvents (n-heptane, cyclohexane, limonene and n-hexane), 10 MPa, 100 °C, and 60 min.	[123]

			PLE: solvents (EtOH/ EToHc: 0, 50, 100 (v/v)), 20 min, 50, 100, 150 °C and 10 MPa by CCD.	
***Moringa oleifera* leaves.**	Fatty acids Phenolics Flavonoids (Quercetin, Kaempferol) Volatiles	Sequential SFE-GXLE-PHWE	SFE-CO_2 GXLE PHWE	[124]
***Isochrysis galbana* microalga.**	Fatty acids Carotenoids Chlorophylls	Sequential SFE-GXLE-PLE-PHWE	SFE: GXLE: PLE: PHWE:	[30]
***Porphyridium cruentum* microalga.**	Phycoerythrin, sulfated polysaccharides, Zeaxanthin, and carotenoids.	Sequential PHWE-PHWE-PLE	PHWE: 25 °C PHWE: 25, 50, 100 and 150 °C. PLE: EtOH, 125 °C, 10 MPa, and 20 min.	[125]
Rowanberry pomace.	Carotenoids (β-carotene). Fatty acids (total lipophilic). Polyphenols (total phenolics).	Sequential SFE-PLE	SFE-CO_2: 25-45 MPa, 40-60 °C and 180 min. PLE: solvents (acetone, EtOH, H_2O), 15 min, 70 °C and 10.3 MPa.	[126]
Passion fruit bagasse.	Piceatannol Scirpusin B	Sequential SFE-PLE	SFE-CO_2 (3 sequential steps): the 1st at 60 °C and 17 MPa, the 2nd at 50 °C and 17 MPa and the 3rd at 60 °C and 26 MPa. PLE: 50, 75, 100 % EtOH in H_2O, 70 °C and 10 MPa.	[127]
Cocoa bean hulls.	Fatty acids. Phenolics.	Sequential SFE-PLE	SFE: 15 and 30 MPa, 7-15 % EtOH, 22 g/min, 120 min, and 40 °C. PLE: 0-100 % EtOH in H_2O, 20 min, 40 and 70 °C and 10 MPa.	[74]

Black mulberry leaves.	Phenolics.	Sequential PLE-SFE	PLE: Pure EtOH, 20 min, 40-200 °C and 10 MPa. SFE-CO_2: 20 and 30 MPa and 40 °C.	[128]
Scenedesmus obliquus.	Lipids Carotenoids Carbohydrates and proteins.	Sequential SFE-GXLE-PHWE	SFE: CO_2, 50 °C, 36 MPa and 120 min. GXLE: CO_2 + 75 % EtOH, 50 °C, 7 MPa and 150 min. PLE: 50 °C, 10 MPa and 45 min.	[75]
Granadilla peel.	Carotenoids: (β-carotene. Lutein). Phenolics. Pectin.	Sequential PLE-PHWE. Sequential GXLE-PHWE.	PLE: EtOH, 30 min, 10 MPa, and 40-60 °C. GXLE: 50 % EtOH, 30 min, 10 MPa, and 40-60 °C. PHWE: 120, 140, and 160 °C and 10 MPa.	[129]
***Galdieria phlegrea* Microalga.**	Carotenoids: zeaxanthin. β-carotene. Lipids.	Sequential PLE-SFE	PLE: Pure EtOH, 100 bar, 50 °C, and 30 min. SFE- CO_2: 350 bar, 60 °C, and 100 min.	[130]

N.A: not apply.

2.2. Plant matrices

Algeria has an important natural potential of medicinal plants with a great diversity, due to its vast territory from north to south with different soil types and climates ranging from Mediterranean in the north to Saharan in the south, a unique environment for this resource.

These plants have been traditionally used by indigenous communities for various medicinal and therapeutic purposes [131][132][133]. However, the Algerian flora has been reported to contain approximately 3164 species from different botanical families and has not been extensively studied in terms of pharmacological aspects as well as phytochemical characteristics [134]. This represents a significant opportunity for researchers and the pharmaceutical industry to explore and develop new therapeutic approaches.

The two-biomass selected for study in the present work, from two different zones (as illustrated in Figure (1.5)), were *Ammodaucus leucotrichus* and *Silybum marianum*. These species were selected in the context of the valorisation of underexplored and unexploited plant resources in Algeria. In the following sections, a brief summary of the characteristics, composition and applications of these plants will be presented.

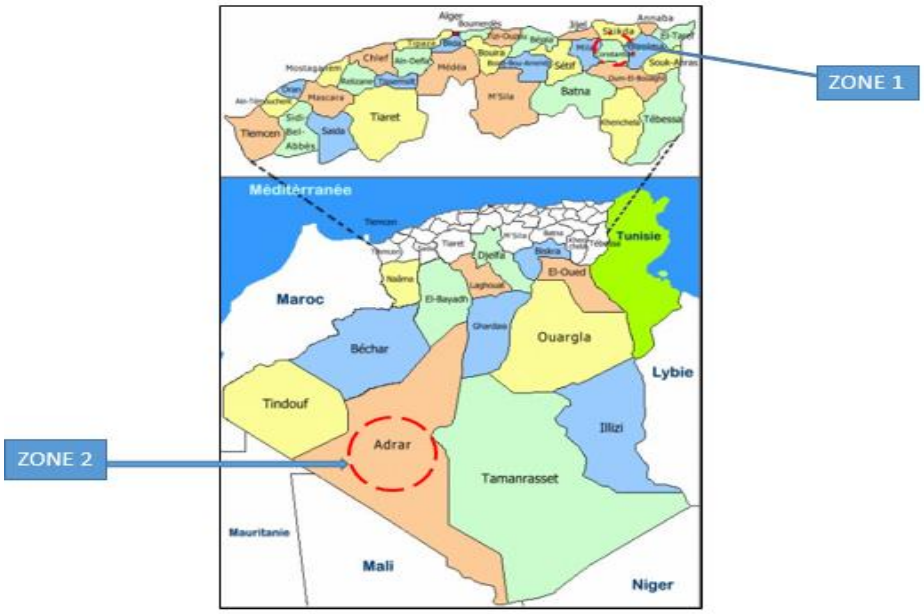

Figure 2.5: Location for sampling areas (Ammodaucus leucotrichus: Adrar, Silybum marianum: Constantine) ([134], Modified by the author).

2.2.1. Ammodaucus leucotrichus

2.2.1.1. Presentation and botanical description

Ammodaucus leucotrichus Coss. & Dur. (*A. leucotrichus*) is a small aromatic endemic plant, distributed throughout the Canary Islands, North and West Africa in the Saharan and sub-Saharan countries, including Morocco, Algeria, and Tunisia extending into Egypt and tropical Africa. It is a monotypic genus belonging to the Apiaceae family and is unknown to the international trade market. It is commonly known as 'hairy cumin' because of the dense silky hairs that cover the fruits. In Algeria, its local name is Oumdriga, Elmanssoufa, Akaman or Kammunes-sofi.

Morphologically, *A. leucotrichus* is a small annual wild or cultivated plant, 10 to 12 cm tall, glabrous with erect, finely striped stems, branched from the base. The leaves are finely lobed and slightly fleshy, forming flat and narrow ridges with sheathing petioles. The

white flowers are grouped in umbels of 2 to 4 branches with 5 free petals. The fruit is a diachene, 6 to 10 mm long and covered with dense, soft, white hairs. The species usually flowers in early spring, between February and April. It grows spontaneously in wadis, on sandy-gravelly soils in arid conditions where the annual rainfall is less than 100 mm.

Figure 2.6: Ammodaucus leucotrichus natural habits (Taked by the author).

2.2.1.2. Phytochemical and pharmacological potential

Numerous ethnopharmacological studies have noted the traditional use of *A. leucotrichus* to treat gastric and digestive disorders (nausea, constipation, diarrhea, vomiting, cramps and colic, intestinal worms, regurgitation) [135][136]. It is also used to treat patients suffering from high blood pressure [137] and diabetes [4][138]. Moreover, the aerial parts of the plant are used in culinary applications as cooking ingredients as a flavouring agent [139].

Most of the previous works about *A. leucotrichus* mainly focused on the chemical composition and pharmacological activities of lipophilic extract like Antioxidant [140][141][142][143], anti-mycotoxin [144], anti-inflammatory [142], neuroprotective [145], antibacterial [146][147][140][141][143], antifungal [147][148][140][143][144], and antitumor [146][143] effects. The GC-MS analysis showed that R-perillaldehyde and

limonene as the main components in EO of *A. leucotrichus* [149][139][145][142][143][150], and to a less extent bornyl angelate, perilla alcohol, ɣ-decalactone and methyl perillate. Most of the previous studies on *A. leucotrichus* were related to the phytochemical composition and the pharmacological activity of the essential oil [142][139][145]. However, only few investigations considered the polar extracts and their bioactivities, such as antimicrobial [143][132], anti-inflammatory [151][132], antioxidant [152][133][153][154], anticancer [132][155], anticholinesterase [151], and anti-diabetes effects [133][154][156]. Phytochemical screening of polar extracts revealed the presence of rhamnazin, naringenin-hexoside, p-coumaroyl-hexoside, isorhamnetin-(malonyl) glucoside, luteolin and their derivatives, apigenin and their derivatives, chrysoeriol and their derivatives [154][157].

2.2.1.3. Earlier published works about Ammodaucus leucotrichus biomass

Table 2.3: Previous published studies about Ammodaucus leucotrichus plant (period 2016-2023) (Created by Author).

Title	Objectives of the study	Main results	Year	ref
Polyacetylenic caffeoyl amides from *Ammodaucus leucotrichus*.	• Isolation and structure elucidation of 14 compounds from an ethyl acetate extract of *A. leucotrichus* fruits using a combination of methods (GC-MS, HPLC-PDA-MS-ELSD, NMR).	• The identification of 7 flavones and two lignans: luteolin, Apigenin, chrysoeriol, apigenin 7-O-β-D-glucopyranoside, chrysoeriol 7-O-β-D-glucopyranoside, (8R,7′S, 8′R)-isolariciresinol 9′-O-β-D-glucopyranoside, (7S,8R)-balanophonin 4-O-β-D-glucopyranoside, and (7R,8S,7′S,8′R)-4,9,4′,7′-tetrahydroxy-3,3′-dimethoxy-7, 9′-epoxylignan. • Six undescribed poly- acetylenic caffeoyl amides are obtained from *A. leucotrichus* fruits.	2023	[157]
Ammodaucus leucotrichus Coss. & Durieu: Anti-hyperglycemic activity via the inhibition of α-amylase, α-glucosidase, and intestinal glucose absorption activities and its chemical composition.	• Investigation of antihyperglycemic activity of aqueous extract *A. leucotrichus* fruits (in vitro, in vivo, in situ).	• The IC_{50} of intestinal α-glucosidase and pancreatic α-amylase were 0.254, 1.81 mg/mL, respectively, in vitro. • In vivo, the oral intake of *A. leucotrichus*	2022	[156]

	• Evaluation of chemical composition using HPLC.	extracts (150 mg/kg) was significantly reduced hyper-glycemia induced by the sucrose, starch, and glucose in the normal and alloxan diabetic rats. • In situ, *A. leucotrichus* extracts significantly decreased intestinal glucose absorption. • HPLC results revealed the presence of vanillin, quercetin, kaempferol, and thymol.		
Nutrients and Main Secondary Metabolites Characterizing Extracts and Essential Oil from Fruits of *Ammodaucus leucotrichus* Coss. & Dur. (Western Sahara).	• Evaluation of the nutritional values of crude *A. leucotrichus* fruits. • Detection and quantification of perillaldehyde and ammolactone-A in decoction and alcoholic extracts from *A. leucotrichus* fruits.	• R-perillaldehyde and limonene as major components present in EO of *A. leucotrichus* fruits. • Crude *A. leucotrichus* fruits showed a high content of fiber, Ca, K and Fe.	2022	[150]
Determination of the polyphenolic content of *Ammodaucus leucotrichus* Cosson and Durieu by liquid chromatography coupled with mass spectrometry and evaluation of the antioxidant and anti-glycation properties.	• Characterization of hydro-alcoholic extract obtained from *A. leucotrichus* seeds by the determination of their polyphenolic content by HPLC-PDA-ESI/MS.	• The phytochemical screening revealed the presence of 16 compounds with Luteolin-glucoside is the most abundant. • High antioxidant activity of hydroalcoholic	2022	[154]

	• Evaluation of the antioxidant and antiglycation activities.	extract from *A. leucotrichus* seeds with IC_{50} = 0.39 mg AAE /ml. • The antiglycation effect of the extract of *A. leucotrichus* was lower than that of metaformin (positive control).		
In Vitro Investigation of the Anticancer Properties of *Ammodaucus leucotrichus* Coss. & Dur.	• Investigation of the genoprotective, proapoptotic, antiproliferative, and cytodifferentiating potential on different human cell models (TK6 and HL60) of ethanolic extract of *A. leucotrichus* fruits and R-perillaldehyde (monoterpene isolated from *A. leucotrichus* fruits).	• *A. leucotrichus* ethanolic extract and Perillaldehyde were able to induce apoptosis and protect from clastogen-induced DNA damage.	2022	[155]
Biological activities of different extracts of *Ammodaucus leucotrichus* subsp. leucotrichus Cosson & Durieu from Algerian sahara.	• Investigation of the chemical composition of different extracts of *A. leucotrichus* and subsp. leucotrichus fruits. • Evaluation of the EO, ethanolic and	• The highest TPC and TFC were observed in the ethanolic extract compared to the aqueous extract. • The IC_{50} results of DPPH assay were 20.64, 337.6 and 97.89 mg/mL for the EO, aqueous and ethanolic extract, respectively.	2020	[153]

	aqueous extracts potential as a source of phenolic and flavonoid compounds as well as a natural antioxidants and antimicrobials.	• The percent inhibition of β-carotene of EO 43.78 % was higher in comparison with the other tested extracts. • The antimicrobial activity of the two tested extracts were significantly lower than that of the EO.		
Antioxidant, α-Glucosidase, and Nitric Oxide Inhibitory Activities of Six Algerian Traditional Medicinal Plant Extracts and 1H-NMR-Based Metabolomics Study of the Active Extract.	• Obtaining different ethanolic extracts from different plants that are traditionally used for the treatment of hyperglycaemia (*P. harmala L., Z. album, A. valentinus L., A. leucotrichus, L. albus*, and *M. vulgare*). • Evaluation of TPC, DPPH, α glucosidase, and nitric oxide (NO) inhibitory activities of different obtained extracts. • Metabolite profiling using 1H-NMR of the active extract.	• The extract of *A. leucotrichus* using ethanol, exhibited the highest DPPH scavenging activity (IC_{50}=26.26 µg/mL). • *A. leucotrichus* showed the least TPC among the samples tested (124.98 µg GAE/mg extract). • The highest NO inhibitory activity was observed with 100 % ethanol extracts of *A. leucotrichus* with 50.53 %.	2020	[133]
Detailed chemical composition and functional properties of *Ammodaucus*	• Determination of the nutritional value and	• High levels of proteins, carbohydrates, PUFA	2019	[132]

leucotrichus Cross. & Dur. and *Moringa oleifera* Lamarck.	chemical composition regarding minerals, free sugars, organic acids, fatty acids, and tocopherols. • Evaluation of the anti-proliferative, anti-inflammatory and antibacterial activities of the decoction and hydroethanolic extracts.	and ashes were present in both plants. • Polyunsaturated fatty acids were predominant in M. oleifera and mono- unsaturated fatty acids in *A. leucotrichus.* • Extracts exhibited significant cytotoxicity on Hela and MCF-7 cell lines. • The anti-inflammatory and antibacterial activities were significantly higher in the hydroethanolic extracts.		
Valorization and identification of bioactive compounds of a spice *Ammodaucus leucotrichus.*	• Identification of phenolic compounds of ethyl acetate and n-butanol fractions of aerial parts (stems, leaves and seeds) of *A. leucotrichus.* • Determination of antioxidant, antibacterial and antifungal activities.	• Chemical analysis allowed the identification of naringenin, quercetin, sinapic, ferulic, p-coumaric and vanillin. • Strong antioxidant activities (DPPH and FRAP) were recorded in the ethyl acetate and n-butanol fractions.	2019	[152]
Chemical composition, antimicrobial, antioxidant and anticancer activities of	• Chemical characterization of EO the	• The principal constituents present in EO were	2019	[143]

essential oil from *Ammodaucus leucotrichus* Cosson & Durieu (Apiaceae) growing in South Algeria.	obtained from the aerial parts of *A. leucotrichus* by GC-MS analysis. • Biological characterization (antioxidant and antimicrobial) of EO. • Evaluation of anticancer activity of EO against HePG2 (Hepatic) and HCT116 (Colon) human cancerous cell lines.	perillaldehyde (64.66 %), and D- limonene (26.99 %). • The EO showed interesting antimicrobial properties, especially on Salmonella enterica and E. coli. • *A. leucotrichus* EO exhibited significant antioxidant effects confirmed by DPPH, cyclic voltammetry as well as a notable anti-cancer activity with respect to the HCT116.		
Chemical profile and bioactive properties of the essential oil isolated from *Ammodaucus leucotrichus* fruits growing in Sahara and its evaluation as a cosmeceutical ingredient.	• Chemical characterization of the EO obtained from *A. leucotrichus* fruits. • Evaluation of the biological properties of the EO (antimicrobial, antioxidant, and anti-inflammatory). • Incorporation of the EO in a base cosmetic and evaluate the biological efficacy along the storage time.	• Perilla aldehyde identified as the main component present in the EO (85.6 % of the total composition). • The E.O showed a strong anti-inflammatory activity (IC_{50}=11.70 µg/mL), and antioxidants potential. • The EO could be considered as a preserving ingredient in cosmetic formulations after having shown bioactivity for 28 days.	2018	[142]

Evaluation of the Antioxidant Activities of Organic Extracts from *Ammodaucus leucotrichus* Coss & Dur Fruit Part Harvested from the Algerian Sahara.	• Determination of the phenolic and flavonoid contents, also, the assessment of the antioxidant activity (DPPH-scavenging, OH-scavenging and β-carotene bleaching) of different organic extracts of the *A. leucotrichus* fruit.	• The highest TPC was observed in ethanol extract (160.61 mg GAE/g), while the highest TFC was recorded in acetone extract (97.38 mg CEQ/g). • The organic extracts exhibited a moderate antioxidant activity.	2018	[158]
The essential oil of Algerian *Ammodaucus leucotrichus* Coss. & Dur. and its effect on the cholinesterase and monoamine oxidase activities.	• Evaluation of the chemical composition of *A. leucotrichus* essential oil by GC-MS analysis. • Determination of the inhibitory potency of the essential oil against cholinesterase (AChE, BChE) and monoamine oxidase MAOs.	• The main constituents of EO were perillaldehyde (58.3%) and limonene (23.33%). • The EO exhibited a promising BChE activity IC_{50}= 95.2 µg/mL, where the perillaldehyde and limonene IC_{50} 42.7 and 66.7 µg/mL, respectively. • The highest MAOs inhibition was observed by the EO (IC_{50} of MAO-A= 112.5, MAO-B= 40.5 µg/mL).	2018	[145]
Anti-mycotoxin Effect and Antifungal Properties of Essential Oil from *Ammodaucus leucotrichus* Coss. & Dur. on Aspergillus flavus and Aspergillus ochraceus.	• Evaluation of the anti-mycotoxin effect and antifungal activities of EO from *A. leucotrichus.*	• The EO possess important antifungal properties. • The EO caused the inhibition of AFB1 and OTA	2017	[144]

	• Determination of the physicochemical parameters and chemical composition of the EO.	synthesis, mycelial growth, germination and sporulation spores. • A total of 19 compounds were identified in the hydro- distilled oil, dominated by perilla aldehyde (81.62 %).		
Chemical composition, antioxidant and antibacterial activities of the essential oils of medicinal plant *Ammodaucus leucotrichus* from Algeria.	• Comparison of the chemical composition of *A. leucotrichus* EO collected in two different locations. • Evaluation of the antioxidant properties (DPPH and β-carotene assays) and antimicrobial activity (Gram-positive and Gram-negative bacteria).	• The EO of different regions (Masaad and Debdeb) are rich in perrilla aldehyde (37.5 and 60.1%), limonene (29.2 and 6.9 %) and perillaalcohol (7.0 and 6.7 %), respectively. • The EO possess strong antibacterial activity and weak antioxidant activity.	2017	[141]
Antimicrobial and antioxidant activity of essential oil of *Ammodaucus leucotrichus* Coss. & Dur. seeds.	• Evaluation of the chemical composition (GC-MS), antimicrobial (Fungi, Yeast, Gram-positive and Gram-negative bacteria) and antioxidant (β-carotene) properties of the seed oil obtained from *A. leucotrichus*.	• The main constituents of EO were perillaldehyde (59.12 %) and limonene (23.89 %). • The EO seemed to have a good antioxidant capacity with 68.66 % of inhibition rate.	2016	[140]

		• The EO possessed an effective antimicrobial activity with MIC values ranged from 0.37 to 0.92 mg/ml.		
Antifungal activity of essential oil from the fruits of *Ammodaucus leucotrichus* Coss. & Dur., in liquid and vapour phase against postharvest phytopathogenic fungi in apples.	• Evaluation of the antifungal activity of EO from *A. leucotrichus* fruits against three phytopathogenic fungi (Botrytis cinerea, Penicillium expansum and Rhizopus stolonifera).	• The EO displayed significant potential of antifungal activity against the tested phytopathogenic fungi (B. cinerea and P. expansum: MIC= 0.125 µL/mL air, and R. stolonifera: MIC = 0.25 µL/mL air).	2016	[148]

2.2.2. Silybum marianum

2.2.2.1. Presentation and botanical description

Silybum marianum (*S. marianum*) is an herbaceous annual or biennial plant that has been used in traditional medicine for more than 2000 years, mainly to treat liver disorders and to protect the liver from toxins. Commonly, it is referred as milk thistle or Mary's thistle. It belongs to the Astraceae family which is native in Mediterranean countries and Western Asia. Milk thistle is also present in America and in south Australia as an invasive plant [159][160].

Figure 2.7: Milk thistle plant [161].

S. marianum herb reaches a height of 40-200 cm with large, green, and prickly leaves, spherical purple flowering heads, and strongly spinescent stems. When broken, the leaves and stems contain a milky sap which gives it its name. The flowering season is from June to September. Each flower head can produce about 190 seeds. The achene is about 5-7 mm long, up to 2-3 mm wide and 1.5 mm thick. Attached to the achene is a long white pappus. Mature plants have hollow stems while mature fruits vary from greyish white to brown and almost glossy with spots [161][162].

The *S. marianum* plant has many health-promoting properties and this is the reason it is currently cultivated as a medicinal plant on large areas in different parts of the world including Austria, Hungary, Germany, Poland, China, Canada, Peru and Argentina. *S. marianum* is one of the most important medicinal crops in Europe and North America [162].

Demand for silymarin ranges from 18 and 20 tonnes per year, resulting in an average annual sale reaching approximatively $8 billion [163]. In 2019, products derived from *S. marianum* seeds were placed in the top ten selling herbal dietary supplements in the natural and health food market and the 23rd top-selling herbal supplements in the mainstream multi-outlet channel market in U.S., with total sales of $ 26 million [164]; consequently, it is an economically important crop worldwide. Yet, in Algeria, this plant is considered as a troublesome weed. This has mainly guided the selection of this plant in view of its valorisation.

2.2.2.2. Phytochemistry

S. marianum seeds are rich in various phytochemicals such as silymarin, lipids (about 25-30 %) (mainly linoleic, oleic and palmitic acids), proteins, and polysaccharides. The active ingredient in milk thistle is silymarin (SLY), which is a mixture of polyphenolic compounds of more than 7 flavonolignans mainly silybin (silibinin) A, silybin (silibinin) B, isosilybin (isosilibinin) A, isosilybin (isosilibinin) B, silychristin (silicristin), and silydianin; and one flavonoid taxifolin [165]. Several factors such as environmental conditions, geography, genetics and the time of sowing and harvesting of the plant, determine the amount of each individual compound in SLY [162]. Figure (2.8) represents the chemical structure of components of silymarin.

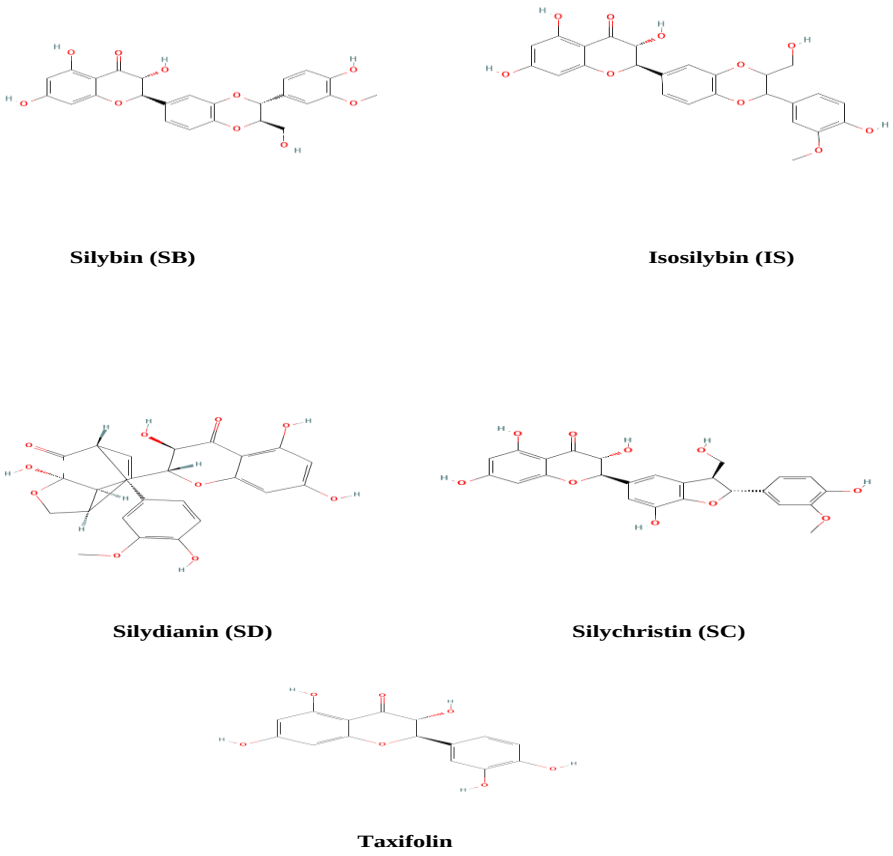

Figure 2.8: Structures of silymarin compounds of interest in our study **(Created by the author).**

2.2.2.3. Silymarin extraction

Several conventional and non-conventional extraction techniques, to extract SLY from *S. marianum* seeds were described in the literature (see Table (2.4)). According to European Pharmacopoeia, the SLY extraction from *S. marianum* seeds requires a two steps process because of the high amount of lipids contained in seeds. The first steps eliminate the high lipids content using hazardous solvent such as hexane or petroleum ether whereas in the second step SLY is extracted from defatted seeds [166]. Consequently, the removal of this defatting step has numerous advantages including reduction of costs and elimination of time-consuming purification steps requiring extensive use of solvents classified as hazardous chemical waste for the people and the environment. Therefore, the present research focused on alternative methods of SLY extraction, using green solvents such as water, ethanol and CO_2 or their combinations, which allows the elimination of traditional approaches drawbacks.

Table 2.4: Previously published results of silymarin and bioactive molecules extractions from Silybum marianum (Created by Author).

Extraction technique	Chromatographic technique	Compounds detected	Maximum yield	References
Enzyme-mediated solvent extraction. Maceration. Cold-pressed extraction	HPLC. GC-MS.	SLY. α-Tocopherol. Fatty acids.	SLY= 1.739 mg/100 mg of oil.	Liu et al. 2023 [167].
Maceration.	HPLC.	SLY.	SLY= 36.23, 38.02 and 40.93 mg/mL from ground whole seeds, whole and ground whole pericarps, respectively.	Gilabadi et al. 2023 [168].
Maceration.	HPLC-ESI-MS/MS. TLC.	SLY, TX, Cyanidin, SC, SD, SB (a), SB (b), IS (a), IS (b).	/	Mukhtar et al. 2023 [169].
Ultrasound assisted extraction followed by maceration.	UPLC-MS/MS.	SLY, SB, IS, SD, TX, SC and SM.	SLY= 15.29 mg/g fresh weight. SB= 6.2 mg/ g PM IS= 1.18 mg/ g PM SD= 3.33 mg/ g PM TX= 1.56 mg/ g PM SC= 5.16 mg/ g PM SM= 1.02 mg/ g PM.	Mahgoub el al.2023 [170].
Ultrasound assisted extraction. Maceration.	RP-HPLC-DAD	SC, SD, SB (a), SB (b), IS (a) and IS (b).	SLY= 100.51 µg/mL	Jabłonowska et al. 2021 [171].

Ultrasound-assisted extraction (UAE). Maceration.	LC-ESI-MS	TX, SC, SD, SB (a), SB (b), IS (a) and IS (b).	SC 2.40 / 0.94 mg/g DW. SD 1.93 / 0.68 mg/g DW. SB (A) 1.06 / 0.11 mg/g DW. SB (B) 8.43 / 1.31 mg/g DW. IS (A) 4.17 / 0.30 mg/g DW. IS (B) 2.29 / 0.06 mg/g DW.	Drouet et al. 2019 [172].
Conventional extraction methods.	HPLC.	TX, SC, SD, SB (a), SB (b), IS (a) and IS (b).	SLY= 100.22 mg/g DW. TX= 9.35 mg/g DW.	Giuliani et al. 2018 [173].
Ultrasound assisted extraction.	HPLC-ESI-MS.	TX, SC, SD, SB (a), SB (b), IS (a) and IS (b).	SB (a)= 10.59 mg/g SB (b)= 8.42 mg/g TX= 2.46 mg/g SC= 13.31 mg/g IS (a)= 5.01 mg/g IS (b)= 2.31 mg/g SD= 1.85 mg/g	Drouet et al. 2018 [174].
Microwave-assisted extraction (MAE). Conventional extraction methods.	HPLC.	TX, SC, SD, SB (a), SB (b), IS (a) and IS (b).	SLY= 263.1 mg/10 g seeds. SB (a)= 53.9 mg/10g SM seeds. SB (b)= 78.1 mg/10g SM seeds.	Saleh et al. 2017 [175].

			SC= 62.1 mg/10g SM seeds. IS (a)= 21.3 mg/10g SM seeds. IS (b)= 6.9 mg/10g SM seeds. SD= 9.3 mg/10g SM seeds. TX= 31.5 mg/10g SM seeds.	
Microwave assisted extraction. Conventional extraction methods (maceration, Soxhlet…).	HPLC.	SLY.	SLY= 1813.3 ppm	Jahan et al. 2016 [176].
Accelerated solvent extraction (ASE).	HPLC. qNMR.	SLY, TX, SC (a), SD, SB (a), SB (b), IS (a) and IS (b).	SLY= 29.94 mg/g DW TX= 1.74 mg/g DW SC (a)= 6.91 mg/g DW SD= 0.79 mg/g DW SB (a)= 6 mg/g DW SB (b)= 8.56 mg/g DW IS (a)= 2.24 mg/g DW IS (b)= 0.46 mg/g DW	Abouzid et al. 2016 [177].
Ultrasound assisted extraction.	LC-MS/MS.	Caffeic acid Ferulic acid Chlorogenic acid Luteolin Miricetin Apigenin Silybin	SB= 9,499 mg/g.	Lucini et al. 2016 [178].

Supercritical CO_2 extraction.	HPLC GC-MS.	SLY, SB, SC, SD and Fatty acids.	SB= 40.98 mg/g SC= 32.31 mg/g SD= 39.32 mg/g	Ben Rahal et al. 2015 [179].
Supercritical CO_2 extraction.	HPLC GC-MS.	SB(a), SB(b) and Fatty acids.	SB(a)= 2.29 mg/g SB(b)= 1.92 mg/g	Çelik et al. 2015 [180].
Pressurized liquid extraction (PLE) Soxhlet extraction.	HPLC.	SLY, SC, SD, SB (a), SB (b), IS (a) and IS (b).	SLY= 19.65 mg/g SC (a)= 3.68 mg/g SD= 7.43 mg/g SB (a)= 3.34 mg/g SB (b)= 5.19 mg/g IS (a)= 2.58 mg/g IS (b)= 1.42 mg/g	Wianowska et al. 2015 [181].
Ultrasonic-assisted enzymatic extraction. Maceration.	Reverse-Phase-HPLC.	SLY, TX, SC, SD, SB (a), SB (b), IS (a) and IS (b).	SLY= 7.94 %	Zhao et al. 2015 [182].

2.2.2.4. Pharmacological effects of silymarin

Previous research demonstrated that SLY extracted from milk thistle seeds could be used in several therapeutic cases, such as liver [183][184][185], cardiovascular [186], neurodegenerative [187][188][189], and diabetes [190][191], diseases. SLY has been used to protect cells from deterioration, helping detoxification, and promote regeneration of damaged cells [161]. It is also considered to be a chemo preventive and anticancer agent for various types of organs such as lung, liver, cervix, breast, bladder, skin, prostate, stomach, pancreas, bladder, and colon [192][193]. Furthermore, its anti-inflammatory [194], antioxidant [195], anti-apoptotic [193], anti-fibrotic [184], anti-lipid per-oxidative,

immunomodulatory, antiviral, and antimicrobial properties [185] have been the subject of various studies in recent years.

2.3. Neuroprotective effect

Neurodegenerative diseases (NDDs) are a group of neurological disorders that cause a gradual and sustained decline in neuronal functioning. This group includes Alzheimer's disease, Parkinson's disease, multiple sclerosis, Huntington's disease, traumatic brain injury, stroke, and cerebral ischemia among others [189]. Alzheimer's disease (AD) is the most common form of NDD, accounting for 75 % of all cases. AD is a condition that affects memory, learning, cognition, and behaviour [196]. AD presently ranks as the fourth leading cause of mortality among older adults, affecting approximately 45 million individuals worldwide. It is projected that this figure will escalate to 80 million by the year 2050 [197][5]. The number of deaths resulting from AD has surged by over 145 % between 2000 and 2019 [198]. The degeneration of neurons leads to alterations in brain morphology and rapid reduction in brain volume, as depicted in Figure (2.9). Moreover, AD imposes a substantial emotional and financial burden on both the patient's family and the wider community, owing to the caregiving responsibilities and the impact on income. [199][200].

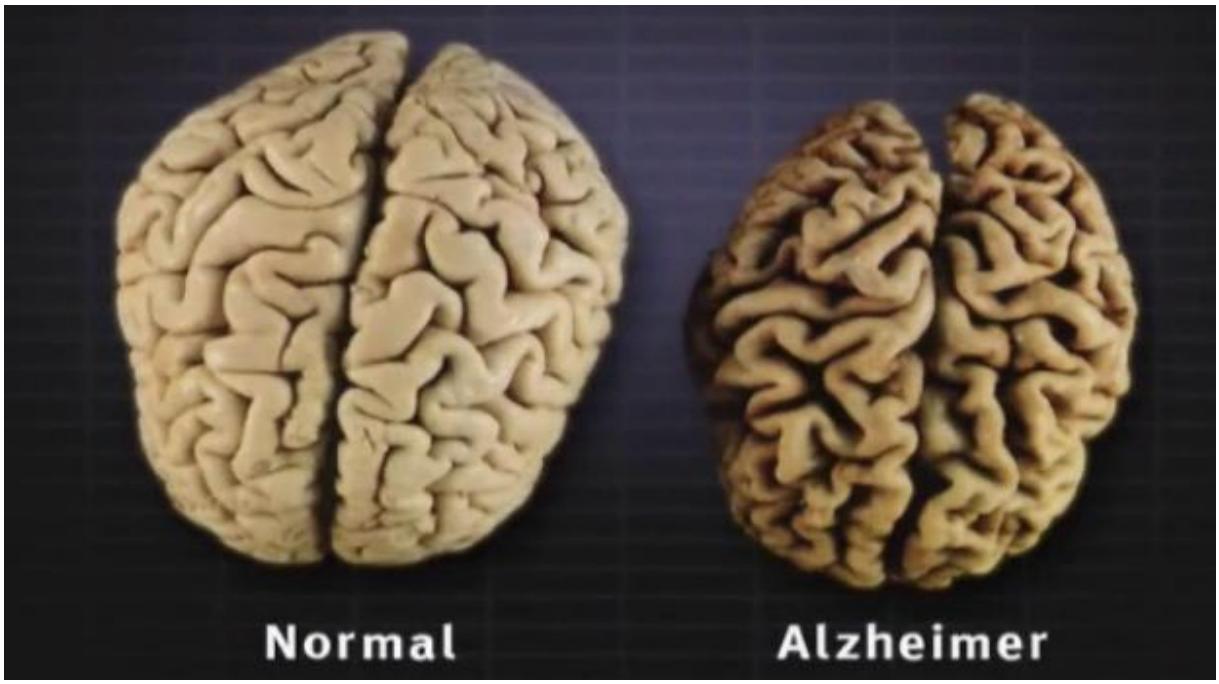

Figure 2.9: Brain morphology change in Alzheimer's disease [202].

The exact cause of AD remains unclear. However, research suggests that a combination of genetic and environmental factors, such as lifestyle and diet, may contribute to its development [201]. This disease can go undetected for many years, as it is believed to begin decades before symptoms appear [202], and these symptoms worsen over time. The complexity of the factors that cause AD has hindered the discovery of a cure. As a result, Alzheimer's disease and other neurodegenerative disorders are currently considered to be progressive and incurable. Therefore, pharmacological interventions only aim to slow down the progression or alleviate the symptoms. Therefore, there is a growing focus on preventative measures such as adopting healthy habits, incorporating dietary components, and exploring therapeutic interventions based on natural substances.

2.3.1. Therapeutic approach in Alzheimer's disease

As stated above, AD is a complex condition with multiple contributing factors, including neuroinflammation, oxidative stress caused by free radicals, and cognitive dysfunction in the brain [203]. The primary neurotransmitter in the brain, acetylcholine (ACh), plays a crucial role, and a decline in its levels has been linked to cognitive impairments. Therefore, the use of acetylcholinesterase (AChE) inhibitors has become the primary treatment for dementia associated with AD. These inhibitors prevent the breakdown of acetylcholine, enhancing its transmission as a neurotransmitter. In contrast, neurodegenerative diseases like AD often exhibit inflammation [204]. Another key enzyme involved in the progression of neuroinflammation is lipoxygenase (LOX). Consequently, LOX inhibitors may be considered as potential therapeutic options for addressing inflammation in neurodegenerative diseases.

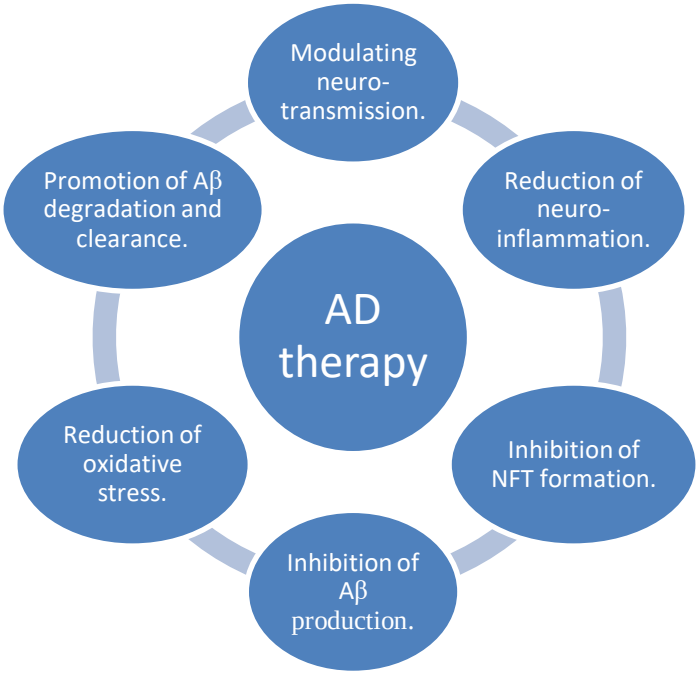

Figure 2.10: Diffrent approaches for AD therapy (Created by the author).

Figure (2.10) illustrates different approaches to treating AD. Researchers have worked for many years to combat AD on multiple fronts, including exploring therapeutic compounds with antioxidant and anti-inflammatory properties, inhibitors of cholinesterase enzymes, and substances with potential protective effects against neurotoxic metabolism associated with the formation of Aβ plaques. These mechanisms function as neuroprotective measures [205].

Most drugs approved for treating mid-to-moderate Alzheimer's disease (AD) are acetylcholinesterase (AChE) inhibitors. These drugs improve the cognitive abilities of AD patients by increasing the concentration of acetylcholine (ACh), which enhances learning and memory [206]. Prominent examples of such drugs include Donepezil, Memantine, Caproctamine, Galanthamine, and Rivastigmine [207].

However, it is important to note that all of these drugs and treatments have various undesirable side effects. Therefore, there is an urgent need to search for new, effective, and natural remedies.

2.3.2. Natural products with neuroprotective potential

Chemical treatments have been used to alleviate certain symptoms, but there is still no cure for AD. Therefore, there is growing interest in developing new dietary supplements

made from natural extracts that can slow down the process of neurodegeneration. It is important to note the growing interest in secondary metabolites as a 'multitarget' therapy for AD. This approach has gained recognition as a significant prospect for managing the ailment in the future. Several natural sources have been identified as potential agents against Alzheimer's disease in both laboratory and living organism experiments [208]. These sources include polyphenols [209], carotenoids [210], polysaccharides [211][212], anthocyanins [197], alkaloids [213] and proteins [214] found in plants and marine organisms [215]. Some examples of these and other natural products extracts tested as anti-AD can be seen in **Table (2.5).**

Table 2.5: Neuroprotective function of different vegetable matrixes and theirs principles bioactive molecules (Created by the author).

Plant matrices	Interested compounds	Neuroprotective assays	References
***Rosmarinus officinalis* l.**	Monoterpenes. Diterpenes.	Antioxidant in vivo. Anti-inflammatory. AChE/BChE.	[216–218]
Phormidium autumnale	Phytosterol.	Antioxidant (ROS). Anti-inflammatory (LOX). AChE.	[219]
Fenugreek	Flavonoids.	Antioxidant in vivo.	[220]
Oat seedlings	Phenolics. Proteins.	Antioxidant. Anti-inflammatory in vivo. Cytotoxicity.	[204]
Thinned peaches	Polyphenolics (4-O-caffeoylquinic acid, isoferulic acid and caffeic acid…).	Antioxidant (RNS, ROS). Anti-inflammatory (LOX). AChE/BChE. PAMPA-BBB.	[221]
Kalanchoe daigremontiana	Phenolic acids.	Antioxidant (RNS, ROS). Anti-inflammatory (LOX).	[222,223]
Nothofagus pumilio	Phenolics. Anthocyanin.	Antioxidant.	[224]
Robinia pseudoacacia	Alkaloids.	AChE/BChE.	[225]
	Flavonoids.	Antioxidant.	[226]
	Acacetin.	Anti-inflammatory in vivo.	[227,228]
***Coffea arabica* l.**	Rutina, Epicatequina, Phenolics acids.	Antioxidant. Anti-inflammatory (LOX, IL-6, TNF). AChE/BChE	[229–231]

	Caffeine. Protocatechuic. Chlorogenic acids.	Anti-inflammatory. Antioxidant (ROS, RNS).	[232]
Orange by-products.	Monoterpenes.	Antioxidant (RNS). AChE/BChE.	[233][234]
	Phenolics.	AChE. Anti-inflammatory (IL-6).	[235–238]
Olive leaves.	Phenolics. Flavonoids.	Antioxidant (ROS, RNS).	[239,240]
	Oleuropeina,	Protection in vivo Aβ42. Antioxidant in vivo (ROS). Anti-inflammatory (IL-1β).	[241–244]
	Triterpenes.	AChE/BChE.	[245,246]
***Dunaliella salina* microalga**	β- carotene.	AChE. Antioxidant in vivo (ROS). Anti-inflammatory in vivo (TNF-α, IL-6).	[247–249]
	Zeaxanthin	Protection in vivo (Aβ42). Anti-inflammatory in vivo (IL 1β).	[250]
Cyphomandra betacea	Phenolics. Carotenoids. Anthocyanins.	Antioxidant. AChE. Anti-inflammatory (TNF-α, IL-1β).	[251–253]
	Phenolic acids. Rutin. Quercetin hexoside.	Antioxidant (RNS, ROS). AChE/BChE. Anti-inflammatory LOX. Cytotoxicity (HK-2, THP-1, and SH-5YSY) cell lines.	[254]
***Cissus sicyoides* l.**	Terpenoids.	Antioxidant DPPH.	[255]

	Phenolics compounds. Flavonoids.	Anti-inflammatory in vivo.

CHAPTER III:

MATERIALS AND METHODS

CHAPTER III: MATERIALS AND METHODS

In this third chapter, we outline the materials and methods used for this experimental work. First, we present the workflow of this dissertation. Next, we describe the various techniques employed to extract bioactive molecules and to characterize them both chemically and functionally. These include chromatographic methods like HPLC and GC-MS, as well as in-vitro assays such as anti-inflammatory, anti-cholinergic and antioxidant activities.

3.1.Work plan: workflow

- ***Ammodaucus leucotrichus***

1. Obtaining enriched extracts from *Ammodaucus leucotrichus* cross. Dur. biomass using pressurized liquids extraction (PLE) using two green solvents at different extraction temperatures.
2. Phytochemical screening of the obtained extracts such as the total phenolic compounds (TPC) and total carbohydrate compounds (TCC).
3. Biological characterization of obtained extracts through a battery of in vitro assays, including the evaluation of inhibitory acetyl-cholinergic enzyme capacity, as well as their anti-inflammatory and antioxidant capacities (through the inhibition of lipoxygenase (LOX) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity, respectively).
4. Metabolic profiling of *Ammodaucus leucotrichus* seeds extracts using ultra high-performance liquid chromatography coupled quadrupole/time-of-flight mass spectrometry UHPLC-q-TOF-MS.
5. Optimization of SFE using a Box-Behnken experimental design, considering pressure, temperature and percentage of co-solvent as experimental variables on the total recovery, total phenolic compounds (TPC), total carbohydrate compounds (TCC), and anti-cholinesterase activity (AChE).
6. Chemical characterization of extracts obtained with SFE in terms of phytochemicals content using gas chromatography coupled with mass spectrometry (GC-MS).
7. Biological characterization of the optimum point (anti-inflammatory (LOX) and antioxidant (DPPH) activities).
8. Application of two steps biorefinery approach to maximize the recovery of bioactives from *Ammodaucus leucotrichus* (under optimum conditions obtained in the previous steps) to obtain extracts enriched with high acetyl-cholinesterase inhibition (AChE).

9. Chemical characterization of the different extracts obtained from GXLE using advanced analytical tools such as liquid chromatography (LC) and gas chromatography (GC) to identify the specific compounds contained in each fraction.

10. Biological characterization of each obtained fraction (anti-cholinesterase, anti-inflammatory and antioxidant activities).

- ***Silybum marianum***

1. Evaluation of gas-expanded liquid extraction kinetics using green solvents: Study of the effect of different compositions of solvents, aqueous ethanol (20 %, 50 % or 80 % (v/v)) at different CO_2 / liquid ratios (25, 50 and 75 %) on the extraction of Silymarin compounds.

2. Phytochemical analysis for the total phenolic content (TPC) of all the obtained fractions.

3. Chemical characterization of extracts obtained in the previous step in terms of Silymarin complex content using ultra high performance liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS) detection.

4. Biological characterizations, including the evaluation of the inhibitory capacity of the acetyl-cholinesterase enzyme, the anti-inflammatory capacity (by the inhibition of the lipoxygenase (LOX) enzyme) and the antioxidant activity (by the free radical scavenging (ABTS).

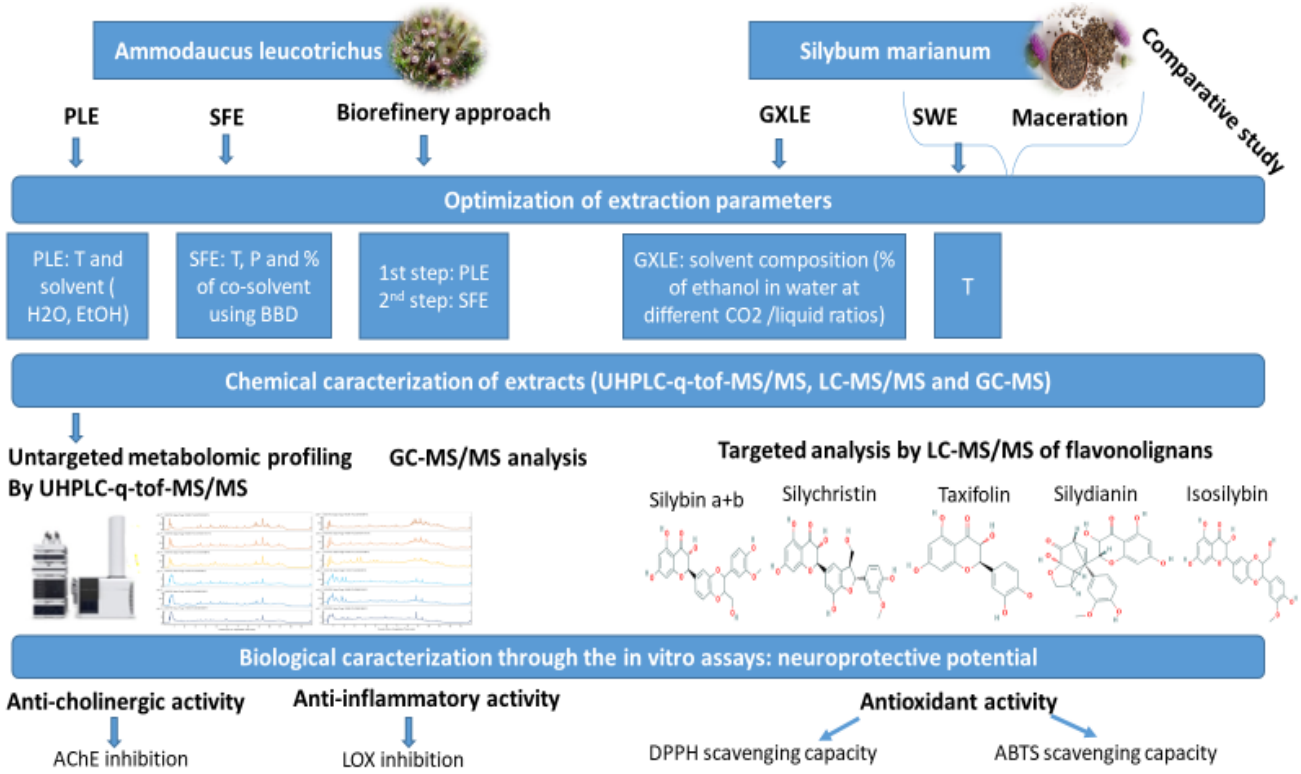

Figure 3.1: Scheme of the work plan followed in this PhD Thesis (Created by the author).

3.2. Plant materials

3.2.1. Ammodaucus leucotrichus

Wild *Ammodaucus leucotrichus* Cross Dur was collected from 2019 harvest in Adrar, a desert area 285 m above sea level, in the southwest of Algeria, as shown in the map in Figure (3.2). The seeds were hand separated from any other parts.

Figure 3.2: Zone of sampling of Ammodaucus leucotrichus plant ([134], Modified by the author).

A cryogenic mill (Cryomill, Retsch, Haan, Germany) was used to ground the plant fruits, as shown in Figure (3.3). The average particle diameter was less than 0.5 mm. The granulated sample was stored at 4 °C, ready for use.

(a) (b)

Figure 3.3: Representative pictures of Ammodaucus leucotrichus fruit; a) raw material, b) ground material (Created by Author).

3.2.2. Silybum marianum

Wild mature *S. marianum* was collected from June 2019 harvest in Ali Mendjli (36.2570 °N, 6.5831 °E, and 766 m above sea level), Constantine, Algeria. The seeds were separated manually from any other parts (Figure (3.4)). Thereafter, the seeds were milled up to a particle diameter less than 0.5 mm using a cryogenic grinding (Cryomill from Retsch GmbH, Haan, Germany). The powder was stored in dark conditions at 4 °C until further use.

Figure 3.4: Mature seeds from Silybum marianum (Taked by the author).

- **Cryogenic grinding**

A mass of 1.0 g of the biomass was ground in a cryomill (Retsch GmbH, Germany), with an integrated cooling system maintained at -196 °C, which uses liquid nitrogen, depicted in Figure (3.5). A metallic ball performed the shaking. The process was carried out

in three steps: precooling (40 sec, 5 Hz), intermediate (60 sec, 25Hz) and final (20 sec, 5Hz) phases.

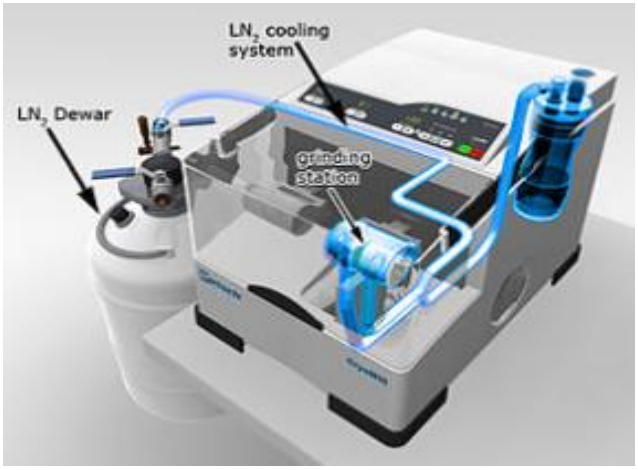

Figure 3.5: The cryogenic grinding instrument (Take it from the Manuel of the instrument).

3.3. Extraction of bioactive molecules

3.3.1. Extraction from Ammodaucus leucotrichus

3.3.1.1. Pressurized liquid extraction apparatus and treatment

Pressurized liquid extractions from dried biomass were carried out using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), which consists of a high pressure pump (between 34 and 206 bar), an oven to heat up the extraction cell (between room temperature and 200 °C), an extraction vessel to hold the considered sample, withstanding high pressure and having a system of valves to keep the extraction conditions stable, a back pressure regulator to keep the P_{ext} and a vial to collect the extract. The extraction procedure was as follows:

1. The extraction cell was fitted with cellulose filter at the inlet and outlet (to maintain the sample inside) and placed into the oven.
2. The cell was filled by pumping the selected solvent, with the pressure was increased to the desired value.
3. Heat up time was fixed depending on the extraction temperature (automatically fixed by the equipment).

4. A static time process was performed with all valves shut for the specified extraction times.
5. A depressurization took place.
6. The cell and the tubing were rinsed (with 60 % of the cell volume using the fresh extraction solvent).
7. Solvent was purged from the cell with N_2 gas during 2 minutes at 10 bar.
8. The dissolved extract in the solvent issuing from the extraction process was collected in a vial and stored in dark at -20 °C until further use.

Prior to the extraction, each solvent was sonicated for 10 to 15 min to prevent oxidation due to air removal induced by micro-cavitation caused by ultrasounds. Between the two successive extractions, a rinse of the complete system was made to overcome any extract carry-over from one experiment into the next.

All parts of the cell were separated and thoroughly cleaned with water, sonicated successively in an aqueous chlorine solution, and dried before a new sample was packed, to avoid carryover from re-use of an extraction cell.

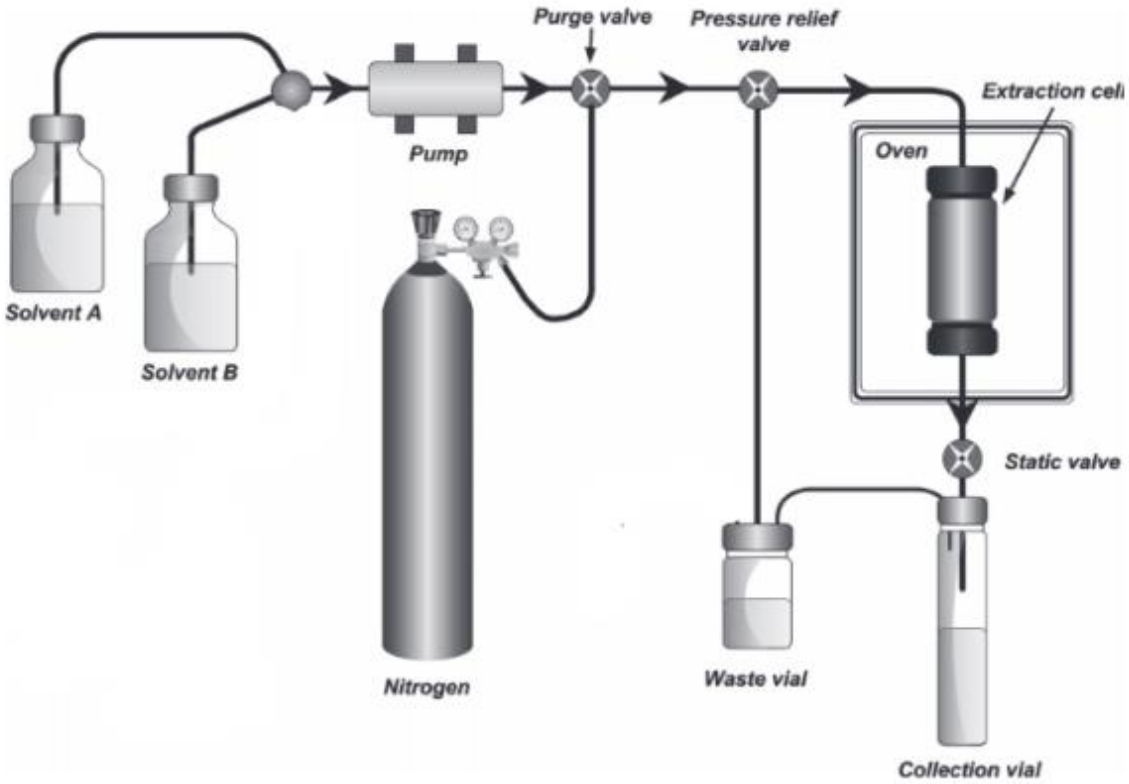

Figure 3.6: Diagram of a Dionex ASE® 200 pressurised liquid extraction apparatus (Created by Author).

- **Pressurized liquid extraction from *Ammodaucus leucotrichus* seeds**

For all the experiments, 1 g of cryogenic grinded seeds was mixed with 4 g of sea sand (dispersive agent) in an 11 mL stainless-steel extraction cell with cellulose filters at both sides (to avoid the passage of suspended particles into the collection vials). Initial experiments were performed at three different times (10, 20 or 30 min) to set the extraction time using ethanol as solvent at 110 °C and 103.4 bar. All assays were done at 105 bar.

Extractions were performed using ethanol and water individually or in mixture, as solvents at different extraction temperatures (40, 110 and 180 °C) to cover a wide range of dielectric constant. All experiments were carried at least in triplicate. Water extracts were lyophilized in a freeze-drier (Lyobeta, Telstar, Terrassa, Spain), while ethanolic extracts were evaporated under nitrogen stream. Samples were stored at −20 °C in dark to prevent degradation until further analysis. The extraction yield was determined according to the following equation:

$$Extraction\ yield\ (\%) = \frac{Mass\ of\ dried\ extract\ (mg)}{Mass\ of\ initial\ biomass} \times 100 \qquad \text{(Eq.3.1)}$$

3.3.1.2. Supercritical fluid extraction apparatus and treatment

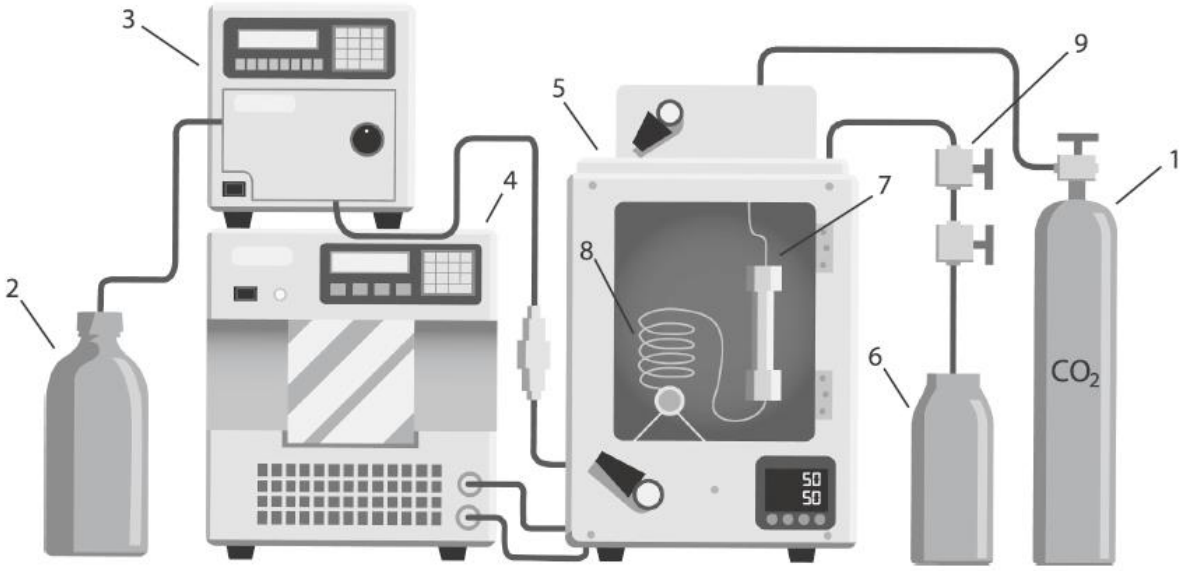

Figure 3.7: A representation of the supercritical fluid extractor used for both green extraction processes: Gas-expanded liquid and supercritical fluid extraction. (With permission of Fagundes et al [66]**)**

In the present work, Supercritical fluid Extraction and Gas-expanded liquid extraction were performed in a homemade compressed fluids extraction system (Figure (3.7)). The equipment consisted of a (1) CO_2 cylinder, (2) solvent Bottle, (3) HPLC liquid pump (PU-2080; Jasco, Hachioji, Japan), (4) high pressure CO_2 pump (PU-2080 Plus CO2; Jasco, Hachioji, Japan), (5) oven with controlled temperature (Finnigan, ThermoFisher Scientific, Waltham, USA), (6) collection vial, (7) hand tight extraction cell, (8) microfluid mixer with serpentine, (9) manual micro-metering needle valve (Vici-Valco Instruments Co. Inc., Houston, USA).

The extraction process started with cooled CO_2 from a cylinder, then compressed by a CO_2 pump and eventually mixed with a co-solvent, pumped from its reservoir through a high-pressure liquid pump. Subsequently, the mixture (co-solvent + CO_2) was preheated and then entered into the oven to go into the extraction cell containing the plant material. The flow rate was set at 4 $mL.min^{-1}$ in the CO_2 pump. The extraction process was performed inside the extraction process, after reaching the set values of the process conditions (temperature and pressure). The pressure was controlled adjusting the opening of two needle valves to bring the solvent to the supercritical state, while the temperature was controlled by the oven. The extraction cell was packed from bottom to top with: glass wool/glass beads/glass wool/sample-dispersant mixture /glass wool. Glass wool helps prevent caking

of biomass in the system lines and inhibits solvent channelling during the extraction. Upon exiting the oven, CO_2 was allowed to return to its gaseous state, hence separated from the extracted compounds at room conditions. The extracts were collected in a plastic tube protected from light and immerged in an ice bath.

- **Supercritical fluid extraction from *Ammodaucus leucotrichus***

Supercritical fluid extraction (SFE) of phytochemicals was carried out by using a home-built extraction system, as described in the previous section. For each experiment, a 1.5 g of sample was loaded into an extraction cell after being mixed with 3 g of sea sand. A constant flow rate of 4 $ml.min^{-1}$ was applied. Selection of extraction time was carried out by performing a kinetic study, under the central conditions of the experimental design, for 240 min and sampling every 20 min. The system was operated in dynamic mode, and different temperatures, pressures and co-solvent amounts were considered. The adopted parameter values are shown in Table (3.1). All experiments were performed at least in triplicate. The ethanol present in the extracts was removed under nitrogen stream and the obtained mass was used to calculate the extraction yield, expressed in weight percentage of dry biomass, according to relation (Eq.3.1). The extracts were protected from light, and stored under refrigeration (−20 °C) until further analysis.

Table 3.1: Codes and levels of the three considered independent variables for Box–Behnken design (Created by the author).

Independent variables	Code	Level correspondence		
		Low(-1)	Medium(0)	High(+1)
Temperature	X1	40 °C	55 °C	70 °C
Pressure	X2	100 bar	200 bar	300bar
Co-solvent	X3	5 %	10 %	15 %

3.3.1.3. Sequential high-pressure extraction process from *Ammodaucus leucotrichus*

A mass of 1 g of dried biomass was mixed with 4 g of sea sand of 0.1- 0.6 mm particle size to fill a stainless-steel extraction cell. A combined extraction mode was performed in

two sequential steps, decreasing the polarity of the solvents, in order to optimize the AChE potential of *A. leucotrichus* extracts. (1) A raw material from seeds was subjected to PLE at optimized conditions, determined in the section (3.3.1.1). The extracts were lyophilized and stored in darkness. (2) The residue and the dried extract of PLE were then subjected to SFE at the optimum conditions determined in the previous section (3.3.1.2). The extracts were dried using N_2 stream, weighed and stored at −20 °C in the dark, until further analysis. The experimental procedures were performed in triplicates.

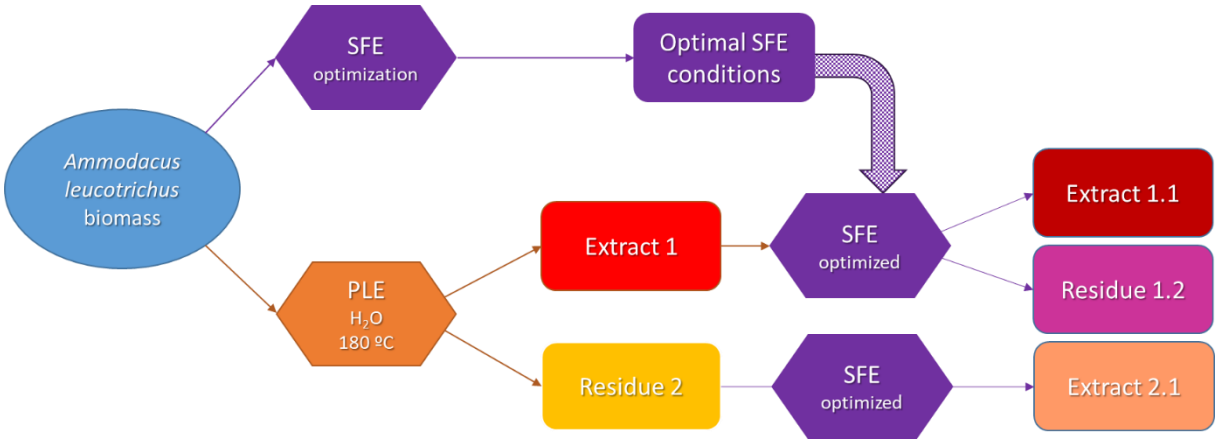

Figure 3.8: Schematic representation of sequential proposed platform for the valorization of A. leucotrichus biomass (Created by the author).

3.3.2. Extraction from Silybum marianum

3.3.2.1. Gas-expanded liquid extraction of silymarin from Milk thistle (*Silybum marianum*) seeds

Silybum marianum extract was prepared in a homemade compressed fluid extractor (illustrated in Figure (3.7)). Briefly, 1.5 g of grinding material was mixed with 3 g of sand. The pressure was set at 90 bar. Previous papers [256][257] showed that the pressure was a non-significant factor in the zone of compressible fluid, hence the required milder working value. However, the temperature was fixed at 40 °C based on previously published works [180][179]. The examined parameter was solvent composition, which consisted of a ternary mixture of CO_2: EtOH: H_2O, at constant flow rate of 4 mL.min^{-1}. A kinetic study was also performed for all the assays, collecting fractions every 20 min for a total extraction time of 160 min. Solvent composition of each experiment can be seen in Table (3.2). Three

percentages of (25, 50, 75 %) were used for CO_2 when volumetrically mixed with Ethanol and Water solvents of different composition (25, 50, 75 % ethanol v/v). Theoretical dielectric constants of the mixtures were based on data from [258] and [259] and calculated using (Eq.3.2) , described by Chien [260]:

$$\varepsilon_{mix} = (\varepsilon_{CO2} \times \varphi_{CO2}) + (\varepsilon_{EtOH} \times \varphi_{EtOH}) + (\varepsilon_{H2O} \times \varphi_{H2O}) \quad \text{(Eq.3.2)}$$

Where ε is the dielectric constant and φ is the volume fraction of solvent.

Extracts were collected in a 50 mL centrifuge tube (falcon type) that was cooled by immersion in an ice bath and protected from light, to prevent degradation. Water fraction was lyophilized, while ethanol fraction was evaporated under nitrogen stream. The obtained dried extracts were stored at −20 °C, until further analysis.

3.3.2.2. Reference extraction procedure: Solid-Liquid Extraction

In order to compare the results obtained by GXLE, a conventional method of extraction was performed as a reference extraction reported by Martinelli et al [261]. This procedure consists of two steps: a defatting using hexane followed by the silymarin extraction using 75 % methanol. Seed sample (1 g) and hexane (40 mL) were mixed in centrifuge tubes and agitated in a thermo-mixer Eppendorf (Wesseling, Germany) at 45 °C for 24 h at 750 rpm. The supernatant was removed, and the precipitate was extracted again with 40 mL of 75 % methanol. The mixture was continuously stirred for 12 h at 45 °C. The residue was filtrated and the extract was completely dried under a stream of nitrogen. Three independent extractions were performed.

3.3.2.3. Reference extraction procedure: subcritical water extraction

Another one-step method using green solvent was chosen as reference extraction for comparison. Subcritical water Extraction (SWE) was performed in order to compare the performance of the chemical analysis with those obtained by gas-expanded liquid extraction. Briefly, 1 g of cryogenically grinded seeds were mixed with 2 g of sand and inserted into a 11 mL stainless steel extraction cell in a Dionex ASE 200 PLE system (Dionex, Sunnyvale, CA, USA). The pressurized water extraction was carried out under the following conditions: pressure of 1500 psi (103.4 bar), extraction time of 10 min and two different temperatures (75 and 175 °C), denoted as experiments 10 and 11 in Table (3.1), with the theoretical

dielectric constant taken from [262]. The residual solvent was freeze-dried at 4°C and 0.08 mbar (Lyobeta, Telstar, Terrassa, Spain). This procedure was performed in triplicate.

Table 3.2: Solvent compositions for silymarin bioactive fractions obtaining from S. marianum using GXL (Created by the author).

	Extraction solvents ratio in percentage (v/v)			Theoretical
#	CO_2	Ethanol	Water	dielectric constant, ε
1	75	5	20	17.0
2	75	12.5	12.5	13.5
3	75	20	5	10.1
4	50	10	40	32.8
5	50	25	25	25.8
6	50	40	10	18.8
7	25	15	60	48.5
8	25	37.5	37.5	38.0
9	25	60	15	27.6
10			100 (75 ºC)	60.5
11			100 (175 ºC)	38.2
12				1.88 (hexane) 44.55 (75 % MeOH)

Experiments 1-9 were performed at 90 bar, 40 °C and for 160 min, while benchmark experiments, 10 and 11, were carried out at 103 bar for 10 min at expressed temperature). Maceration experiment (12) was performed at room conditions.

3.4. Phytochemical analysis

3.4.1. Total phenolic content

A spectrophotometric method was used to determine the total phenolic content (TPC), based on their characteristic absorbance, as previously described [263], with some modifications. A volume of 10 µL of an extract solution (10 mg mL−1 in EtOH) was mixed and agitated with 600 µL of water milli-Q and 50 µL of Folin–Ciocalteu reagent (undiluted

Folin–Ciocalteu reagent). After 1 min, 150 µL of 20 % (w/v) Na_2CO_3 was added and the volume was adjusted to 1 mL with water. Then, the mixture was submitted to vortex for 120 min in darkness at room temperature. A volume of 300 µL of each mixture was placed in a 96-well microplate spectrophotometer reader, Bio-Tek instruments (Winooski, VT, USA). The absorbance was measured at 760 nm. The total phenolic content was calculated from a calibration curve using Gallic acid as standard (from 0.031 to 2 g/mL). The data were expressed as milligram Gallic acid equivalents (mg GAE/g extract).

3.4.2. Total flavonoid content

The total flavonoid content (TFC) was determinated using aluminium chloride colorimetric method described by Woisky and Salatino [264]. A volume of 100 µL of extract solution (2 mg mL^{-1} in methanol) was mixed with 140 µL of methanol and 60 µL of aluminium chloride at 0.2 % (w/v). The mixture was incubated for 30min in dark at room temperature. The absorbance was measured at 425 nm using a microplate spectrophotometer reader. An external standard calibration curve of quercetin (0.2–20 µg mL^{-1}) was used to calculate the total flavonoids content. Total flavonoids were expressed as milligram quercetin equivalent per gram of extract (m QC /g extract).

3.4.3. Total carbohydrate content

The total carbohydrate content (TCC) was performed using the phenol-sulfuric acid method reported by Dubois et al. [265]. The assay solution consists of 278 µL of extract (diluted in Milli-Q water at known concentration), 167 µL of 5 % phenol, and 1000 µL of concentrated sulphuric acid (H_2SO_4). The mixture was agitated and incubated for 30 min at room temperature in the dark. Later, 300 µL of each reaction mixture was transferred to a 96-well microplate. The absorbance was read at 490 nm. The calibration curve was constructed using glucose as an external standard (6.25–100 µg mL^{-1}). Results were expressed as mg carbohydrate per g of extract.

3.5. In vitro bioactivity assay

3.5.1. Anti-inflammatory activity

The LOX inhibition activity was measured by fluorescence assay based on enzyme kinetics and according to the method by Whent et al. [266]. The assay consists in first determining K_M of the enzyme, which is the concentration of substrate that permits the

enzyme to achieve half $V_{max.}$ and the value of which was measured by mixing 100 µL of LA (6.5 mM) in EtOH/H_2O (1:1, v/v), 100 µL of EtOH/H_2O (1:1, v/v), 75 µL of fluorescein (1 µM) in buffer and 60 µL of LOX 208 U/µL in buffer, in each well. Then, the inhibition assay solution consisted of 100 µL of extract sample at the different concentrations (100 µg mL^{-1}– 1000 µg mL^{-1}) in EtOH/H_2O (1:1, v/v), 75 µL of fluorescein (1 µM) in buffer (150 mM Tris-HCl pH 9), 60 µL of LOX 208 U/µL in buffer and Linoleic acid, in a concentration studied in K_M, prepared in EtOH/H_2O (1:1, v/v), were placed in each well.

Fluorescence was measured at λ excitation of 485 nm and λ emission of 530 nm every minute for 15 min at 25 °C. Positive (quercetin) and negative (without addition of extracts/standard) controls were included for both LOX assays. The calibration curves were built to obtain LOX inhibition percentage, which was calculated according to Equation (3.3). Results were expressed as IC_{50} (%).

$$DI\ (\%) = \frac{(V_0 - V_1)}{V_0} \times 100 \qquad \text{(Eq.3.3)}$$

Where V_1 and V_0 are mean velocity obtained for LOX in presence and absence of inhibitors, respectively.

3.5.2. Anti-cholinesterase activity

The acetylcholinesterase inhibition assay was investigated using Ellman´s method, modified by fluorescent enzyme kinetics reported by Sanchez-Martínez et al. [89]. Previously, K_M Michaelis-Menten constant was measured to fix the substrate concentration at which the reaction rate was half of the maximum velocity rate. Mixtures of 100 µL of buffer (150 mM Tris-HCl at pH 8.0), 50 µL of acetylthiocholine at a concentration of the K_M value in ultrapure water, 100 µL of the extract at different concentrations (50 to 500 µg/mL for AChE in EtOH/H_2O (1:1, v/v) were considered. After 10 min of incubation, 25 µL of ABD-F (125 µM) in buffer was added and 25 µL of AChE was diluted at 0.8 U/mL in the buffer. The fluorescent parameters were given as: temperature = 37 °C, wavelength excitation = 389 nm and emission = 513 nm. A runtime of 15 min at intervals of 1 min was adopted. Eq. (3.3) represents the percentage of inhibition of sample compared to negative control, where V_1 and V_0 are mean velocities obtained for AChE in presence and absence, respectively, of inhibitors. Galantamine, a drug used to treat Alzheimer disease, was used as positive control.

3.5.3. Antioxidant activities

3.5.3.1. DPPH$^{\bullet}$ radical scavenging capacity

The free radical scavenging activity was determined spectrophotometrically by the DPPH assay reported by W.Brand-Williams et al [267]. A stock solution of DPPH with a concentration of $6*10^{-5}$ M in methanol was prepared. This mother solution was stable for one month when stored at -20 °C protected from light. This solution was further diluted 1:10 with methanol. Each well was filled with different volumes from 10 µL to 100 µL of sample, starting with a concentration of 1.5 mg/mL, were mixed with 150 µL of DPPH solution ($6*10^{-5}$ M in MeOH). Absorbance measurements were recorded at 517 nm by the above-mentioned microplate reader, after 30 min of reaction in the dark, at room temperature. The results were expressed as IC_{50}, the concentration required to reduce 50 % of the initial DPPH concentration. It was calculated by linear regression from a graph where the abscissa represented the extract concentration and the ordinate the free radical scavenging activity. Therefore, the higher antioxidant capacity, the lower IC_{50} value. Solvent (used to dissolve the samples) plus plant extract solution were used as a blank, while the mixture of DPPH solution (150 µL; $6*10^{-5}$ M) and solvent was used as a negative control. BHT was used as positive control.

3.5.3.2. ABTS^{+} radical scavenging capacity

The scavenging activity towards the ABTS$^{\bullet+}$ radical was carried out according to R. Re et al [268]**,** with slight modifications. ABTS stock solution was generated through the reaction of 7 mM ABTS with 2.45 mM potassium persulfate and under darkness, at room temperature, for 16 h before use. The aqueous ABTS$^{\bullet+}$solution was diluted with 5 mM phosphate buffer (pH 7.4) until absorbance reached values of 0.70 (± 0.02) at 734 nm. Briefly, 100 µL of the extract sample at different concentrations from 0.014 to 0.0014 µg/mL in 50 % aqueous EtOH and 250 µL ABTS$^{\bullet+}$radical solution were placed in a 96-well microplate. The Absorbance was measured at 734 nm after an incubation of 45 min at room temperature in the absence of light. Results were expressed as TEAC (Trolox equivalent antioxidant capacity) values (µmol trolox/g sample) using Trolox as reference standard employing four different concentrations of each extract giving a linear response between 20

and 80 % compared with the initial absorbance. $ABTS^{\bullet+}$ inhibition percentage was calculated according to the formula (3.4):

$$\%\ inhibition = \frac{A_{ABTS\ control} - (A_{sample} - A_{sample\ blank})}{A_{ABTS\ control}} \times 100 \quad \text{(Eq.3.4)}$$

Where A_{ABTS} control is the absorbance of $ABTS^{\bullet+}$ radical in buffer at t = 0 min; A sample, is the absorbance of an $ABTS^{\bullet+}$ solution mixed with extracts and A blank sample is the absorbance of samples with- out $ABTS^{\bullet+}$. Comparisons between extracts were made by IC_{50} value which represents the concentration (µg/mL) of the extract that inhibited 50 % of the $ABTS^{\bullet+}$ radical.

3.6. Chemical characterization

3.6.1. Untargeted analysis for Ammadaucus leucotrichus extracts by UHPLC-q-TOF-MS/MS

An ultra-high performance liquid chromatography system UHPLC (1290 Agilent Technologies, Santa Clara, CA, USA) coupled with quadrupole time-of-flight mass spectrometry q-TOF (Agilent 6540) equipped with an orthogonal electrospray ionization source ESI (Agilent Jet Stream, AJS, Santa Clara, CA, USA) was used for the chemical profiling analyses of Ammadaucus leucotrichus seeds extracts. A Zorbax Eclipse Plus C18 column (2.1 × 100 mm, 1.8 µm particle diameter, Agilent Technologies, Santa Clara, CA) with a Zorbax Eclipse Plus C18 column (2.1 × 5 mm, 1.8 µm particle diameter) from Agilent, like a column of security was used for chromatographic separation. Analyses were conducted under positive and negative ionization modes. The flow rate was 0.5 mL/min; the injection volume was 2 µL. The mobile phases were composed of A (0.01 % formic acid in water) and B (0.01 % formic acid in acetonitrile) for acquisition in negative ionization mode (ESI-), while, A (0.1 % formic acid in water) and B (0.1 % formic acid in acetonitrile) were used for acquisition in positive ionization mode (ESI+). The elution gradient was as follows: 0 min, 0 % B; 7 min, 30 % B; 9 min, 80 % B; 11 min, 100 % B; 13 min, 100 % B; 14 min, 0 % B.

The mass spectrometer was operated in MS and MS/MS modes using the following parameters: capillary voltage, 3 KV; nebulizer pressure, 40 psi; drying gas flow rate, 11 L/min; gas temperature, 300 ◦C; skimmer voltage, 45 V; fragmentor voltage, 110 V. The MS and Auto MS/MS modes were set to acquire m/z values ranging between 50–1100 and 50–

800, respectively, at a scan rate of 5 spectra per second. Auto MS/MS mode was operated selecting 4 precursor ions per cycle at a threshold of 200 counts, as reported by Ballesteros-Vivas et al [269]. The reference compound solution for internal mass calibration of the q/TOF at mass spectrometer contained 5 μM of purine ([C5H5N4]+ 121.050873 m/z) and 2.5 μM HP-0921, hex-S-5akis(1H,1H, 3H-tetra- fluoropropoxy) phosphazine ([C18H19O6N3P3F24]+ at 922.009798 m/z) in acetonitrile-water (95:5, v/v) from Agilent.

The Agilent Mass Hunter Workstation software 4.0 (Agilent, Santa Clara, CA, USA), was used for post-acquisition data processing. All the data were converted to the mzXML file format. The MS/MS data of all the samples of each mode were uploaded and processed by Global Natural Products Social Molecular Networking (GNPS) (https: //gnps.ucsd.edu, accessed 3 September 2021) [270]. The parameters of GNPS were set to a cosine score of > 0.7 with a minimum requirement of 6 ions to match, precursor mass tolerance of 0.02 Da and the fragment ion mass tolerance of 0.02 Da. Additionally, identification of detected compounds was verified, also using the following databases: PubChem; HMDB; PhytoHub; and/or Massbank. Semi-quantitative analysis was done just for comparison purposes of detected compound area among extracts.

3.6.2. UHPLC-ESI-MS/MS analysis for quantification of the flavanolignans from Silybum marianum extracts

The flavanolignans and the taxifolin were quantified by UHPLC-ESI-MS/MS using an Accela (Thermo Scientific, San Jose, CA) equipped with thermostatted autosampler injector, binary gradient solvent pump, column oven and online degasser (Santa Clara, CA, USA) coupled to a triple quadrupole mass spectrophotometer (TSQ Quantum, Thermo Electron Corporation, San Jose, CA) with an ESI (Electrospray Ionization) interface. The UHPLC separation was performed at 35 °C with 10 μL injection volume on a Core-Shell C18 100 ˚A column (Kinetex, 50 ×2.1 mm i.d.; 1.7 μm; Phenomenex, Torrance, USA). The mobile phases were A (0.1 % formic acid in water) and B (0.1 % formic acid in acetonitrile), The elution gradient at constant flow rate of 0.25 mL/min was as follows: from 80 % to 40 % A (20 min), an isocratic hold at 40 % A (5 min), from 40 % to 80 % A (2 min), and then maintained isocratically for 5 min.

MS analysis was operated using the following parameters: capillary voltage 2 kV; drying temperature, 350 ºC; vaporizer temperature, 400 ºC; drying gas flow rate, nebulizer

gas pressure, 45 psi; 7 L/min. Operating in negative ionization mode The transitions of the compounds (including parent > product ion): SB a + b = 481.94 > 301.018 m/z, SC = 4 81.14 8 > 325.026 m/z, SD = 481.189 > 151.043 m/z, IS = 481.184 > 257.029 m/z, and TX = 303.039 > 285.045 m/z.

Data were acquired and processed with Thermo Fisher Scientific Xcalibur 2.0 software. The amount of different flavonolignan in different samples was calculated using calibration curve of individual compounds prepared at different concentrations (0.01-0.5 mg ml-1). All samples were dissolved in pure ethanol at an appropriate concentration (1–10 mg mL-1) and filtered using 0.45 μm nylon filters before analysis. Analyses were carried out by duplicate, and the results are expressed as mg of compound per g of extract.

3.6.3. GC-MS analysis of Ammodaucus leucotrichus extracts

The analysis was carried out on a GC MS-QP plus system from Shimadzu (Kyoto, Japan). The separation was achieved using a DB-5ms Column (30 m × 0.25 mm I.D. × 0.25 μm df, Quadrex Corporation, Woodbridge, CT). Helium was used as carried gas at a constant flow rate of 1.0 mL/min. The injection volume was 1.0 μL, using a split mode with a ratio of 30. Temperature was programmed from 60 to 240 °C at a rate of 4 °C/min, where it was held for 5 min. MS parameters were the following: interface temperature, 280 °C; source temperature, 300 °C; mass range, 50-500 m/ z; scan speed, 1250 amu/ s. The data collection and handling were performed using the GCMS solution (ver. 2.50 SU3, Shimadzu) software. The identification of the compounds was carried out using the National Institute of Standards and Technology (NIST) and WILEY libraries with the GC-MS Solution software (Shimadzu, Kyoto, Japan). The last classification of the compounds was performed using NPClassifier [271].

3.7. Statistical analysis

Response surface experimental design and its statistical assessment were conducted using Statgraphics Centurion XVI software (StatPoint Technologies, Inc., Warrenton, VA, USA).

IBM SPSS Statistics software V15 (New York, USA) was employed for statistical analysis using a significance level of 95 %. One-way analysis of variance (ANOVA), together with Tukey’s test were employed. All extraction procedures and in vitro analysis

were carried out in triplicate. The results were expressed as the mean ± standard deviation (SD).

Principal Component Analysis (PCA) of *Ammadaucus leucotrichus* extracts, obtained by pressurized liquid extraction, was performed to explain the relationships between in vitro neuroprotective activities and chemical composition. Principal component analysis (PCA) and cluster analysis were performed using Minitab 17 statistical software (Minitab, LLC, State College, PA USA).

- **Experimental design**

Response surface methodology (RSM) was used to analyse the responses of a system in order to investigate the effects due to the variations of selected parameters. Supercritical fluid extraction from *A. leucotrichus* seeds was optimized using a Box Behnken design studying the effects of temperature (40-70 °C); pressure (100-300 bar and co-solvent (5-15 %) ethanol on the extraction yield (expressed as the percentage of dry extract weight per initial plant weight), total phenolic content (expressed as mg of GAE per g of extract), total carbohydrate content (expressed as mg of carbohydrates per g of extract) and Acetylcholinesterase inhibitor capacity (IC_{50} µg/mL). A total of 17 experimental data points were conducted in a randomized run order, including five central points (table (3.3)).

Experimental design and its statistical assessment were created using Statgraphics Centurion XVI software (StatPoint Technologies, Inc., Warrenton, VA, USA). The second order polynomial model proposed for each response variable (Yi) is as follows:

$$Y_i = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + \varepsilon \quad \text{(Eq.3.5)}$$

Where X_1 is the temperature, X_2 is the pressure, X_3 is the percentage of co-solvent, a_0 is the intercept, a_1, a_2 and a_3 are the linear coefficients, $a_{1,1}$, $a_{2,2}$ and $a_{3,3}$ are the quadratic coefficients, a_{12},a_{13},a_{23} are the interaction coefficients and ε is the error. For each response, the effect of each experimental parameter and its statistical significance were analysed from the standardized Pareto chart, accepting the significances at $p \leq 0.05$. The suitability of the model was evaluated by the correlation coefficient (R^2) and the analysis of variance (ANOVA). Additionally, multiple response optimization was performed by the combination of different experimental parameters, in order to maximize the desirability function.

Table 3.3: The experimental design for supercritical fluid extraction from Ammodaucus leucotrichus fruit (Created by the author).

Run	Factors		
	P(bar)	T(°C)	Co-solvent (%)
1	100	40	10
2	300	40	10
3	100	70	10
4	300	70	10
5	100	55	5
6	300	55	5
7	100	55	15
8	300	55	15
9	200	40	5
10	200	70	5
11	200	40	15
12	200	70	15
Central point	200	55	10
Optimum point	300	70	15

CHAPTER IV:

RESULTS AND DISCUSSION

CHAPTER IV: RESULTS AND DISCUSSION

The present chapter is dedicated to the presentation of all the results and the corresponding discussions. It is important to note that this chapter is divided into three distinct sections, each focusing on one of the three extraction processes considered: Pressurized Liquid Extraction (PLE), Supercritical Fluid Extraction (SFE), and Gas Expanded Liquid Extraction (GXLE). In order to increase the overall extraction yields and bioactivities of the extracts, a sequential approach was proposed and tested. This approach, based on the biorefinery concept, includes the use of both pressurized liquid and supercritical extraction. The chemical characterization of the extracts obtained by these three different extraction techniques is also presented and discussed.

In order to evaluate the effectiveness of the proposed approach and the reliability of the results obtained, a comparison was made with values obtained from previously reported sources.

4.1. Use of compressed fluids for the extraction of bioactive molecules from Ammodaucus leucotrichus seeds

Considering the limited amount of information available in the existing literature regarding the phytochemicals and bioactivities of *Ammodaucus leucotrichus*, the primary aim of the present study was to evaluate the possible neuroprotective effects of extracts obtained by pressurized liquid extraction (PLE). This evaluation was performed using a variety of in vitro assays, including investigations of antioxidant (DPPH), anti-inflammatory (LOX), and anti-cholinergic (AChE) activities. In addition, the use of ultra-performance liquid chromatography-quadrupole time-of-flight tandem mass spectrometry (UHPLC-q-TOF-MS/MS) techniques was employed to determine the metabolic profiling of these extracts. Furthermore, the secondary objective of this research was to optimize the process parameters of SFE (Supercritical Fluid Extraction) through the implementation of Response Surface Methodology (RSM). In addition, the effects of these parameters on yield, total phenolic content (TPC), total flavonoid content (TCC) and acetylcholinesterase (AChE) inhibition were evaluated. The chemical profile analysis was performed using gas chromatography-mass spectrometry (GC-MS) and UHPLC-q-tof-MS/MS techniques. Finally, the third objective of this study was to asses de viability of biorefinery process based on sequential extractions, combining PLE in the first step and SFE in the second step, with

the aim of obtaining valuable extracts that have the potential to exhibit neuroprotective activity from the seeds of *A. leucotrichus*.

4.1.1. Pressurized liquid extraction from *Ammodaucus leucotrichus* seeds

4.1.1.1. Extraction of phytochemicals

In order to determine the appropriate duration of the extraction process, a comprehensive study of the extraction yield was conducted at three different time intervals: 10, 20, and 30 minutes. These studies were carried out using pressurized ethanol at a temperature of 110 °C, which is the mean temperature of the experimental design, and a pressure of 103.4 bar (1500 psi, standard pressure for PLE). The statistical analysis performed, as shown in Figure (4.1), indicated that there were no significant differences in extraction yield after extended extraction times. Consequently, based on these results, a 10 min extraction time was considered appropriate for the extraction process under the above conditions. This decision was made in order to avoid any undesirable reactions that may occur due to longer extraction times. Furthermore, selecting a shorter extraction time of 10 min would also contribute to energy conservation during the extraction process.

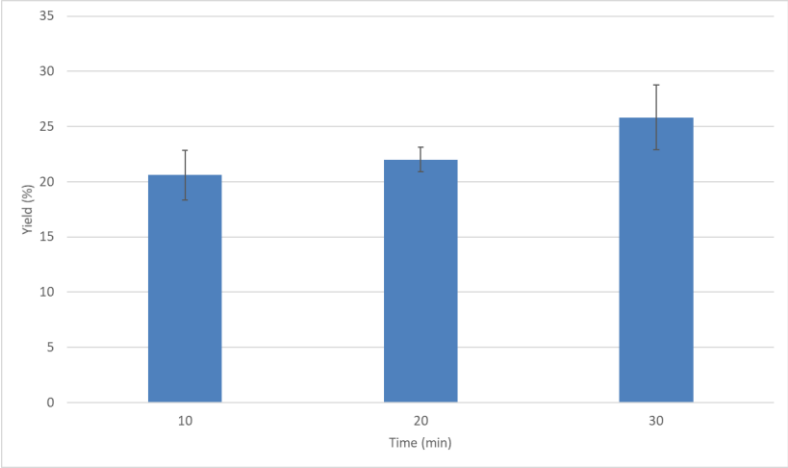

Figure 4.1: Extraction recoveries of Ammodaucus leucotrichus by pressurized ethanol at 110 °C and 103.4 bar at different extraction times. Mean values with different superscript letters are significantly different ($p < 0.05$) (Created by the author).

Due to the fact that Water and Ethanol possess significantly low environmental impacts, they were chosen and employed as the extraction solvents during the experiment,

wherein a range of varying temperatures were utilized to facilitate the extraction process for the bioactive molecules from the seeds of *A. leucotrichus*. The results are reported in Figure (4.2) and Table (S2).

As expected, and despite the use of a solvent, it was observed that the extraction yield experienced a notable increase with temperature. Furthermore, the most notable increase in extraction yield with temperature was observed when water was used at an extraction temperature of 180 °C (see Figure (4.2)). Nevertheless, the maximum yield obtained reached a value of 44.44 %, which is three times higher than the minimum yield of 15.55 % obtained at a relatively cooler temperature of 40 °C using EtOH, resulting in a lower yield compared to water. This particular pattern of behaviour can also be explained by the enhanced mass transfer properties, resulting in improved solubilities at elevated temperatures. These results are consistent with those documented in references [37] [8].

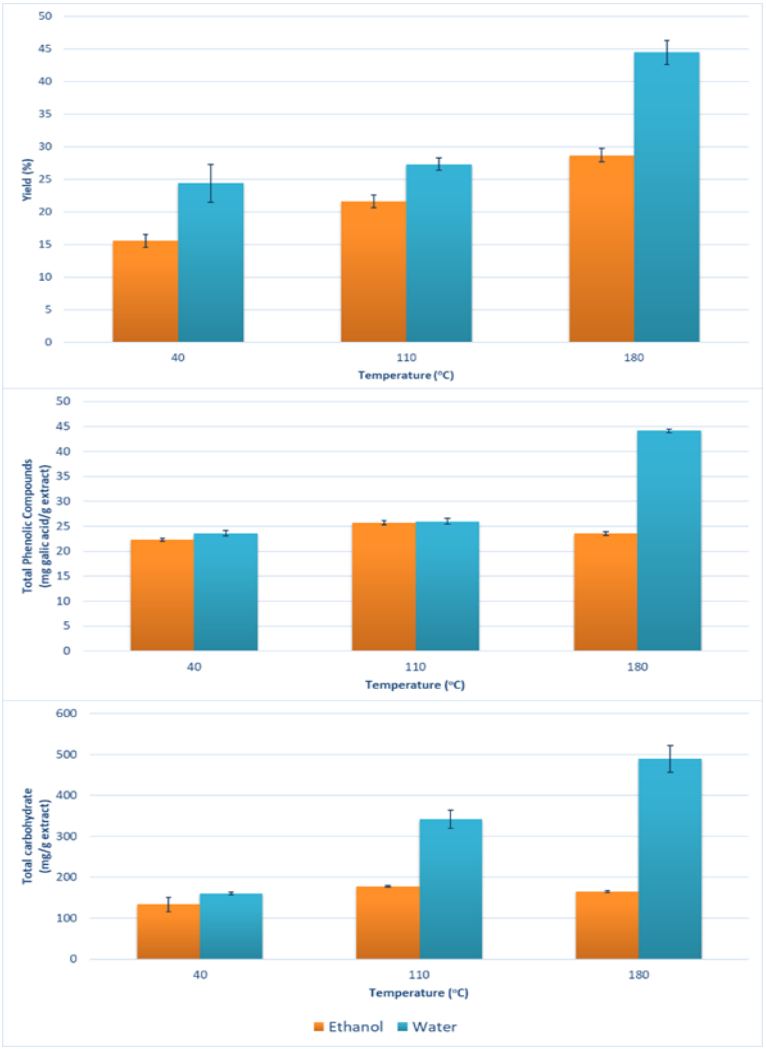

Figure 4.2: Extraction yield, total phenolic (mg GAE/g extract) and total carbohydrate (mg/g extract) determined in the obtained extracts of Ammodaucus leucotrichus using 10 min pressurized liquid extraction at the indicated conditions (Created by the author).

The highest total phenolic content, 43.5 ± 0.8 mg GAE/g extract, and total carbohydrate content, 489.36 ± 6.64 mg/g extract, were found when the process was conducted at a temperature of 180 °C using water. It is important to note that these conditions also gave the highest total yield. On the contrary, when ethanol was used at a temperature of 40 °C, the resulting total phenolic and total carbohydrate contents were significantly lower, 22.3 ± 0.3 mg GAE/g extract and 133.63 ± 13.42 mg/g extract, respectively. The influence of temperature was observed to have a positive effect on the total phenolic and total carbohydrate contents when water was used, while no significant effect on these values was observed when ethanol was used.

In a previous study conducted by Ziania et al. [132], it was also found that the hydroethanolic extracts of *A. leucotrichus* contained a high amount of carbohydrates, especially soluble sugars such as glucose, fructose and sucrose. These carbohydrates were present at a concentration of about 65 g/100 g dw (equivalent to 650 mg/g extract). In comparison, *Moringa oleifera* was found to have a slightly lower concentration of carbohydrates, measured at 56.6 g/100g dw (equivalent to 566 mg/g extract). The researchers considered *A. leucotrichus* to be a valuable source of high energy due to its carbohydrate content.

The high values found with water at 180 °C may be due to the higher solubility of compounds at higher temperature, as well as a possible complex protein and carbohydrates degradation to smaller compounds that could have been easily extracted within 10 min extraction time. Furthermore, small peptides can be detected using the Folin-Ciocalteau test. In addition, starch hydrolysis using pressurized hot water had been proposed and successfully used by previous researchers [272][273].

4.1.1.2. In vitro bioactivity assays

Table (4.1) shows the results of acetylcholinesterase (AChE) inhibition, anti-inflammatory (LOX) and antioxidant capacity (DPPH radical scavenging) of *A. leucotrichus* extracts. It's important to highlight that all the results are presented in terms of IC_{50} (µg/ml), indicating that superior activities are obtained with lower IC_{50} values. Consequently, the extract obtained by using water at a temperature of 180 °C showed the most favourable values.

Table 4.1: IC50 (µg/mL) values from in vitro assays of different Ammodaucus leucotrichus extracts using AChE, LOX, DPPH assays (Created by the author).

Samples	AChE	LOX	DPPH
ETOH-40°C	n.d	197.621 ± 5.646^{f}	287.699 ± 1.816^{f}
ETOH-110°C	n.d	140.076 ± 9.076^{e}	92.200 ± 6.067^{c}
ETOH-180°C	300.458 ± 18.275^{d}	107.973 ± 14.001^{e}	60.482 ± 0.176^{b}
H2O-40°C	316.817 ± 36.329^{d}	536.985 ± 7.255^{d}	129.711 ± 7.146^{e}
H2O-110°C	222.329 ± 32.459^{c}	342.311 ± 5.510^{c}	111.184 ± 4.176^{d}
H2O-180°C	55.598 ± 7.724^{b}	39.373 ± 4.783^{b}	58.513 ± 4.756^{b}
Positive control*	4.061 ± 0.310^{a}	14.298 ± 1.748^{a}	18.714 ± 1.301^{a}

n.d: not determined (maximum level of inhibition below 50 %).The values are means ± sd. Different superscripts (a, b, c, d, e, f) indicate significant differences ($p \leq 0.05$). * Chemical standards used as positive controls of each test: Galantamine for AChE, Quercetin for LOX and BHT for DPPH.

Regarding the neuroprotective activity measured by the acetylcholinesterase inhibition assay, it is clear from the data of Table (4.1) that 180 °C provided the highest values of inhibition for both ethanol and water solvents. In fact, at lower temperatures it was not possible to determine IC_{50} for ethanol extracts. The best values obtained with water indicated the higher polarity of compounds responsible for this activity, whose composition would be seen later. Following the steepest ascent of AChE with temperature, 200 °C was tested using pressurized water to confirm whether the combined effect of higher temperature could improve AChE activity. However, the IC_{50} value obtained at 200 °C was higher than that obtained at 180 °C, with 189.430 µg/mL at 200 °C vs. 55.598 µg/mL at 180 °C, respectively. This demonstrated that it was not necessary to increase the temperature to the maximum value tested initially. Previous researchers found relations between the biological activity of extracts obtained from natural matrices at a high temperature using water with the formation of new compounds from Maillard and caramelization reactions [274][275][276]. In fact, the appearance of brown colour, which we found at 200 °C with just a visual estimation, estimated the presence of Maillard reaction products.

Sadaouia et al. studied the inhibitory potential against acetylcholinesterase of the *A. leucotrichus* aerial parts essential oil, and they could not achieve an IC_{50} value for the AChE activity in their extracts–they only provided value for certain pure compounds present in the extract [145]. Therefore, in the present paper, and to the best of the authors' knowledge, it was the first time the AChE inhibitory activity of *A. leucotrichus* extracts was measured. On the contrary, the antioxidant capacity measured by DPPH radical scavenging capacity did not show the same effect with the solvent, but rather showed a dependency on temperature (higher temperature, higher activity). This trend was similar to the one found in the TPC assay, where in fact, the reaction of reduction of molibdotungstate (Folin reagent) was another way to express antioxidant activity. It is common to find the same trend in both values when the main antioxidant compounds present in the sample are phenolic compounds, as will be seen in the following section.

This last effect was also seen in the anti-inflammatory activity measured by lipoxygenase inhibition test (LOX). However, better values were found in ethanol, except

for the extract obtained with water at 180 °C, which provided again the best value. In fact, no significant difference was observed between the extract obtained at 180 °C using water and quercetin, which was used as a positive control.

The best results found for in vitro activities for water at 180 °C were in complete agreement with the highest values of total phenolic and total carbohydrate contents, in the same conditions (180 °C and water); even then, it was the extraction condition that provided the best yield. Their composition will be seen in the following section. To summarize these findings put into evidence the potential of *A. leucotrichus* extracts as inhibitors of AChE, LOX and antioxidants. Thus, the most interesting multi-bioactive extracts of *A. leucotrichus* would be those extracted with water at a high temperature.

4.1.1.3. Chemical profiling by UHPLC-q-TOF-MS/MS: untargeted analysis

One of the objectives of this study was to determine the profile of the compounds present in *A. leucotrichus* seed extract. Thus, the untargeted analysis of all the extracts was carried out by UHPLC-q-TOF-MS/MS. It can be clearly seen in Figure (4.3) and Table (4.2) that the qualitative profile varied depending on the considered sample.

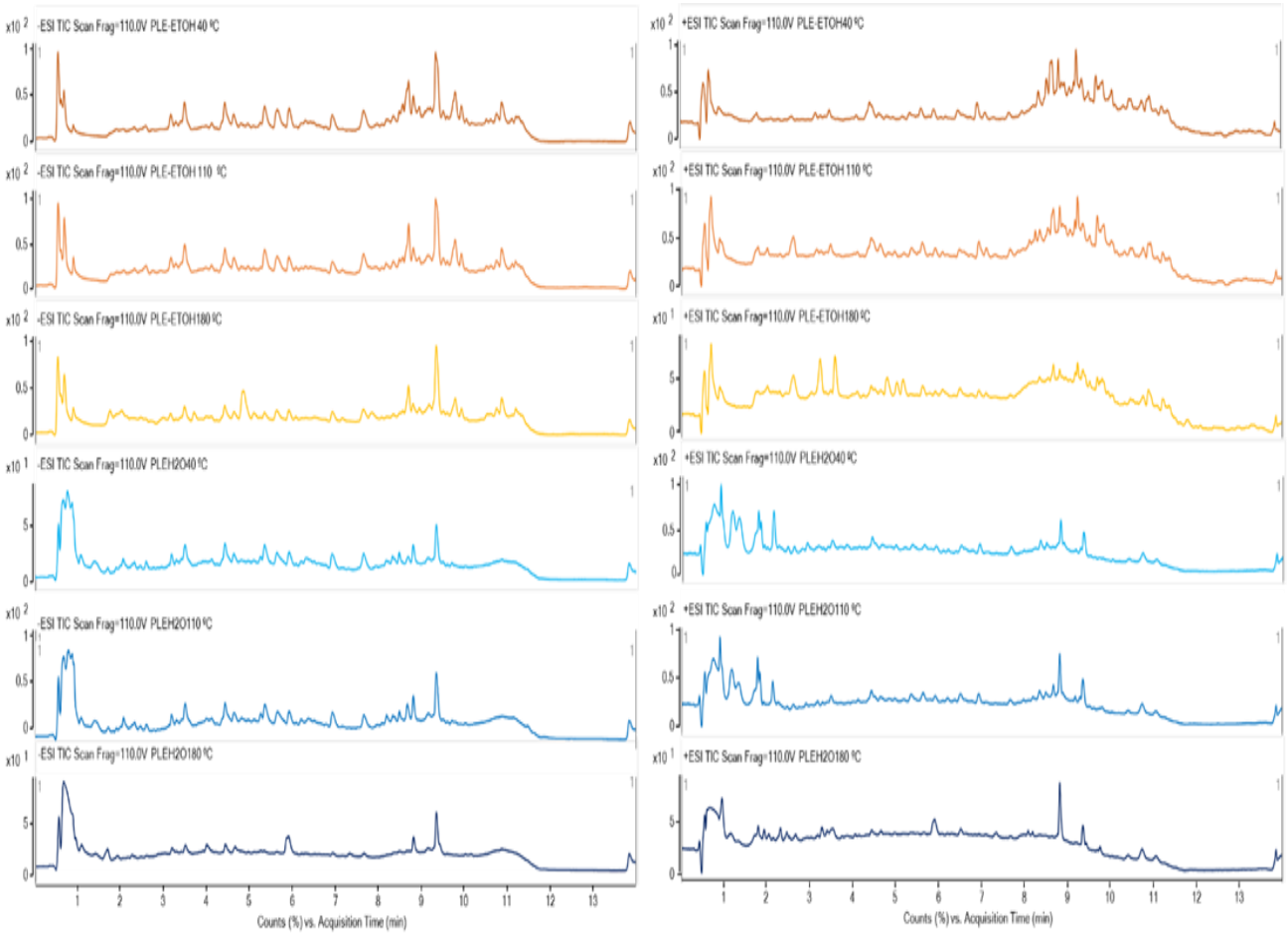

Figure 4.3: UHPLC-ESI-qTOF chromatograms (Total Ionic Current, TIC) of the pressurized liquid extracts of Ammodaucus leucotrichus seeds obtained using 10 min of extraction time at indicated temperatures. Left side negative polarity, right side positive polarity. Orange chromatograms correspond to ethanolic extracts, blue chromatograms correspond to water extracts. Extraction temperature is indicated above each chromatogram (Created by the author).

Table 4.2: Identification and mean areas found in the UHPLC-ESI-qTOF Chromatograms of the pressurized liquid extracts of Ammodaucus leucotrichus seeds obtained using 10 min of extraction time at indicated temperatures and solvents. [M+H]+ indicate positive ESI polarity, [M-H]- indicate negative ESI polarity (Created by the author).

#	Rt (min)	Tentative identification	Molecular formula	Monoisotopic mass	[M+H]+	[M-H]-	Area counts						Ref
							H_2O-180°C	H_2O-110°C	H_2O-40°C	ETOH-180°C	ETOH-110°C	ETOH-40°C	
1	0,547	D-mannitol	C6H14O6	182.079	183.0866	/	-	-	-	256913.06	-	160672.51	[277]
2	0,592	Citric acid	C6H8O7	192.027	/	191.0223	353378.81	195974.47	4210281.72	-	-	-	[278]
3	0,629	Melezitose	C18H32O16	504.169	522.2039	/	-	-	-	29584.98	27162.81	28271.3	
4	0,634	Isomaltulose	C12H22O11	342.1162	360.1487	/	-	-	-	-	-	135561.23	
5	0,666	Trehalose	C12H22O11	342.1162	/	341.1067	-	-	-	-	-	32729.68	
6	0,69	N-fructosyl pyroglutamate	C11H17NO8	291.0954	/	290.0859	-	-	34060.24	299069.54	826707.56	382906.83	
7	0,707	Adenine	C5H5N5	135.0544	136.0615	/	-	-	-	46760.03	-	-	
8	0,727	5-deoxy-5-(methylsulfinyl)adenosine	C11H15N5O4S	313.0844	314.0934	/	35673.29	120821.03	112437.96	27709.55	-	75402.01	

9	0,73	Adenosine	C10H13N5O4	267.0967	268.1035	/	-	-	-	383507.74	-	121233.07	
10	0,759	N-fructosyl isoleucine	C12H23NO7	293.1474	294.153	/	-	-	-	-	-	67835.07	
11	0,768	Adenine hydrochloride	C5H6ClN5	171.0311	136.0607	/	-	-	-	-	2073.85	95037.61	
12	1,089	His-pro l-histidyl-l-proline	C11H16N4O3	252.1222	235.1213	/	63766.14	-	-	-	-	-	
13	1,125	5-methylcytosine	C5H7N3O	125.0589	126.0669	/	-	231184.42	-	-	-	-	
14	1,139	Isoleucine	C6H13NO2	131.0946	132.101	/	625928.34	-	-	-	-	-	[278]
15	1,329	Meglutol (aka 3-hydroxymethylglutaric acid)	C6H10O5	162.0528	/	161.0447	-	1271229.98	-	-	-	-	
16	1,698	Phe-arg	C15H23N5O3	321.1800	322.1921	/	-	-	44413.58	-	-	-	
17	1,774	α-adenosine	C10H13N5O4	267.0967	268.1077	/	-	-	1136956.03	-	-	-	

18	1,832	2-deoxyadenosine	C10H13N5O3	251.1018	252.1112	/	-	754326.63	-	-	-	-	
19	1,892	Guanosine	C10H13N5O5	283.0916	/	282.0869	-	607330.63	525654.93		22931.34	-	
20	2,174	Leu-leu-arg l-leucyl-l-leucyl-l-arginine	C18H36N6O4	400.2798	401.2916	/	-	-	20611.77	-	-	-	
21	2,178	2`-o-methylguanosine	C11H15N5O5	297.1073	298.1144	/	-	16989.46	-	-	-	-	
22	2,408	Pantothenic acid	C9H17NO5	219.1106	/	218.1017	-	-	-	-	-	40563.84	
23	2,479	Chlorogenic acid	C16H18O9	354.0950	/	353.0908	-	26798.15	-	-	-	-	[279]
24	2,617	Succinoadenosine	C14H17N5O8	383.1077	384.1193	/	-	42590.91	32102.49	-	-	-	
25	2,646	N2_n2-dimethylguanosine	C12H17N5O5	311.1229	312.1334	/	-	30971.86	-	-	-	-	
26	2,881	Tryptophan	C11H12N2O2	204.0898	205.0976	/	-	170148.31	233265.5	-	-	36387.87	[280]
27	2,901	Abrine	C12H14N2O2	218.1055	188.0734	/	-	-	82874.54	-	-	-	
28	3,044	Xanthurenic acid	C10H7NO4	205.0375	206.0457	/	-	9935	-	-	-	-	

29	3,152	Salidroside	C14H20O7	300.1209	318.1544	/	-	-	-	-	3928.57	14741.76	
30	3,696	Olivil 4-o-glucoside	C26H34O12	538.205	556.238	/	-	-	13104.4	29531.1	25746.5	12247.41	[281]
31	3,735	Oleuropein	C25H32O13	540.1843	/	537.202	5069.04	-	-	-	-	-	
32	3,78	Magnolioside	C16H18O9	354.0950	355.1068	/	-	9854.92	-	-	-	-	
33	3,948	Licoagroside b	C18H24O12	432.1267	433.1344	/	-	-	-	-	89624.35	-	
34	4,107	Icariside f2	C18H26O10	402.1526	420.1873	/	-	-	-	-	94678.37	-	
35	4,115	Norharmane	C11H8N2	168.0687	169.0776	/	60800.27	-	-	-	-	-	
36	4,321	Leu-phe l-phenylalanine. L-leucyl-	C15H22N2O3	278.1630	279.171	/	-	-	14156.73	-	-	-	
37	4,616	(-)-Erythro-anethole glycol 2-glucoside	C16H24O8	344.1471	/	343.1375	-	216892.16	210475.47	-	349268.58	348151.05	
38	4,79	Melibiose	C12H22O11	342.1162	/	341.1111	-	-	-	1714.93	-	5932.14	
39	4,812	Isomaltulose	C12H22O11	342.1162	/	341.1099	-	-	-	-	10017.86	-	

40	4,862	2-phenylethyl 6-o-[(2s,3r,4r)-3,4-dihydroxy-4-(hydroxymethyl)tetrahydro-2-furanyl]-beta-d-glucopyranoside	C19H28O10	416.1682	434.2057	/	-	14756.56	12440.55	13111.45	30206.48	19273.33	
41	5,232	Scopoletin	C10H8O4	192.0422	193.0499	/	-	-	-	-	92930.27	135092.42	[282]
42	5,244	Rosiridin	C16H28O7	332.1835	350.2176	/	-	-	-	-	16601.91	8557.74	
43	5,433	Secoisolariciresinol diglucoside	C32H46O16	686.2785	/	731.2734	10303.7	15431.41	-	18553.63	17717.47	21842.93	
44	5,473	2-(hydroxymethyl)-6-[4-[(2s.3s)-3-(hydroxymethyl)-5-[(e)-3-hydroxyprop-enyl]-7-methoxy-2.3-dihydro-benzofuran-2-yl]-2-methoxyphenoxy]oxane-3.4.5-triol	C26H32O11	520.1944	538.23	/	-	50564.3	-	46097.94	108018.97	97312.73	

45	5,546	Phe-pro l-phenylalanyl-l-proline	C14H18N2O3	262.1317	245.1287	/	7327.25	-	-	-	-	-	
46	5,6	Luteolin-4-o-glucoside	C21H20O11	448.1005	449.1122	/	-	-	174044.54	-	16414.09	673537.14	[132]
47	5,613	Luteolin	C15H10O6	286.0477	287.0557	/	13212.81	64365.75	21756.23	-		81465.26	[283]
48	5,628	Luteolin-7-o-glucoside	C21H20O11	448.1005	/	447.0951	69065.61	249806.7	80599.32	472705.88	491488.83	709743.1	[132]
49	5,789	Artselaeroside a	C19H28O10	416.1682	/	415.1618	-	13343.51	-	-	-	-	
50	5,895	Secoisolariciresinol	C20H26O6	362.1729	327.1594	/	-	-	133503.9	108794.11	-	-	
51	6,22	Apigetrin	C21H20O10	432.1056	433.1127	/	-	-	-	46851.97	75765.67	-	
52	6,228	Aloenin	C19H22O10	410.1213	433.1107		-	-	-	-	-	74898.28	[284]
53	6,243	Apigenin-7-o-glucoside	C21H20O10	432.1056	/	431.1014	-	50006.98	-	72551.76	107889.58	1381177.72	[285]
54	6,272	Apigenin	C15H10O5	270.0528	271.0587	/	-	-	-	-	-	5461.85	[283]
55	6,411	Kaempferol 3-o-(6"-malonyl-glucoside)	C24H22O14	534.1009	/	533.093	18989.85	175483.87	19501.18	29675.95	38321.12	52268.82	

56	6,439	Peonidin 3-galactoside cation	C22H23ClO11	498.0928	463.1305	/	11870.52	107973.97	33700.05	-	159609.31	22946.27	
57	6,474	Hispidulin 4'-glucoside	C22H22O11	462.1162	/	461.1059	-	36257.29	-	61747.79	104199.27	112174.81	
58	6,583	Kaempferol-o-acetylhexoside	C23H22O12	490.1111	491.1219	/	-	-	-	-	26539.4	-	
59	7,013	Malonylgenistin	C24H22O13	518.106	519.1185	/	-	24689.1	-	-	-	-	
60	7,096	(2e)-5-(2.3-dimethyltricyclo[2.2.1.0~2.6~]hept-3-yl)-2-methyl-2-pentenoic acid	C15H22O2	234.1619	235.1692	/	-	-	17717.68	-	-	-	
61	7,118	Atractyligenin (2-o-beta-glucopyranosyl-)	C26H40O8	480.2723	481.4027	/	-	-	-	17160.03	-	22946.27	
62	7,538	Z-ajoene	C15H24O3	252.1725	235.1681	/	-	-	-	-	-	25738.6	
63	7,573	Luteolin	C15H10O6	286.0477	/	285.0402	91524.94	327175.86	651690.56	1057626.37	1337640.84	1276508.69	[283]
64	7,715	Kahweol	C20H26O3	314.1882	332.2072	/	-	-	-	-	16518.87	-	
65	7,844	6-o-acetylgenistin	C23H22O11	474.1162	475.1237	/	-	-	20071.13	-	-	-	

66	8,237	Apigenin	C15H10O5	270.0528	/	269.0461	-	18256.97	40402.51	35777.26	65299.66	99271	[283]
67	8,312	10,15-octadecadienoic acid, 9,12,13-trihydroxy-	C18H32O5	328.2249	/	327.2156	-	-	34544.7	-	-	54520.82	
68	8,341	Diosmetin	C16H12O6	300.0633	301.0746	/	-	42782.71		42127.41	98510.86	107058.01	[123]
69	8,503	9.12.13-trihydroxy0-octadecenoic acid	C18H34O5	330.2406	/	329.2328	32165.95	215421.58	302602.83	101190.5	253994.81	286558.35	
70	8,672	Caffeic acid	C9H8O4	180.0422	163.0397	/	-	-	-	-	56612.35	-	[283]
71	8,929	Phytosphingosine	C18H39NO3	317.2929	318.3048	/	-	-	-	-	123447.37	-	
72	9,118	Vicine	C10H16N4O7	304.1019	304.3039	/	-	-	-	-	15155.3	-	
73	9,424	1-palmitoyl-2-linoleoyl pe	C39H74NO8P	715.5152	/	714.5089	114762.55	11800.26			25952.95	65464.86	
74	9,674	Coumaroyl glucose (p-)	C18H15O4P	326.0707	327.0754	/	-	-	-	-	28967.57	947.77	
75	9,675	Alpha-dimorphecolic acid	C18H32O3	296.2351	/	295.2278	-	94220.93	-	240380.24	363755.92	453266.03	

76	9,716	Vernolic acid	C18H32O3	296.2351	279.2357	/	-	-	-	-	52633.25	-	
77	9,723	Linolenic acid	C18H30O2	278.2245	279.2357	/	-	-	-	37580.27	-	-	[278]
78	9,746	1-palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine	C21H44NO7P	453.2855	/	452.2765	-	-	-	-	33150.36	55686.05	
79	9,807	1-(9z-octadecenoyl)-sn-glycero-3-phosphoethanolamine	C23H46NO7P	479.3011	/	478.2894	-	-	-	-	-	25958.79	
80	9,918	(-)-Isolongifolol	C15H26O	222.1983	205.1984	/	-	-	-	186569.19	192776.99	-	
81	10,009	6-paradol	C17H26O3	278.1882	279.1602	/	-	64549.93	87230.6	78706.47	128249.87	160475.23	
82	10,069	Lyso-pc(16:0) 1-palmitoyl-sn-glycero-3-phosphocholine	C24H50NO7P	495.3324	496.3432	/	-	-	-	89909.81	-	-	
83	10,219	1-(9z-octadecenoyl)-sn-glycero-3-phosphocholine	C26H52NO7P	521.3481	522.3549	/	43610.79	-	-	92824.45	236486.76	-	

84	10,233	Linoleoyl ethanolamide	C20H37NO2	323.2824	324.2902	/	-	-	-	36625.55	66053.78	59444.5	
85	10,251	Lpc 18:1 1-oleoyl-sn-glycero-3-phosphocholine	C26H52NO7P	521.3481	/	566.3447	-	-	-	-	50191.37	56596.86	
86	10,593	16-hydroxypalmitic acid	C16H32O3	272.2351	/	-	-	-	-	-	154253.85	-	
87	10,662	N-oleoylethanolamine	C20H39NO2	325.298	326.3026	/	-	-	-	-	46449.9	2167.11	
88	10,761	Oleanolic acid	C30H48O3	456.3603	439.3563	/	-	-	-	-	39705.62	-	
89	10,831	9-octadecenamide. (z)-	C18H35NO	281.2718	282.2799	/	-	-	-	729370.61	775760.87	597323.34	
90	11,083	Pheophorbide a	C35H36N4O5	592.2685	593.2706	/	-	-	-	32435.93	102694.61	-	
91	11,633	Dihydroperillic acid glucuronide	C20H24N2O2	326.10016	/	325.1848	1870.22	-	-	-	-	-	
92	11,841	Resveratrol 5-o-glucoside	C20H22O8	390.13146	391.2838	/	-	-	-	-	6691.07	-	
93	13,074	MGMG (16:3)	C25H42O9	486.282885	531.4067	/	-	-	-	9813.91	11807.11	14406.67	

94	13,902	Pi 34:2 phosphatidylinositol(34:2)	C43H79O13P	835.06799	/	833.5184	-	-	-	-	1344.31	956.12	

In total, a staggering number of 94 compounds have been tentatively identified, mainly comprising unbound and glycosylated phenolic compounds in addition to lipids and organic acids. Given the paucity of available literature on the phytochemical investigation of the composition of *A. leucotrichus* seed extracts, it becomes a difficult task to draw comparisons with the results obtained in the present study and previous literature results. Ziania et al. [132] studied aerial parts composition of *A. leucotrichus* , and they confirmed the presence of some phenolic compounds such as Apigenin-6,8-C-diglucoside, Luteolin- 7-O-glucoside, Di-O-caffeoyl-malonylquinic acid, Luteolin-O-(malonyl-hexoside) isomer and Di-O-caffeoyl-dimalonylquinic in the ethanolic extract (80 % v/v) of *A. leucotrichus* seed. Therefore, the detected presence of the Flavonoid derivatives in *A. leucotrichus* seed extract was not surprising [132]. Different studies have shown that the Flavonoid glycosides possess a wide variety of pharmacological activities, such as antioxidant and anti-inflammatory [286][287]. Other compounds identification was based on the MS data; only some of them could be confirmed by previous research in this plant or taxonomical members [277][278][279][280][281][282][283][284][285] [123]. The bioactivity cannot be assigned by a single compound, but in synergy between several compounds. In order to find correlations between the composition and the obtained bioactivities (AChE and LOX), multivariate statistical analysis (combining principal component analysis (PCA) and cluster analysis) was performed using Minitab statistical software. Although 94 individual compounds were detected, not all of them were present in all samples, so these correlation studies were performed considering families of compounds using the normalized areas. Furthermore, considering that the best activities give lower IC_{50} values, the ones used are the inverse of those in Table (4.2). The graphical results of these multivariate analyses are shown in Figure (4.4).

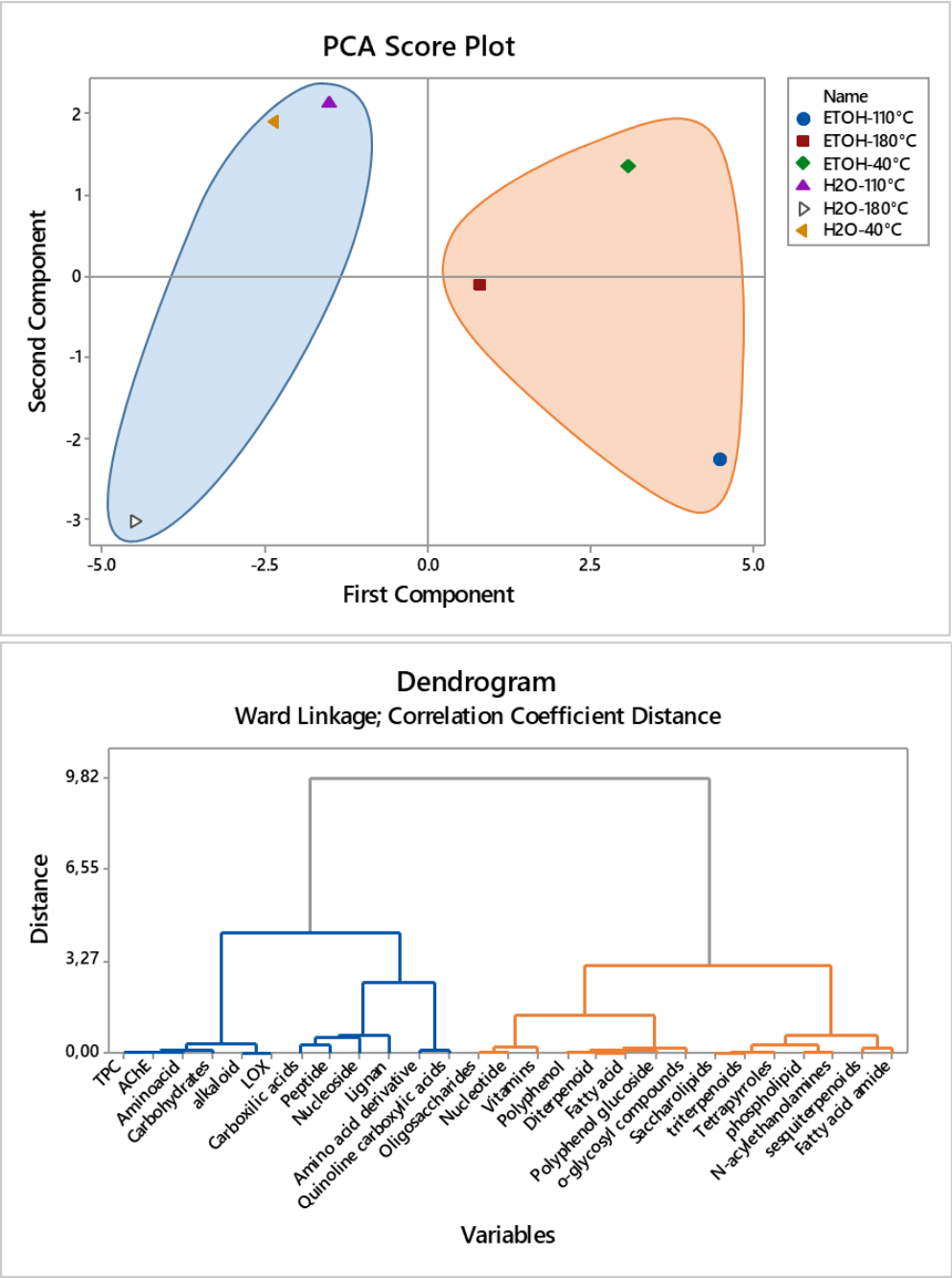

Figure 4.4: Multivariate analyses of Ammodaucus leucotrichus extracts. Score plot obtained in Principal Component Analysis (PCA) and, below, dendogram combining bioactivities and composition of extracts classified by families obtained. Note: Principal component 1 and 2 are the first two components from PCA that capture 67.3 % of variation (Created by the author).

The PCA allowed the classification in two main groups using only two components, which could capture 67.3 % of the encountered experimental variations. With four components, the total variability of the data was accounted for. It could be easily seen that

the first component PC1 could classify extracts regarding the extraction solvent obtaining negative and positive values of PC1 for water (blue region) and for ethanol extractions (orange region), respectively. Regarding the second component of the multivariate analysis (PC2), it allowed to classify the extracts in terms of their anti-inflammatory capacity measured by LOX, giving negative PC2 values to the samples with higher potential. The main point of this analysis was the possibility to discriminate the sample with higher bioactivity (water 180 °C) in terms of anti-inflammatory (LOX) and anti-neurodegenerative (AChE); this sample is the only one whose values of PC1 and PC2 were negative.

The results of the cluster analysis were similar. Two main clusters were obtained as shown in Figure (4.4) (lower graph). On the left, the blue cluster gathers the fractions with higher correlation with bioactivity, as well as high amounts of total carbohydrates and phenolic compounds, and free amino acids, peptides and other compounds. This composition could be found in the *A. leucotrichus* extracts obtained with water at 180 °C.

4.1.2. Optimization of supercritical fluid extraction from Ammodaucus leucotrichus seeds

4.1.2.1. Supercritical fluid extraction optimization

First, the kinetic study of the supercritical fluid extraction process was performed at the central point of the experimental conditions (extraction temperature, 55 °C; extraction pressure, 200 bar; and co-solvent percentage, 10 %) to determine an appropriate extraction duration. The extract was collected every 20 min for a total extraction time of 240 min.

As can be seen in Figure (4.5), the extraction curve shows three-time intervals; a period of increasing extraction intensity up to 80 min (controlled by the convection mechanism), followed by a period of decreasing extraction intensity for approximately 40 min (controlled by the combination of convection and diffusion mechanisms), after which there was no significant escalation in the number of soluble solids extracted (controlled by the diffusion mechanism). Thus, in the current study, the extraction time was set at 100 min, with a recovery of more than 80 % of the extractable material.

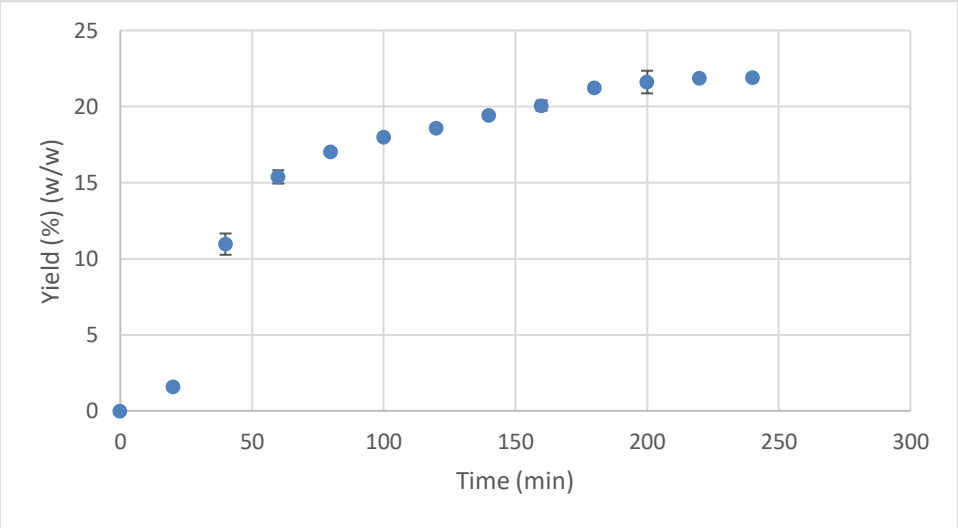

Figure 4.5: Kinetic study of Ammodaucus leucotrichus extraction by supercritical CO2 at 55 °C and 200 bar using a 10 % of ethanol like a co-solvent. Results expressed as means ± SD (n=3) (Created by the author).

A Box-Behnken experimental design was adopted and included three main independent factors: temperature [40 - 70 °C], pressure [100 - 300 bar], and the percentage of co-solvent used during extraction [5 – 15 %], all of which could potentially affect the extractability of bioactive molecules from *A. leucotrichus*. The pressure and temperature ranges were chosen based on the limitations of the supercritical fluid extraction apparatus and to avoid thermal decomposition of bioactive compounds. Ethanol was the most commonly used co-solvent because it is low cost, readily available in high purity, fully biodegradable, and GRAS (Generally Recognized as Safe) for the food, cosmetic, and pharmaceutical industries. An ethanol percentage range of no more than 15 % is also the most commonly reported in the literature for similar studies. However, a very low co-solvent concentration is always desirable in order to preserve the important and green aspect of SFE, which consists in avoiding the use of organic solvents, which are planned to be banned in 2050, in sensitive areas such as pharmaceutical, cosmetic and food industries. Other factors involved in the extraction were kept constant, such as extraction time (100 min), sample amount (1.5 g) and flow rate (4 mL/min). Four response variables were considered: total yield, total phenolic content (TPC), total carbohydrate content (TCC) and anti-cholinesterase activity (AChE). The results obtained for all response variables are summarized in Table (4.3).

Table 4.3: Extraction yield, total phenolic content, total carbohydrate content and AChE inhibition of the extracts obtained from Ammodaucus leucotrichus seeds (Created by the author).

Run	Factors			Response variables			
	P (bar)	T (°C)	Co-solvent (%)	Yield (%)	TPC (mg GAE/g extract)	Total Carbohydrates (mg/ g extract)	AChE IC_{50} (ug/ mL)
1	100	40	10	15.23	15.59	273.52	503.65
2	300	40	10	18.31	19.90	423.23	388.69
3	100	70	10	13.39	28.83	646.56	344.53
4	300	70	10	26.56	32.42	810.56	338.47
5	100	55	5	13.32	13.57	391.96	441.48
6	300	55	5	13.22	18.26	114.85	427.22
7	100	55	15	16.66	27.96	1076.56	332.33
8	300	55	15	19.21	24.56	1257.89	457.74
9	200	40	5	14.13	11.74	112.85	887.36
10	200	70	5	15.03	20.44	161.74	455.24
11	200	40	15	17.39	23.26	522.41	403.10
12	200	70	15	19.88	26.89	674.56	371.65
Central point	200	55	10	17.27	25.29	243.52	425.87
				16.53	25.63	245.52	432.62
				17.25	25.17	242.19	424.95

To better understand the effects of each experimental parameter and their interactions on the response variables, the standardized Pareto plots of these variables and the surface responses are shown in Figures (4.6) and (4.7), respectively. The ANOVA (Analysis of Variance) test provides the significance of the effect induced by the independent factors and their interactions (see Table 4.4).

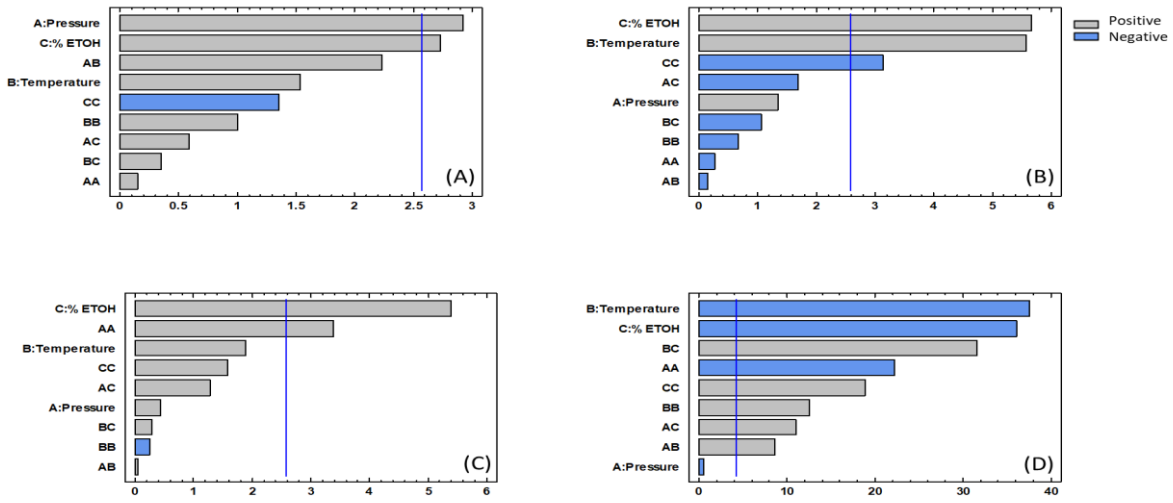

Figure 4.6: Pareto diagram for effect of temperature, pressure and percentage of co-solvent in extraction yield (A), TPC (B), TCC (C), IC50 AChE (D) of Ammodaucus leucotrichus obtained by SFE. The vertical line defines the region of statistical significance (right side) at 95 % confidence level; Blue and grey bars show negative and positive effects of each factor, respectively (Created by the author).

Figures (4.7) and (4.6) show different behavior of the response variables. The yields ranged from 13.22 % in run 4 (300 bar, 70 °C and 10 % EtOH) to 26.56 % in run 6 (300 bar, 55 °C and 5 % EtOH). Temperature had a non-significant positive effect. The influence of the other two single factors (P and % EtOH) was significant, with P being the most important, followed by % EtOH with a positive effect on the response. This result was expected considering that an increase in the percentage of EtOH in the solvent composition induces a greater polarity in favor of the solubility of a wide range of analytes, thus a clear effect of the co-solvent [288]. In addition, the effect on the density of supercritical CO_2 in the presence of EtOH as a co-solvent was mainly due to the pressure, which greatly influenced its solubility and therefore its solubility for the extract components obtained by means of the supercritical extraction process [289][93]. Moreover, the extraction yields obtained by SFE were higher than those obtained by classical methods, being 3.80, 6.58, 10.02 and 7.22 % for water, ethanol, methanol and acetone, respectively [150][155][158]. However, these values were lower than those previously obtained using PLE, with 44 % being the highest yield value obtained.

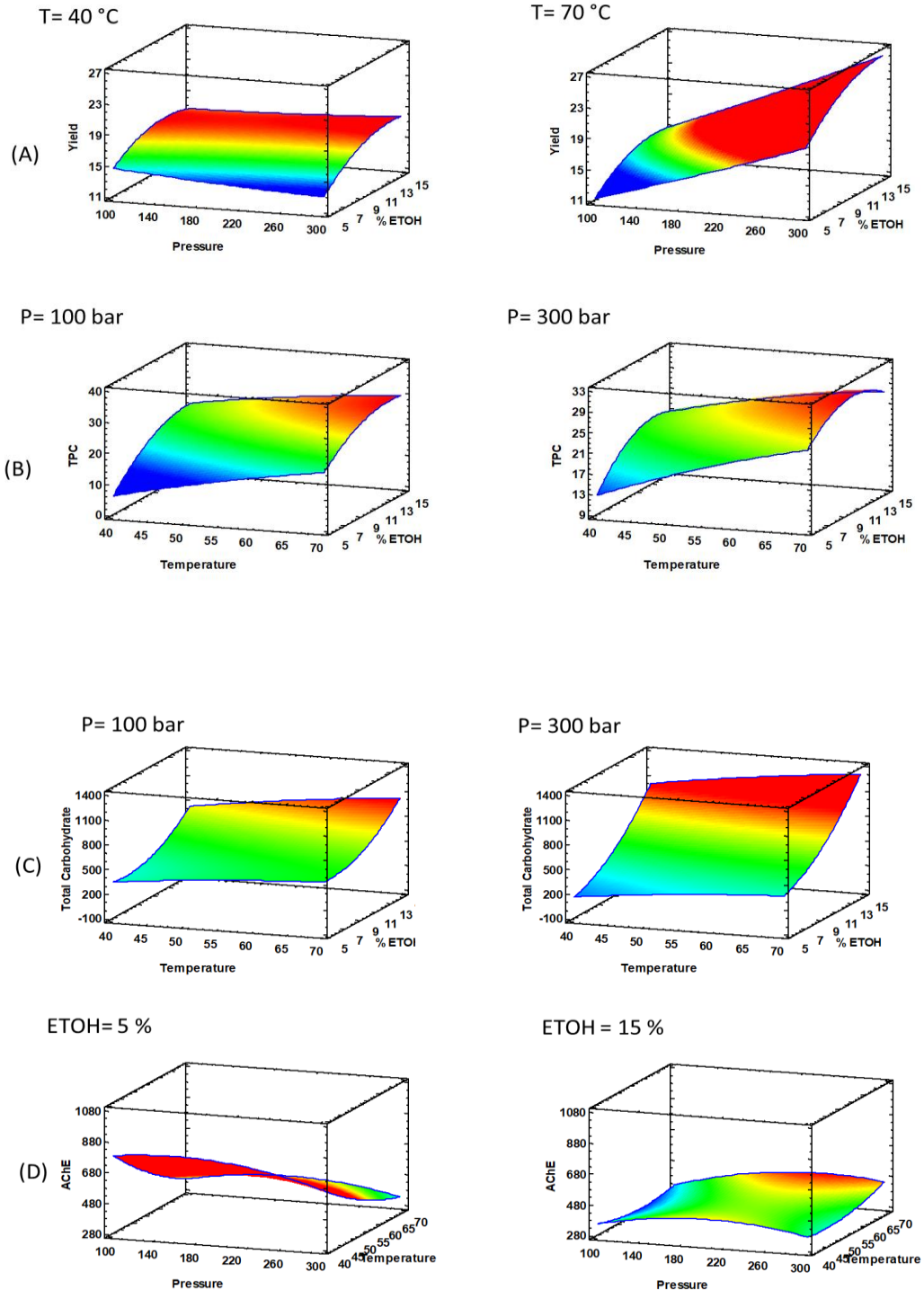

Figure 4.7: Estimated response surface plot for (A) extraction yield, (B) TPC, (C) TCC, (D) IC50 AChE for the optimization of Ammodaucus leucotrichus SFE extracts (Created by the author).

Table 4.4: ANOVA for response surface modeling showing linear, quadratic and interaction relations of each response and coefficients of regression for model prediction (Created by the author).

Response variables	Factor	Sum squares	Degree of freedom	F-value	*p*-value		Estimated regression Coefficients
Extraction yield (%)	X_1	43.6837	1	8.58	0.0327*	β_0	37.77
	X_2	12.0234	1	2.36	0.1850	β_1	-0.09
	X_3	38.0803	1	7.48	0.0411*	β_2	-0.88
	X_1X_1	0.113492	1	0.02	0.8872	β_3	1.15
	X_1X_2	25.4848	1	5.00	0.0755	$\beta_{1,1}$	1.75×10^{-5}
	X_1X_3	1.75271	1	0.34	0.5830	$\beta_{1,2}$	0.00
	X_2X_2	5.13787	1	1.01	0.3613	$\beta_{1,3}$	0.00
	X_2X_3	0.638241	1	0.13	0.7378	$\beta_{2,2}$	0.01
	X_3X_3	9.35513	1	1.84	0.2334	$\beta_{2,3}$	0.01
	Total error	25.4687	5			$\beta_{3,3}$	-0.06
	R^2 = 84.37 %						
	Adjusted R^2 = 56.23 %						
TPC (mg GAE/ g)	X_1	10.5699	1	1.82	0.2351	β_0	-51.16
	X_2	181.27	1	31.22	0.0025*	β_1	0.07
	X_3	186.757	1	32.17	0.0024*	β_2	0.92
	X_1X_1	0.411683	1	0.07	0.8006	β_3	5.86
	X_1X_2	0.131298	1	0.02	0.8863	$\beta_{1,1}$	-0.00
	X_1X_3	16.3592	1	2.82	0.1541	$\beta_{1,2}$	-0.00
	X_2X_2	2.60196	1	0.45	0.5329	$\beta_{1,3}$	-0.00
	X_2X_3	6.43865	1	1.11	0.3405	$\beta_{2,2}$	-0.00
	X_3X_3	57.3081	1	9.87	0.0256*	$\beta_{2,3}$	-0.02
	Total error	29.0287	5			$\beta_{3,3}$	-0.16
	R^2 = 94.06 %						
	Adjusted R^2 = 83.38 %						
TCC (mg/ g)	X_1	5941.32	1	0.18	0.6868	β_0	1280.69
	X_2	115539.	1	3.55	0.1181	β_1	-14.89
	X_3	945377.	1	29.08	0.0030*	β_2	15.76

	X_1X_1	374759.	1	11.53	0.0193*	β_3	-114.44
	X_1X_2	51.0796	1	0.00	0.9699	$\beta_{1,1}$	0.03
	X_1X_3	52562.7	1	1.62	0.2594	$\beta_{1,2}$	0.00
	X_2X_2	2102.5	1	0.06	0.8094	$\beta_{1,3}$	0.23
	X_2X_3	2665.71	1	0.08	0.7861	$\beta_{2,2}$	-0.11
	X_3X_3	80889.0	1	2.49	0.1755	$\beta_{2,3}$	0.34
	Total error	162521.	5			$\beta_{3,3}$	5.92
	R^2 = 90.60 %						
	Adjusted R^2 = 73.67 %						
AChE (IC_{50} mg/mL)	X_1	12.1796	1	0.30	0.6370	β_0	2482.86
	X_2	56601.0	1	1410.78	0.0007*	β_1	1.22
	X_3	52241.6	1	1302.12	0.0008*	β_2	-42.79
	X_1X_1	19860.0	1	495.01	0.0020*	β_3	-153.32
	X_1X_2	2964.53	1	73.89	0.0133*	$\beta_{1,1}$	-0.01
	X_1X_3	4877.21	1	121.56	0.0081*	$\beta_{1,2}$	0.02
	X_2X_2	6299.1	1	157.00	0.0063*	$\beta_{1,3}$	0.07
	X_2X_3	40134.7	1	1000.36	0.0010*	$\beta_{2,2}$	0.18
	X_3X_3	14267.8	1	355.62	0.0028*	$\beta_{2,3}$	1.34
	Lack-of-fit	44732.8	3	371.65	0.0027	$\beta_{3,3}$	2.49
	Total error	80.2408	2				
	R^2 = 81.72 %						
	Adjusted R^2 = 48.83 %						

* Significant.

In terms of TPC, the highest value (32.42 mg GAE/g extract) was observed at 10 % EtOH, 70 °C and 300 bar. It was mainly influenced by the effect of % EtOH (linear and quadratic) and temperature (linear and positive). This effect was also observed by Suárez-Montenegro et al. [90] during the extraction of tamarillo by PLE using ethanol or and water at different temperatures. The values obtained are lower than those reported by other authors for A. leucotrichus, such as Selama et al. Selama et al. [154], Hellal et al. [133] and Louail et al. [153], who reported TPC values as high as 83.07 mg GAE/g DW, 124.98 mg GAE/g extract and 146.18 ± 5.82 mg GAE/g DW for the aerial parts and seeds of hydroalcoholic extracts, respectively. Similar values of total phenolic content (ranging from 22.3 to 43.5 mg GAE/g extract) were obtained from the same batch using PLE. However, the model is able

to explain 94.06 % (R^2 = 0.94063) of the variability in TPC with respect to pressure, temperature and co-solvent.

As it can be clearly observed, the results of total carbohydrate content (TCC) covered a wide range of values, varying from 112.85 to 1257.98 mg glucose equivalents/g extract, which can be considered as high compared to a maximum of 650 mg/g extract reported in previous studies [17][154]. Glucose was used as a standard for calibration in this assay; other carbohydrates may be present in extracts that may respond differently to the phenol-sulfur quantification method, explaining why results are above 1000 glucose equivalents/g extract. Previous studies have used SFE-CO_2 to extract carbohydrates from different natural sources [290][291][292]. Ziani et al. [132] reported that carbohydrates were the predominate compounds in *A. leucotrichus* with a value of 65.0 ± 1.0 g/100 g dw, in agreement with the present results. The TCC variables were mainly influenced by the linear effect of ETOH % and the quadratic effect of pressure. Both temperature and pressure had a non-significant effect (linear and positive). The R^2 coefficient indicates that the fitted model explains 90.60 % of the variability of the results.

In contrast, AChE assay results were expressed as the effective dose of extract per milliliter (µg/mL) required to increase the inhibition concentration by 50 % (EC_{50}). Consequently, the higher the EC_{50} value, the less potent the inhibition potential, as a greater amount of extract is required to inhibit 50 % of AChE enzymatic activity. Therefore, optimizing this performance metric means minimizing its value. As shown in Figure (3.6), according to the Pareto analysis, temperature had the predominant influence on AChE inhibition, followed by the percentage of ethanol (negative, linear relationship), while pressure had no discernible effect. It is worth mentioning that all the examined samples showed a relatively low AChE inhibition capacity compared to galantamine. Based on the general categorization of the efficacy of natural extracts reported by Santos et al. [293], the AChE inhibition capacity (IC_{50} values) of AL extracts isolated by supercritical fluid extraction showed a moderate potency (200 < IC_{50} < 1000 µg/mL). Previous studies had investigated the anti-acetylcholinesterase activity of *A. leucotrichus* aerial part extracts without obtaining an IC_{50} value for the AChE activity [145][151]. In terms of AChE inhibition as a single response variable, no acceptable mathematical model could have been obtained (the lack of fit value was significant) to explain its variability in terms of P, T and co-solvent %. This lack of fit can be justified taking into consideration the different

quantitative data obtained in terms of phytochemicals presented on the different extracts and their synergy.

Table 4.5: Predicted and experimental values of the responses were obtained at the optimum conditions of the independent variables. The experimental data are given as the mean ± SD (n=3) (Created by the author).

	Pressure (bar)	Temperature (°C)	Co-solvent (%)	Yield (%)	TPC (mg GAE/g extract)	TCC (mg/g extract)	AChE IC_{50} (ug/mL)
Predicted values	300	69.9937	14.433	26.0812	28.1846	1235.07	432.649
Experimental values	300	70	15	19.8142 ± 0.3759	21.7070 ± 0.6840	1007.5573 ± 3.3562	416.932 ± 13.5982

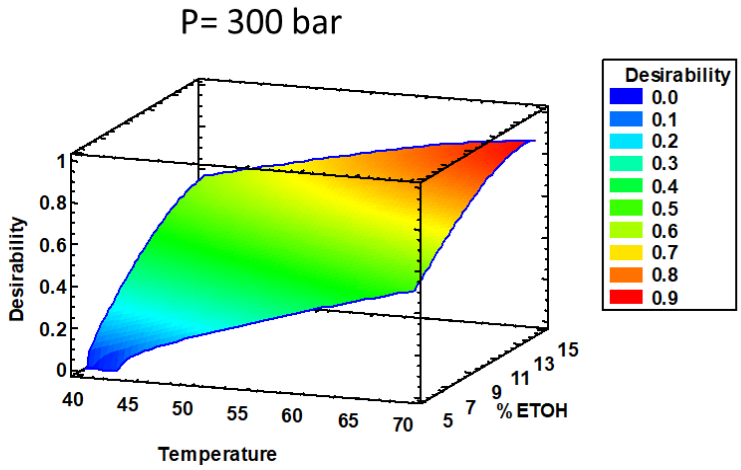

Figure 4.8: Estimated surface response obtained for the multiple response optimization for SFE extracts (Created by the author).

The Multiple Response Optimization (MRO) is a technique used in Response Surface Methodology (RSM) to optimize multiple response variables simultaneously. The MRO is a powerful tool for solving complex optimization problems with multiple objectives to be optimized [294]. In the RSM, the goal is to identify the optimal conditions for a process or system by manipulating one or more input parameters and measuring the corresponding

changes in output responses. The MRO extends this approach by considering multiple response variables, each with its own objective function. The goal is to find the combination of input parameters that maximizes or minimizes the overall response vector [295]. In this case, an MRO was performed to obtain maximum extraction yield with maximum TPC, TCC and minimum IC_{50} of AChE. As can be seen in Table (4.5), the experimental results for the extraction performed according to the optimal SFE conditions (300 bar, 70 °C and 15 % EtOH) were similar to those theoretically predicted by the model.

4.1.2.2. Sequential high-pressure extraction process from Ammodaucus leucotrichus

Process integration and process intensification are two related but distinct concepts in chemical engineering. Both terms refer to the optimization and improvement of chemical processes, but they differ in their focus and approach. Process integration refers to the holistic approach of combining multiple unit operations into a single, integrated process flow sheet. The goal of process integration is to create a seamless transition between individual units to ensure efficient use of resources, minimal waste generation, and improved economic performance. This approach requires a deep understanding of the entire process, including material balances, heat and mass transfer, and process control. Process intensification, on the other hand, is a more focused approach that seeks to improve specific aspects of a process, typically by increasing the intensity of one or more processes. Sequential extraction processes can be considered intensified processes if the extraction step is repeated several times in succession, with each cycle providing additional purification or concentration of the target compound. Each cycle uses the same feedstock and produces a smaller volume of product, resulting in higher productivity and lower costs compared to traditional batch processes.

The aim of this work consisted in the intensification of a green extraction process to obtain different fractions with the most interesting compounds from *A. leucotrichus* biomass. As shown in Figure (3.8), two sequential steps were applied: PLE-H_2O followed by SFE-CO_2, where the 2nd step was applied to the extract and residue of the 1st step. The conditions of the 1st step (10 min and 10.3 MPa at 180 °C) were selected within the PLE, with better yield, TPC, TCC and IC_{50} of AChE results. The 2nd step conditions (15 % EtOH and 300 bar at 70 °C) were selected within the SFE as described in the previous section.

Table 4.6: Yield, TPC, TCC and IC50 values (µg/ mL) for antioxidant (DPPH), anti-inflammatory (LOX) and inhibition of acetylcholinesterase (AChE) obtained from Ammodaucus leucotrichus seeds extracts using biorefinery extraction approach (Created by the author). Sample names are the same as Figure (3.8).

Sample	TPC (mg GAE/ g of extract)	TCC (mg/ g of extract)	IC_{50} (µg/mL)		
			AChE	**LOX**	**DPPH**
Opt SFE	21.7070 ± 0.6840	1007.5573 ± 3.3562	416.932 ± 13.5982	193.2636 ± 12.7687	90.5779 ± 6.3601
Extract 1[§]	48.2840 ± 0.9647	507.2236 ± 14.0744	55.598 ± 7.724	39.3733 ± 4.7834	58.5134 ± 4.7563
Extract 1.1	89.2673 ± 1.6861	263.4461 ± 1.0826	100.96 ± 12.6704	68.7954 ± 2.3125	34.8545 ± 1.3124
Residue 1.2	42.1158 ± 0.6840	516.3317 ± 4.9218	275.0329 ± 5.209	123.2816 ± 13.0539	130.1908 ± 3.4554
Extract 2.1	17.3189 ± 0.3286	10.1150 ± 0.7578	n.d	185.5946 ± 24.2020	103.0407 ± 12.3587
Positive control *	-	-	4.061 ± 0.310	14.2983 ± 1.7482	18.7144 ± 1.3013

Results are expressed as the means ± SD (n=3). n.d: not determined (maximum level of inhibition below 50%). *Chemical standards used as positive controls of each test: Galantamine for AChE, Quercetin for LOX and BHT for DPPH.

The total phenolic content (TPC) of the different extracts (Table 4.6) varied from 17 mg GAE/g (SFE extract from the residue of PLE) to 89 mg GAE/g (SFE extract from the extract after PLE). In terms of TCC, SFE using CO_2 and ethanol extract showed the highest total carbohydrate content compared to extracts from PLE and combined methods. This was in agreement with the study by T.Gong et al. which showed that the yield of carbohydrates extracted by SC-CO_2 was significantly higher than that extracted by other methods [292]. Nevertheless, the use of SFE as a second extraction step left a significant amount of carbohydrates in the 1.2 residue, while the 1.1 extract was enriched in phenolic compounds compared to the initial PLE extract.

In terms of bioactivity, the results of the AChE assay of *A. leucotrichus* seed extracts showed low AChE inhibitory capacity compared to galantamine, which was used as a reference. PLE extract showed the best anticholinesterase performance (IC_{50} of 55.59 µg/mL) compared to extracts from SFE and combined methods. This may be explained by

the synergistic effect in terms of AChE inhibition between those extracted by SFE and those not extracted. It was not possible to determine the IC_{50} of extract 2.1 because after sequential extraction the extract could not inhibit 50 % of acetylcholinesterase activity. Alike AChE results, single PLE extraction showed higher anti-inflammatory activity (LOX IC_{50} of 39.37 µg/ mL) compared to single SFE extraction and all the fractions obtained from the combination PLE-SFE. On the other hand, the lowest activity was found in SFE extract (IC_{50} of 193.26 µg/ mL), this result was found to be better than the one reported by Miguel et al., who use the aerial parts of the plant (IC_{50} of 621.26 µg/ mL) [151]. All the fractions were also tested for their antioxidant activities by DPPH assay. The highest inhibition of the DPPH radical was obtained from SFE extract 1.1 (34.85 µg/ mL), emphasizing the importance of separating the lipid fraction. Very similar inhibition capacity was reported by Hellal et al. for *A. leucotrichus* using ethanolic extracts [133].

The results in Table (4.6) suggest a significant synergistic effect of the compounds responsible for the bioactivity of *A. leucotrichus* extracts obtained with water (Extract 1) on acetylcholinesterase (AChE) and lipoxygenase (LOX) activities. While the enrichment of phenolic compounds found in supercritical fluid extraction (SFE) extract 1.1 resulted in an increase in antioxidant activity as measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. This finding highlights the potential of these extracts as a source of natural antioxidants with improved efficacy compared to individual compounds. However, it is important to note that further research is needed to fully understand the mechanisms underlying these synergistic effects and to identify the involved specific compounds.

4.1.2.3. Chemical characterization

a. Chemical analysis by GC-MS of SFE extracts

The relationship between the supercritical carbon dioxide (SC-CO_2) extraction conditions and the chemical composition of the extracts was investigated using gas chromatography-mass spectrometry (GC-MS) (Table (4.7)). A total of 12 compounds were detected and tentatively identified, mainly belonging to the terpenoid class. As an interesting result, the analysis revealed that perillaldehyde was the major compound present in the extracts, closely followed by β-caryophyllene. These results were consistent with previous reports on the chemical composition of *A. leucotrichus* essential oils collected from different

regions [150][296]. A comparison of the results with those from the literature, showed that five of the detected compounds including perillaldehyde, perillyl alcohol, β- caryophyllene, limonene, α-humulene had been previously identified in *A. leucotrichus* seeds and fruits extracts [143][150][146]. Probably, the observed high concentration of perillaldehyde could be attributed to the optimal extraction conditions used, such as temperature, pressure and solvent composition, which might have played a crucial role in the selective separation and isolation of this compound. The chromatogram of the optimum point illustrated in Figure (S.2). The solubility of the various compounds varied with the tested experimental conditions (temperature, pressure and percentage of co-solvent) in SC-CO_2 with ethanol as co-solvent, while, similar composition profiles were obtained but significantly different quantitatively.

Table 4.7: Tentatively identified compounds by GC-MS/MS analysis from SFE extracts of Ammodaucus leucotrichus seeds (Created by the author).

#	Rt (Min)	FAMILY	Tentative identification	FORMULA	MATCH FACTOR (%)	MONOISOTOPIC MASS	MAIN FRAGMENTS (M/Z)	SFE1	SFE2	SFE3	SFE4	SFE5	SFE6	SFE7	SFE8	SFE9	SFE10	SFE11	SFE12	SFE (optimum point)	SFE (central point)	ref
1	7.11		Benzene, nitroso- (cas) nitrosobenzene	C6H5NO	77	107	77, 51, 50	-	-	-	671900	-	-	-	-	20614	-	-	-	13040	55490	
2	14.87	Monoterpenes	Perillaldehyde	C10H14O	88	150	135, 122, 107, 79, 77, 53	11376805	10905882	9478017	28469688	14627507	17218247	10466812	11354628	21182100	37955157	35964133	26048167	62801633	31033108	[150]
3	15.68	Monoterpenes	Perilla alcohol	C10H16O	82	152	119, 93, 91, 79, 77, 67, 55	151932	122425	154056	61118	274306	300036	208748	148994	299734	559004	494416	599410	607649	503428	[145]

4	17.75	Sesquiterpenes	Beta-caryophyllene	C15H24	80	204	119, 107, 105, 91, 79, 55	1690812	1703674	4889985	11088894	1249130	3174388	3497008	1684852	816000	5978982	7901800	1114679	25189066	3536858	[139]
5	18.71	Sesquiterpenes	4-(1,5-dimethyl-4-hexenyl)-3-cyclohexen-1-one	C14H22O	81	206	79, 67, 65, 55, 53	-	-	-	-	-	-	-	-	-	154515	131660	103236	396996	99783	
6	18.89	Monoterpenes	Limonene	C10H16	78	136	121, 93, 91, 79, 68, 53	441522	322614	4492276	27423	464436	624275	411962	431108	629746	1056754	1123710	1122700	1680473	1075601	[146]
7	19.09	Aldehydes	5-methyl furfural	C6H6O2	79	110	53, 51	21691	-	-	-	12643	38021	-	-	47578	62052	28330	42344	99599	85980	[297]
8	21.22	Lactones	Gamma-DODECALACTONE	C12H22O2	91	189	85, 69, 57, 55	23521	-	-	-	38928	45782	43416	41193	88200	85034	123633	125878	123586	119680	[298]

9	21.75	Sesquiterpenes	Alpha-humulene	C15H24	83	204	121, 117, 107, 105, 93, 91, 79, 77, 67	173044	268924	6329 64	12 5505	151151	709920	867745	328715	162220	1670888	2179546	3331843	9523613	1245110	[141]
10	28.04	Alcohol	3-butyn-2-ol (cas) 1-butyn-3-ol	C4H6O	80	70	55, 53, 51	50392	50460	7538 1	-	102973	99059	126599	67380	97930	152838	241528	226254	391393	312768	
11	47.64	Fatty acid	2-oxobutanoic acid	C4H6O3	100	102	57	-	-	-	-	-	24075	-	-	-	-	19840	41239	42768	21823	
12	48.31		Unknown				55	-	-	-	-	-	40404	-	-	-	-	-	-	-	68214	

GC-MS analysis of sequential extraction fractions

PLE extracts before and after SFE treatment (1.1, 1.2, and 2.1) were analyzed by GC-MS/MS. The library search was carried out using NIST and Wiley GC–MS libraries, as well as comparisons of the fragmentation pattern of the mass spectra with data available in the literature. A total of 32 compounds were detected, 24 of which were tentatively identified. The results of this study are summarized in Table (4.8). Where it can be observed that perillaldehyde and 2-oxobutanoic acid were found in all fractions. Extract 1.1 was the fraction with a greater variety of compounds. In addition, the extracts obtained by sequential extraction had a chemical profile very different compared to those from the SFE. On the other hand, pivalyl chloride, 2-furoic acid, tuberolactone, 2-hydroxypyridine, α-terpineol, 2-methyl-3-isobutenyl-4-penten-2-ol, 1-germa-2-silabutane and isopentyl acetate were found only in the extract 1.1 fraction obtained by combination of between PLE-SFE. Previous research by LI et al. had shown that pivalyl chloride was a molecule obtained by enzymatic hydrolysis of rhodioloside D, a monoterpene glycoside isolated from the roots of Rhodiola rosea [299]. As far as it is known, the compounds 4,6-heptadiyn-3-one, diphenyl ether, pivalyl chloride, 2-furoic acid, tuberolactone, 2-hydroxypyridine, 2,3-diamino-2-cyanosuccionitrile, 5-methylfurfural, 2-methyl-3-isobutenyl-4-penten-2-ol, 2-oxobutanoic acid, 1-germa-2-silabutane, 2-nonen-1-ol, isopentyl acetate, and 4-(1,5-dimethyl-4-hexenyl)-3-cyclohexen-1-one were reported for the first time in *A. leucotrichus* extracts. The presence of specific compounds can be attributed to reactions during the extraction procedures. Due to the considerable amount of carbohydrates, a caramelization reaction may occur in the PLE, giving rise to new molecules. Moreover, after the SFE, additional molecules may be enriched due to the fractional separation of the above-mentioned reaction by-product.

Table 4.8: Tentatively identified compounds by GC -MS/MS analysis from extracts of Ammodaucus leucotrichus seeds obtained using biorefinery approach and SFE extract under optimum conditions (Created by the author).

Peak	Rt (Min)	Family	Name	Formula	Match factor (%)	Monoisotopic mass	Main fragments (m/z)	Extract 1 (PLE)	Extract 1.1	Extract 2.1	Residual 1.2	Ref
1	7.05		4,6-Heptadiyn-3-one	C7H6O	83	106	78, 77, 50	-	-	50779	-	[300]
2	7.1	Phenols	Diphenyl ether	C12H10O	73	170	78, 77, 51	30183	192101	46548	139165	
3	7.2		Unknown				77	20208	29152	-	-	
4	7.26	Carboxylic acid	Pivalyl chloride	C5H9ClO	76	120	77, 57, 51	-	48098	-	-	[299]
5	7.79	Carboxylic acid	2-Furoic acid	C5H4O3	97	112	95	-	57888	-	-	
6	8.41	Lactones	Tuberolactone	C10H14O2	100	166	97	-	18092	-	-	
7	8.99	Pyridines	2-hydroxypyridine	C5H5NO	91	95	95, 67, 55, 51	-	131260 4	-	-	
8	10.76		Unknown	-	-	-	55	-	12364	-	-	
9	10.94		Unknown	-	-	-	267, 73	-	83458	-	-	
10	11.31		Unknown	-	-	-	139, 111, 53	-	60005	-	-	

11	12.05	Monoterpenes	.Alpha.-terpineol	C10H18O	82	154	121, 93, 77, 67, 59	-	441505	-	-	[141][301]
12	13.44		2,3-Diamino-2-cyanosuccionitrile	C5H5N5	81	135	83, 55, 53	-	-	291436	-	
13	14.86	Monoterpenes	Perillaldehyde	C10H14O	88	150	135, 122, 107, 79, 67, 53	15974757	45963362	1319246	86235	[150]
14	15.18	Organic compounds	Benzene, 1,1'-oxybis- (CAS) Phenyl ether	C12H10O	76	170	91, 77, 51	39718	273826	-	-	
15	15.66	Monoterpenes	Perilla alcohol	C6H16O	82	152	119, 105, 91, 79, 67, 53	15801	1132934	-	-	[139]
16	16.78		Unknown	-	-	-	73	-	464182	-	-	
17	17.24		Unknown	-	-	-	67, 53	-	21921	-	-	
18	17.74	Sesquiterpenes	B-Caryophyllene	C15H24	82	204	119, 105, 91, 79, 67, 53	-	31653862	-	-	[302]
19	18.71	Sesquiterpenes	4-(1,5-Dimethyl-4-hexenyl)-3-cyclohexen-1-one	C14H22O	81	206	91, 79, 77, 68, 67, 53, 51	-	165303	-	-	
20	18.89	Monoterpenes	Limonene	C10H16	78	136	121, 93, 91, 79, 68, 53	-	954586	200246	-	[150]
21	19.09	Aldehydes	5-methyl furfural	C6H6O2	73	110	91, 69, 67, 55, 53	-	71138	-	-	[297]
22	19.42		Unknown	-	-	-	79, 53, 51	-	19356	-	-	

23	20.07		2-methyl-3-isobutenyl-4-penten-2-ol	C10H18O	71	154	93, 91, 81, 79, 67, 59, 53, 51	-	373453	-	-	
24	20.69	Monoterpenes	Myrcenol	C10H18O	81	154	121, 107, 79, 77, 59, 53, 51	482672	3509200	-	42872	[150]
25	21.19	Lactones	Gamma-dodecalactone	C12H22O2	81	198	85, 69, 57, 56, 55	-	450070	-	-	[298]
26	21.74	Sesquiterpenes	Alpha-humulene	C15H24	82	204	121, 107, 105, 93, 91	-	14509215	-	-	[141]
27	22.38		Unknown	-	-	-	281, 73	-	155760	-	-	
28	23.11	Hydrocarbon	Benzene, (1-ethylpropyl)	C11H16	70	148	91, 55,50	-	895765	108366	-	
29	24.45	Alcanes	1-germa-2-silabutane	C2H10GE SI	100	136	59	-	18751	-	-	[303]
30	27.47	Alcohols	Isopentyl acetate	C7H14O2	100	130	73	-	59379	-	-	
31	46.85	Alcohols	2-Nonen-1-ol	C9H18O	97	142	57, 55	-	-	242552	-	[304]
32	47.62	Fatty acids	2-oxobutanoic acid	C4H6O3	100	102	57, 55, 53, 51	180323	160518	66563	21193	

b. Untargeted screening analysis by UHPLC-q-TOF-MS

For further identification of the secondary metabolites present in the extracts, the UHPLC-q-TOF-MS/MS technique was used. Hence, the tentative identification of compounds was carried out through MS1 and MS2 data with the spectral reference library available on GNPS. For this, the MS/MS data obtained from UHPLC-q-TOF-MS were converted and uploaded to the GNPS platform [270]. Considering the challenges involved in the putative identification of compounds present in natural products, GNPS facilitates the identification of molecular structures by annotating spectra of sets of MS/MS data using sophisticated algorithms that cross-reference the uploaded data with its repository [305]. Moreover, as a collaborative tool containing different libraries, GNPS permits more accurate, up-to-date, and cross-referenced identification, increasing confidence in the results. On the other hand, chemical information about compounds already identified in *A. leucotrichus* were considered for checking potential misleading annotations, reinforcing the validation of the tentative identification of each metabolite. By applying such approach, a total of 41 phytochemicals were detected and tentatively identified at the optimal point of SFE extract (s1) and the different fractions of biorefinery extracts from *A. leucotrichus* seeds. The retention time (min), tentatively identified compounds, exact mass, molecular formula, error (Δppm) and main MS/MS fragments are shown in Table (4.9). In general, the fraction obtained using only SFE under the optimal conditions was the most concentrated in bioactive compounds compared to the fraction obtained by the combined methods. Probably the high value found in the total carbohydrate analysis was related to the high amount of many compounds with sugar moieties as can be seen in Table (4.9). Among these compounds with carbohydrates moieties eight of them worth mentioning: 5'-deoxy-5'-(methylsulfinyl) adenosine, adenosine, 2'-deoxyadenosine, guanosine, N-(1-deoxy-1-fructosyl) phenylalanine, scopoletin 7-O-glucoside, icariside F2, and sayaendoside; five flavonoids including luteolin 4'-O-glucoside, luteolin, luteolin 7-O-glucoside, luteolin 3'-methyl ether, and apigenin 4'-glucoside. and rosiridin, (3S,3AS,6E,9S,10E,11aS)-3,6,10-trimethyl-2-oxo-2,3,3a,4,5,8,9,11a-octahydrocyclodeca[b]furan-9-yl-beta-d-glucopyranoside, (2R, 3R,4S,5S,6R)-2-[(2E)-4-ethenyl-2,5-dimethylhexa-2,5-dienoxy]-6-(hydroxymethyl) oxane-3,4,5-triol, (2R,5S,9R,12R)-13,13-dimethyl-7-oxatetracyclo[7. 5.0.01,5.02,12] tetradecane-4,8-dione, (2R,3R,4S,5S,6R)-2-[(2E)-4-ethenyl-2,5-dimethylhexa-2,5-dienoxy]-6-(hydroxymethyl) oxane-3,4,5-triol, austinoneol and globulol as terpenoids and 6-methoxy-7-hydroxycoumarin as coumarins were detected. Compounds such as scopoletin 7-O-

glucoside, caffeic acid, apigenin 4'-glucoside, aloe-emodin and pheophorbide A were identified only in the SFE extract.

On the other hand, there were also compounds like: N-(1-deoxy-1-fructosyl)phenylalanine, (+)-Cycloolivil glucoside, 2-beta-D-Glucopyranosyloxy-4-methoxy-cis-cinnamic acid, Sayaendoside, 2,3-Divanillyl-1,4-butanediol, Apigenin-4'-glucoside, (3S,3AS,6E,9S,10E,11aS)-3,6,10-trimethyl-2-oxo-2,3,3a,4,5,8,9,11a-octahydrocyclodeca[b] furan-9-yl beta-d-glucopyranoside, N-cyclohexyl-2-benzothiazol-amine, Lauryldiethanolamine, Austinoneol and Pheophytin A that had been tentatively identified in *A. leucotrichus* for the first time in this work. Nevertheless, their identity should be confirmed by other methods.

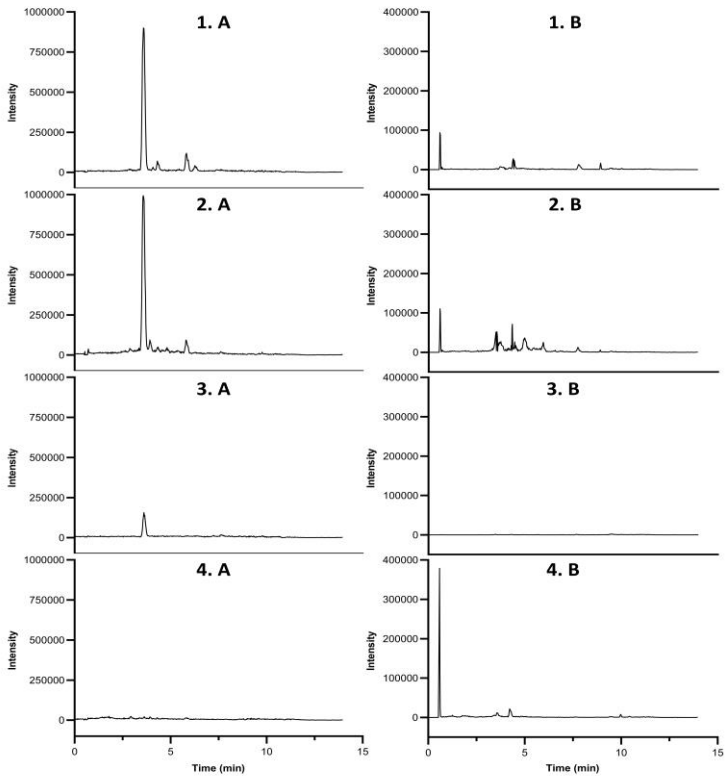

Figure 4.9: UHPLC-ESI-qTOF Chromatograms (Total Ionic Current, TIC) obtained using biorefinery approach of Ammodaucus leucotrichus seeds. Left side positive polarity, right side negative polarity (Created by the author).

Table 4.9: Tentatively identified compounds by LC-q-TOF-MS/MS analysis from extracts of Ammodaucus leucotrichus seeds obtained using biorefinery approach (Created by the author).

#	Rt (min)	Tentative identification	Molecular formula	Molecular ion	Measured mass (Δppm)	MS/MS product ions (m/z)	SFE (optimum point)	Extract 1 .1	Extract 2.1	Residual 1.2	Chemical Subclass	Ref
1	1.28	5'-Deoxy-5'-(methylsulfinyl) adenosine	C11H15N5O4S	[M+H]+	314.0900 (-5.66)	136.06 (100), 97.03 (68)	X	X	-	X	carbohydrates and carbohydrate derivatives	
2	1.31	Adenosine	$C_{10}H_{13}N_5O_4$	$[M+H]^+$	268.1030 (-4.02)	136.06 (100), 119.03 (63)	-	X	-	X	carbohydrates and carbohydrate derivatives	
3	1.51	2'-Deoxyadenosine	$C_{10}H_{13}N_5O_3$	$[M+H]^+$	252.1090 (-0.3)	136.06 (100)	-	X	-	X	carbohydrates and carbohydrate derivatives	
4	2.05	Guanosine	$C_{10}H_{13}N_5O_5$	$[M+H]^+$	284.1000 (3.6)	152.06 (100), 135.03 (73)110.02 (50)	X	X	-	X	carbohydrates and carbohydrate derivatives	
5	2.60	N-(1-deoxy-1-fructosyl)phenylalanine	$C_{15}H_{21}NO_7$	$[M+H]^+$	328.1400 (2.81)	120.08 (100), 132.08 (66)	X	X	-	X	carbohydrates and carbohydrate derivatives	

6	2.89	L-Tryptophan	$C_{11}H_{12}N_2O_2$	$[M+H]^+$	205.0970 (-0.86)	146.06 (100), 77.04 (75), 160.08 (69)	-	-	-	X	ShikimatesSmall peptides and Phenylpropanoids	[280]
7	4.05	2-[4-[4-[hydroxy-(4-hydroxy-3-methoxyphenyl)methyl]-3-(hydroxymethyl)oxolan-2-yl]-2-methoxyphenoxy]-6-(hydroxymethyl)oxane-3,4,5-triol	$C_{26}H_{34}O_{12}$	$[M+NH_4]^+$	556.2380 (-1.47)	137.06 (100), 157.06 (78), 187.07 (70)	X	X	-	-	Lignans	
8	4.07	(+)-Cycloolivil glucoside	$C_{26}H_{34}O_{12}$	$[M+H]^+$	538.205 (-3.32)	341.13 (100), 311.12 (86), 189.09 (69)	X	X	-	X	Lignans	
9	4.11	Scopoletin 7-O-Glucoside	$C_{16}H_{18}O_9$	$[M+H]^+$	354.0951 (-3.8)	193.05 (100), 133.02 (51), 178.02 (42)	X	-	-	-	Carbohydrates	
10	4.23	Caffeic acid	$C_9H_8O_4$	$[M-H]^-$	180.0423 (-2.13)	135.05 (100)	X	-	-	-	Carboxylic acids	
11	4.35	2-beta-D-Glucopyranosyloxy-4-methoxy-cis-cinnamic acid	$C_{16}H_{20}O_9$	$[M-H]^-$	356.1107	134.04 (100),	X	X	-	X	Phenylpropanoids (C6-C3)	

					(-0.44)	178.03 (32)						
				$[M+H]^+$	356.1107 (-1.45)	177.06 (100), 195.06 (32)	X	X	-	X	Phenylpropanoids (C6-C3)	
12	4.37	Icariside F2	$C_{18}H_{26}O_{10}$	$[M+H]^+$	402.1526 (-3.37)	91.05 (100), 85.02 (91), 115.04 (86)	X	X	-	X	carbohydrates and carbohydrate derivatives	
13	5.12	Sayaendoside	$C_{19}H_{28}O_{10}$	$[M+NH_4]^+$	434.2010 (-2.35)	115.04 (100), 133.05 (72), 145.05 (45)	X	-	-	X	carbohydrates and carbohydrate derivatives	
14	5.46	6-Methoxy-7-hydroxycoumarin	$C_{10}H_8O_4$	$[M+H]^+$	192.0423 (-2.77)	94.04 (100), 133.03 (93), 81.03 (84)	X	X	-	-	Coumarins	
15	5.50	Rosiridin	$C_{16}H_{28}O_7$	$[M+NH4]^+$	332.1835 (-3.78)	107.08 (100), 135.11 (100),	X	X	-	X	Terpenoids	

						93.07 (92)						
16	5.78	2-(Hydroxymethyl)-6-[4-[(2S,3S)-3-(hydroxymethyl)-5-[(E)-3-hydroxyprop-1-enyl]-7-methoxy-2,3-dihydro-1-benzofuran-2-yl]-2-methoxyphenoxy]oxane-3,4,5-triol	$C_{26}H_{32}O_{11}$	$[M+NH_4]^+$	538.2270 (-2.45)	137.06 (100), 311.12 (73), 175.07 (39)	X	X	-	-	Lignans	
17	5.97	Luteolin 4'-O-glucoside	$C_{21}H_{20}O_{11}$	$[M+H]^+$	448.1006 (-4.11)	287.05 (100), 153.01 (5)	X	X	-	X	Flavonoids	
18	6.01	Luteolin	$C_{15}H_{10}O_6$	$[M+H]^+$	286.0477 (-3.57)	153.01 (100), 69.00 (44), 89.04 (32)	X	X	X	X	Flavonoids	[154]
19	6.04	Luteolin-7-O-glucoside	$C_{21}H_{20}O_{11}$	$[M-H]^-$	448.1006 (-3.1)	285.05 (100)	X	X	-	-	Flavonoids	[132]
20	6.18	2,3-Divanillyl-1,4-butanediol	$C_{20}H_{26}O_6$	$[M-H_20+H]^+$	345.1690 (-3.49)	137.06 (100), 122.03 (25), 163.07 (14)	X	X	-	-	Lignans	
21	6.57	Apigenin-4'-glucoside	$C_{21}H_{20}O_{10}$	$[M+H]^+$	432.1056	271.06 (100),	X	-	-	-	Flavonoids	

					(-4.46)	272.06 (31)						
22	7.23	(3S,3AS,6E,9S,10E,11aS)-3,6,10-trimethyl-2-oxo-2,3,3a,4,5,8,9,11a-octahydrocyclodeca[b]furan-9-yl beta-d-glucopyranoside	$C_{21}H_{32}O_8$	$[M+NH_4]^+$	430.2410 (-5.86)	159.12 (100), 233.15 (50), 131.09 (38)	X	X	-	-	Terpenoids	
23	7.81	(2R,3R,4S,5S,6R)-2-[(2E)-4-ethenyl-2,5-dimethylhexa-2,5-dienoxy]-6-(hydroxymethyl)oxane-3,4,5-triol	$C_{16}H_{26}O_6$	$[M+H]^+$	314.1729 (0.73)	93.07 (100), 107.08 (93)	X	-	-	-	Terpenoids	
24	8.40	Aloe-emodin	$C_{15}H_{10}O_5$	$[M+H]^+$	271.0600 (-0.28)	69.01 (100), 153.02 (95), 119.05 (75)	X	-	-	-	Polyketides Polycyclic aromatic polyketides	[306]
25	8.41	N-cyclohexyl-2-benzothiazol-amine	$C_{13}H_{16}N_2S$	$[M+H]^+$	233.1110 (1.39)	151.03 (100), 55.05 (48)	X	X	X	-	Alkaloids	
26	8.52	Luteolin 3'-methyl ether	$C_{16}H_{12}O_6$	$[M+H]^+$	300.0634 (-2.17)	258.05 (100), 229.04 (31), 153.01 (20)	X	X	-	-	Flavonoids	[154]

27	8.84	Lauryldiethanolamine	$C_{16}H_{35}NO_2$	$[M+H]^+$	274.2740 (-0.28)	43.05 (100), 70.06 (78), 57.07 (78), 88.07 (67)	X	X	X	X	Fatty acids	
28	8.92	(2R,5S,9R,12R)-13,13-dimethyl-7-oxatetracyclo[$7.5.0.0^{1,5}.0^{2,12}$]tetradecane-4,8-dione	$C_{15}H_{20}O_3$	$[M+H]^+$	249.1480 (-1.91)	145.10 (100), 203.14 (52)	X	X	-	X	Terpenoids Sesquiterpenoids	
29	9.14	Tetradecyldiethanolamine	$C_{18}H_{39}NO_2$	$[M+H]^+$	302.3050 (-1.25)	57.07 (100), 70.06 (99), 284.29 (55)	-	-	-	X	Fatty acids	
30	9.20	Austinoneol	$C_{24}H_{30}O_6$	$[M+H]^+$	415.2100 (-3.56)	119.08 (100)	-	-	-	X	Terpenoids Meroterpenoids	
31	9.35	Globulol	$C_{15}H_{26}O$	$[M-H_20+H]^+$	205.1940 (-8.3)	55.05 (100), 107.08 (89), 93.07 (91), 149.13 (65)	X	-	X	-	Terpenoids Sesquiterpenoids	[145]

32	10.19	1-(9Z-Octadecenoyl)-sn-glycero-3-phosphocholine	$C_{26}H_{53}NO_7P$	$[M+H]^+$	522.3540 (-2.64)	104.11 (100), 184.07 (43)	X	X	X	X	Fatty acids	[307](55)(56)(55)(55)
33	10.22	Linoleoyl ethanolamide	$C_{20}H_{37}NO_2$	$[M+H]^+$	324.2890 (-2.09)	62.06 (100)	X	X	X	-	Fatty acids	
34	10.34	16-Hydroxypalmitic acid	$C_{16}H_{32}O_3$	$[M-H]^-$	271.2278 (-0.09)	225.22 (100)	X	-	X	-	Fatty acids	
35	10.55	Palmitoyl ethanolamide	$C_{18}H_{37}NO_2$	$[M+H]^+$	300.2900 (1.08)	62.06 (100)	-	X	X	-	Fatty acids	[308]
36	10.63	Hydroquinidine	$C_{20}H_{26}N_2O_2$	$[M-H]^-$	325.1947 (7.92)	183.01 (100), 197.03 (41)	X	X	X	-	Alkaloids	
37	10.72	N-Oleoylethanolamine	$C_{20}H_{39}NO_2$	$[M+H]^+$	325.2981 (-1.12)	62.06 (100), 44.05 (54), 55.05 (23)	X	X	X	-	Amino alcohols	
38	10.72	6-decanolide	C10H18O2	[M+H]+	171.1380 (0.14)	57.06 (100), 73.03 (36)	X	X	X	X	Fatty acids	
39	10.78	9-Octadecenamide, (Z)-	C18H35NO	[M+H]+	282.2780 (-4.17)	55.05 (100), 43.05	X	X	X	X	Fatty acids	

						(73), 57.07 (76)						
40	11.04	Pheophorbide A	$C_{35}H_{36}N_4O_5$	$[M+H]^+$	592.2686 (-3.13)	533.25 (100), 460.22 (33), 447.21 (26)	X	-	-	-	Chlorophyl	
41	12.87	Pheophytin A	$C_{55}H_{74}N_4O_5$	$[M+H]^+$	870.5659 (-3.66)	593.27 (100), 533.25 (77)	-	X	-	-	Chlorophyll	[309]

4.2. Gas-Expanded liquid extraction of the Silymarin compounds from Silybum marianum seeds

For the first time a green, efficient and sustainable Gas Expanded Liquid (GXL) extraction method was used to isolate the silymarin complex from the seeds of *S. marianum* with the aim of replacing conventional methods and avoiding the defatting step. For this reason, an extraction kinetics study was carried out for a total time of 160 min, with samples taken every 20 min, to investigate whether it would be possible to carry out a fractionation throughout the extraction time to improve the specific recovery of the different flavonolignans. Thus, collected extracts were analysed by UHPLC-ESI-MS, in order to quantify the silymarin mixture. Extraction conditions were chosen considering previously published works. Temperature was fixed at 40 °C, and showed to be an optimum temperature for the extraction of oil and flavonolignans [179] [180]. While pressure was set at 90 bar, previous works [310] [256] [257] showed that pressure was not a significant factor when working in the zone of compressible fluid and it could be mild, thus, to minimize operational costs. Regarding co- solvent, several researchers reported that the utilization of the water–ethanol mixtures, in the supercritical fluid extraction as a co-solvent had been established to be more efficient for the extraction of phenolic compounds than the corresponding single solvent system [311] [312] [51]. The solubility of water in SC-CO_2 is very low, while the solubility of ethanol is very high in SC-CO_2 [313]. According to the classification of GXL's which reported by Jessop and Subramaniam, water is classified as class I, since it has not dissolved compressed liquid CO_2, whereas ethanol is class II solvents, because it can dissolve the compressed CO_2 [60]. Therefore, this work was carried out using two phases GXL [58] according to equilibrium depicted by Fornari et al. [314] by means of the Group Contribution Equation of State (GC-EoS). Using the same ternary mixture as the GXL proposed in the present work but working in a phase equilibria equipment with a glass window, Fornari et al. found that the mixture could be divided in two different phases: one supercritical formed mainly by CO_2 and ethanol and a liquid phase formed by water and ethanol. Two-phases GXL have different transport properties such as viscosity, density or self-diffusion coefficients as described by Cunico & Turner 2017 [59].

Under these conditions, the effect of solvent composition on silymarin extraction yield from *S. marianum* seeds was evaluated (Figure (4.10). The obtained extracts were also characterized in terms of extraction yield and total phenolic.

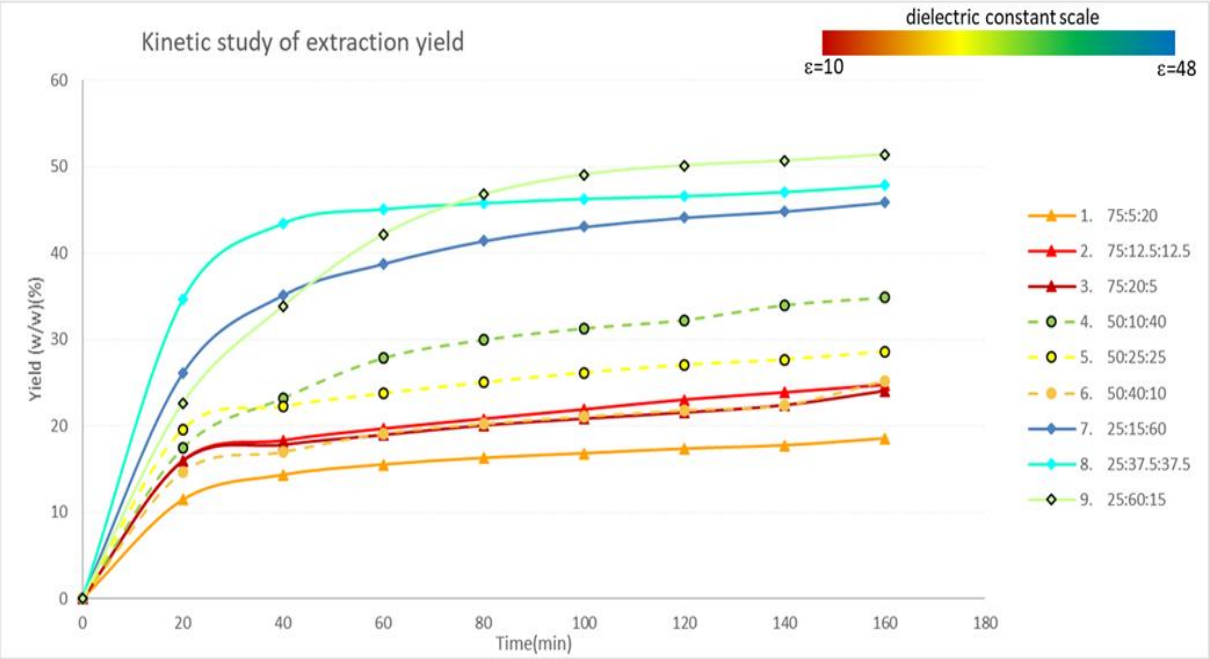

Figure 4.10: Kinetic study of extraction yield obtained from Silybum marianum seeds with different solvent composition (CO2: EtOH: H2O) mixtures at 40 °C and 90 bar. Δ =75 % CO2, ○ =50 % CO2 and ◇= 25 % CO2. Colour of each line correspond to their colour in the shown dielectric constant scale (Created by the author).

As can be seen in Figure (4.10), total yield did not increase remarkably after 80 min extraction time. Maximum yields were achieved in the more polar (higher dielectric constant) extraction conditions experiments (#7, 8 and 9) that was the lower CO_2 ones. Nevertheless, among them #9 (25:60:15), provided the maximum extraction yield with a value of 51.39 % (g extract /100 g seeds). Also, no significant differences were found between the different tests with the percentage of CO_2 at 25 %, 50 % and 75 %, Table (4.10). Besides, experimental data showed that increasing the amount of water in the composition of solvent produced a negative effect on the total extract recovery, when the percentage of SC-CO_2 was constant. All the GXL experiments provided higher extraction yield than the PLE carried out at 75 °C (experiment 10), whose yield was 8.71 % ± 0.38. Meanwhile the PLE carried out at 175 °C (experiment 11) provided a yield 22.71 % ± 0.39, close to the less polar GXL conditions. Pressure and temperature have also an effect on the ionic product and the density according to Kritzer et al. [315]. Polarity is not the only factor and besides it, the ionic strength, pH and viscosity are also important factors in mass transfer. Moreover, the

extracted compounds are also a factor to take into account. The extract obtained in the less polar GXL condition was composed by silymarin in more than 50 % while 5 % only for the water extract. In this sense, to date there are no studies of solubility of flavonolignans in ternary mixtures CO_2: EtOH: H_2O that can explain this effect.

Table 4.10: Kinetic study of extraction yield obtained from S. marianum seeds with different solvent composition (CO2: EtOH: H2O) mixtures at 40 °C and 90 bar. Results expressed as Yield % ± SD (Created by the author).

Time (min)	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Exp 6	Exp 7	Exp 8	Exp 9
	75:5:20	75:12.5:12.5	75:20:5	50:10:40	50:25:25	50:40:10	25:15:60	25:37.5:37.5	25:60:15
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20	26.09 ± 1.56	34.61 ± 1.10	22.65 ± 2.31	17.42 ± 2.03	19.59 ± 3.26	14.67 ± 1.34	11.44 ± 1.03	15.97 ± 0.50	15.94 ± 1.31
40	35.13 ± 0.86	43.43 ± 0.64	33.87 ± 1.23	23.21 ± 1.62	22.26 ± 3.02	16.96 ± 1.40	14.31 ± 0.92	18.30 ± 0.51	17.83 ± 1.39
60	38.76 ± 1.75	45.06 ± 0.78	42.16 ± 1.45	27.85 ± 0.00	23.76 ± 2.93	19.11 ± 1.86	15.51 ± 0.50	19.65 ± 0.68	18.97 ± 1.26
80	41.40 ± 1.47	45.76 ± 0.73	46.77 ± 2.19	29.96 ± 1.42	25.03 ± 2.86	20.22 ± 1.76	16.27 ± 0.35	20.79 ± 1.21	20.07 ± 1.45
100	43.03 ± 0.55	46.26 ± 0.59	49.08 ± 3.14	31.28 ± 2.36	26.16 ± 2.82	21.05 ± 1.78	16.80 ± 0.31	21.88 ± 1.94	20.85 ± 1.56
120	44.10 ± 0.10	46.58 ± 0.57	50.13 ± 3.77	32.24 ± 3.04	27.06 ± 2.79	21.78 ± 1.79	17.33 ± 0.37	23.01 ± 2.18	21.56 ± 1.55
140	44.82 ± 0.53	47.05 ± 0.89	50.68 ± 3.87	33.98 ± 4.69	27.68 ± 2.76	22.38 ± 1.77	17.72 ± 0.39	23.84 ± 2.42	22.41 ± 1.64
160	45.85 ± 0.42	47.81 ± 0.89	51.39 ± 4.24	34.83 ± 4.75	28.61 ± 3.09	25.20 ± 3.38	18.52 ± 0.67	24.72 ± 2.52	24.06 ± 2.75

With regard to the experiment conducted using the traditional extraction technique with one stage for lipid removal and another for extraction (Experiment 12), the final yield of the extract was 10.18 % ± 1.65.

It is important to emphasize that the present investigation yielded higher extraction efficiencies than previously published studies, such as Çelik [180], who used pure supercritical CO_2 at a higher pressure than in the present study and achieved a yield of 32.7 %, and Ben Rahal [179], who used ethanol as a co-solvent and culminated in a yield of 31.83

%. In addition, the extraction rates of the various compounds within the silymarin mixture were examined to elucidate the overall kinetic behaviour observed.

4.2.1. Kinetic study and chemical composition

The composition of the silymarin complex was analyzed by HPLC-ESI-MS/MS (triple quadrupole). Figure (4.11) shows the chromatogram with the different m/z transitions studied.

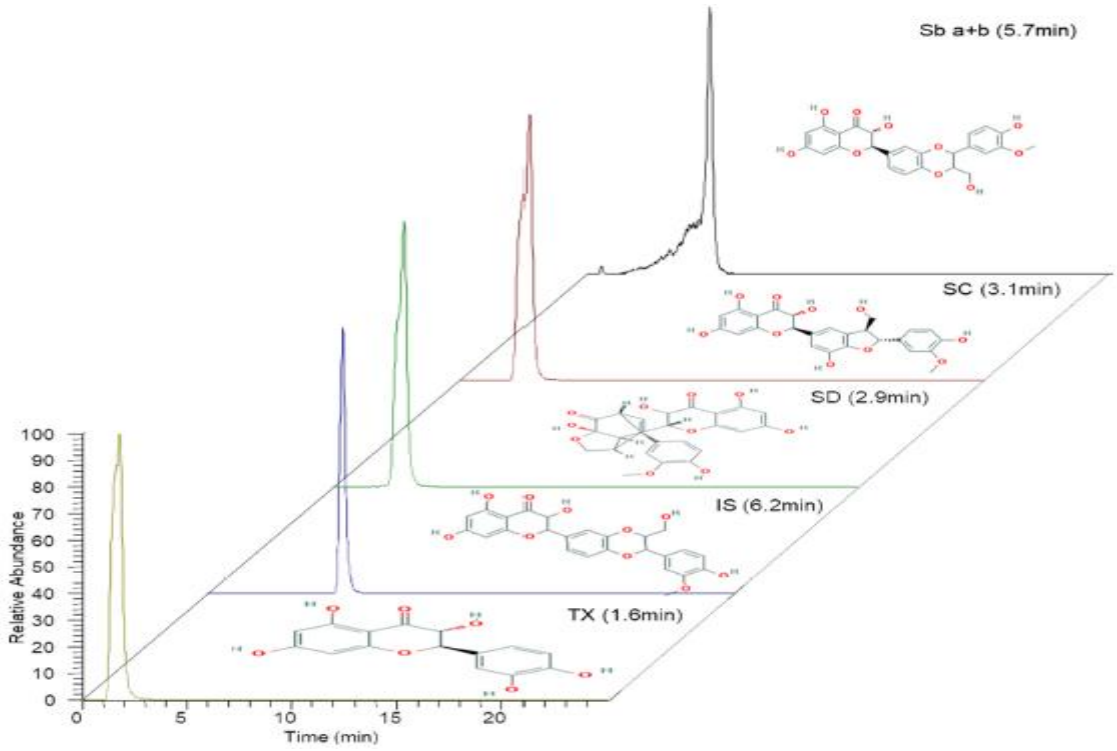

Figure 4.11: Chromatogram of the target silymarin compounds and taxifolin in GXL extracts of Silybum marianum by LC-ESI-MS/MS analysis. The transitions of the compounds (including parent > product ion): SB a+b = 481.94 > 301.018 m/z, SC = 481.148 > 325.026 m/z, SD = 481.189 > 151.043 m/z, IS = 481.184 > 257.029 m/z, and TX = 303.039 > 285.045 m/z (Created by the author).

The analysis of individual composition of the SLY of the obtained fractions from the kinetic study at 20, 40, 60, 80, 100, 120, 140 and 160 min was determined. The extraction rate of each individual compound was strongly affected by extraction solvent and they were different from the global extraction yield as can be seen in Table (4.11) and Figure (S.4) Compounds were quantified using external standard calibration in a UHPLC- ESI-MS/MS

(triple quadrupole) equipment (see Figure (S.3)). Analyzed compounds were Silybin (A + B) (SB_{a+b}), Silychristin (SC), Silydianin (SD), Isosilybin (IS) and Taxifolin (TX). Besides, in Table (4.11) are shown the results for quantification using other extraction methods for comparison such as maceration (solid-liquid extraction) using two-step procedure previously tested by Martinelli et al. [261].

Silybin (A + B) (SB_{a+b}) was the predominant compound among the flavonolignans identified in this work. It was detected under each extraction conditions and its content ranged from 182.02 (mg/g of extract) (Experiment 3, Table (3.2)) to 545.73 (mg/g of extract) (Experiment 5, Table (3.2)). Additionally, the SB_{a+b} values from PLE were lower than those obtained by GXL, with 1.51 (mg/g of extract), 4.60 (mg/g of extract) for 75 °C (run 10), 175 °C (run 11), respectively. The amount of SB_{a+b} and other flavonolignans in extract obtained using two-step method were in accordance to those previously found by Martinelli et al. [261], SB_{a+b} being the most abundant. It is important to mention that higher SB_{a+b} recovery values were obtained using GXL compared to other methods previously published.

Silychristin (SC) was detected in all extraction conditions and its content ranged from 18.263 (mg/g of extract) (Experiment 3) to 110.696 (mg/g of extract) (Experiment 5, Table (3.2)). SC was not detected in the PLE extraction for both temperatures (75 and 175 °C). Silydianin (SD) was detected under each extraction conditions and its content ranged from 0.434 (mg/g of extract) (Experiment 3, Table (3.2)) to 21.216 (mg/g of extract) (Experiment 5, Table (3.2)). No SD was detected in the extracts obtained by PLE (Runs 10 and 11). Regarding isosilybin (IS) it was not detected for two extraction assays (Experiments 2 and 3, Table (3.2)), with a small detected quantity (0.429 mg/g of extract) (Experiment 1) using CO_2: EtOH: H_2O (75:5:20) for 140 min of extraction. All these assays presented a high percentage of CO_2 (75 %) and a high amount of H_2O. Besides, IS was not detected in the extracts obtained by PLE. However, the highest concentration of IS (34.264 mg/g of extract) was achieved using CO_2: EtOH: H_2O (25:37.5:37.5) mixture. The effect of solvent composition in taxifolin was noticeable, since it was the only compound of the silymarin complex preferentially extracted with high amount of CO_2 (Experiments 1-3). Whereas Taxifolin (TX) was not detected for 5 extraction assays (Experiments 4, 6, 7, 8 and 9), with just a small amount detected (0.261 mg/g of extract) in Experiment 5. These assays presented a high fraction of EtOH (50 %, 80 %). The highest TX content was 12.273 (mg/g of extract) of a sample obtained at 40 °C and 90 bar using a mixture of CO_2: EtOH: H_2O (75:5:20).

There was no detection of TX in the extracts obtained by PLE (Runs 10 and 11). Furthermore, 75 % of CO_2 resulted in higher amount of TX compared to smaller amounts (25 % and 50 % of CO_2). Certain amount of TX was found in maceration two-steps experiments, 10.4 mg/g extract, a level in accordance with the one found previously [261] and [173]. However, the content of TX obtained in this work using GXL was higher than the values previously reported [316] [172].

In addition to the total extraction rates of each compound, in terms of extraction kinetics, each compound showed a different profile depending on the solvent. For example, silydianin was preferentially extracted during the first minutes using 50:40:10 (Experiment 6). However, using 25:15:60 (Experiment 7), it was mainly extracted during the last part of the extraction. This may be a consequence of the competitive effect between the different compounds. Curiously, the extraction kinetics of IS using 75% CO_2 showed that it was preferentially extracted after 140 min, while it was preferentially extracted before 100 min using 25% CO_2.

As a result of the experiments, it can be clearly seen that the main compound in the extracts obtained with GXL was silybin (A + B), in agreement with the results of previous studies reported in the literature [184][173]. It is also important to note that the compounds analyzed represented up to 70 % of the total composition of the extracts, confirming the ability of GXL to isolate the SLY complex in a single step with high purification rates using only green solvents. Extraction condition #5 (50:25:25) provided the highest extraction rates of silybin (A + B), silychristin and silydianin. This condition was not the best in terms of yield; however, it provided the highest selectivity rates towards these compounds. This means that the one-step extraction method approach had demonstrated very good performance compared to previous works.

Table 4.11: Kinetic study of the evolution in the extraction of Silymarin and taxifolin in Silybum marianum extracts quantified by liquid chromatography mass spectrometry (UHPLC-ESI-MS/MS). (SB a+b): Silybin (A+B), (SD): Silydianin, (SC): silychristin, (IS): isosilybin and (TX): taxifolin (Created by the author). Results expressed in mg of compound per g extract. Composition of extraction solvent expressed as volumetric ratios of CO2: EtOH: H2O following the same order as Table (3.2).

Compound	Time (min)	Exp. 1 75:05:20	Exp. 2 75:12.5:12.5	Exp. 3 75:20:05	Exp. 4 50:10:40	Exp. 5 50:25:25	Exp. 6 50:40:10	Exp. 7 25:15:60	Exp. 8 25:37.5:37.5	Exp. 9 25:60:15
SB a+b	20	N.Q.	N.Q.	N.Q.	13.646	14.446	50.610	20.774	12.907	4.128
	40	16.723	26.899	11.584	38.120	70.051	78.428	15.755	30.829	21.206
	60	23.992	38.083	22.727	58.514	75.872	64.722	39.300	60.723	23.434
	80	43.340	43.036	21.385	77.871	77.463	70.223	42.509	75.502	34.640
	100	53.259	48.072	33.621	76.429	72.944	63.417	57.562	91.772	52.733
	120	47.435	40.833	40.480	77.371	76.880	67.857	66.545	100.773	67.814
	140	63.367	39.547	29.507	42.228	79.000	69.063	76.000	68.691	83.033
	160	46.886	49.912	22.721	74.857	79.079	10.621	61.812	50.567	56.780
SC	20	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	4.088	N.Q.	N.Q.	N.Q.
	40	N.Q.	2.609	N.Q.	1.472	13.253	20.435	N.Q.	1.547	N.Q.
	60	0.267	5.921	N.Q.	9.043	14.892	13.653	2.787	10.387	N.Q.
	80	8.020	7.166	N.Q.	14.343	17.143	12.325	5.244	12.982	3.039
	100	8.735	9.107	4.809	14.325	12.871	11.758	9.752	16.923	8.045

	120	8.008	5.107	7.015	11.445	13.763	10.801	11.083	20.457	13.159
	140	13.599	3.150	5.250	4.477	19.613	9.206	14.772	12.381	18.376
	160	7.731	9.063	1.190	10.589	16.910	N.Q.	10.934	6.474	9.556
SD	20	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.
	40	N.Q.	N.Q.	N.Q.	N.Q.	1.741	4.649	N.Q.	N.Q.	N.Q.
	60	N.Q.	N.Q.	N.Q.	1.079	3.185	2.612	N.Q.	0.490	N.Q.
	80	N.Q.	N.Q.	N.Q.	2.632	3.287	2.598	N.Q.	2.209	N.Q.
	100	1.580	0.924	N.Q.	2.317	1.532	0.165	N.Q.	4.242	N.Q.
	120	N.Q.	N.Q.	0.434	0.988	2.645	0.073	1.255	5.374	1.849
	140	1.749	N.Q.	N.Q.	N.Q.	4.258	0.474	2.737	2.239	4.484
	160	0.612	N.Q.	N.Q.	0.706	4.568	N.Q.	8.946	0.407	0.598
IS	20	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	0.968	N.Q.	0.212	N.Q.
	40	N.Q.	N.Q.	N.Q.	N.Q.	2.791	6.978	N.Q.	N.Q.	N.Q.
	60	N.Q.	N.Q.	N.Q.	1.684	4.067	2.622	N.Q.	1.117	N.Q.
	80	N.Q.	N.Q.	N.Q.	4.927	1.749	3.522	N.Q.	4.596	N.Q.
	100	N.Q.	N.Q.	N.Q.	2.695	2.830	2.452	1.477	7.332	N.Q.
	120	N.Q.	N.Q.	N.Q.	2.906	4.192	2.269	1.857	9.557	0.295
	140	0.430	N.Q.	N.Q.	N.Q.	5.431	2.178	6.564	2.558	7.565
	160	N.Q.	N.Q.	N.Q.	2.185	5.603	N.Q.	2.611	8.893	1.295
TX	20	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.
	40	N.Q.	0.225	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.
	60	N.Q.	1.503	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.
	80	1.916	1.978	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.
	100	2.951	2.626	0.763	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.
	120	2.054	N.Q.	2.281	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.
	140	3.386	N.Q.	2.031	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.
	160	1.965	0.999	1.167	N.Q.	0.261	N.Q.	N.Q.	N.Q.	N.Q.
Total mg/g extract	358.004	336.761	206.966	546.848	702.321	588.767	460.275	622.140	412.029	

Table 4.12: Silymarin and taxifolin in Silybum marianum extracts obtained in maceration experiment and PLE quantified by liquid chromatography mass spectrometry (UHPLC-ESI-MS/MS). (SB a+b): Silybin (A+B), (SD): Silydianin, (SC): silychristin, (IS): isosilybin and (TX): taxifolin (Created by the author). Results expressed in mg of compound per g extract.

Compound	10 PLE water 75 °C	11 PLE water 175 °C	12 two step hexane+methanol
SB a+b	1.508 ± 0.271	4.599 ± 0.821	104.347 ± 1.459
SC	n.d.	n.d.	5.325 ± 0.844
SD	n.d.	n.d.	2.777 ± 0.664
IS	n.d.	n.d.	3.048 ± 0.925
TX	n.d.	n.d.	10.398 0.600

4.2.2. Total phenolic compounds (TPC)

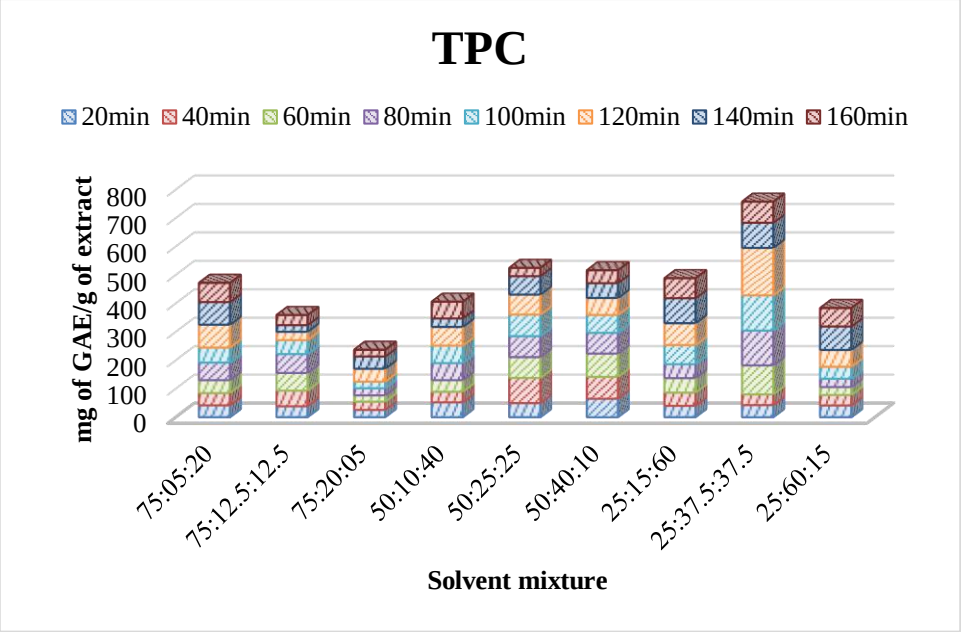

Figure 4.12: Extraction kinetic of Total phenolic content found in extracts of Silybum marianum seeds using Gas Expanded Liquid extraction at different experimental conditions (Created by the author).

As can be seen in Figure (4.12), the TPC showed a different behaviour depending on the extraction conditions. The lowest total phenolic content value (236.93 mg GAE/g extract) was obtained by using (75:20:5) (CO_2: EtOH: H_2O), Experiment 3, and the highest value (757.66 mg GAE/g extract) was 3 times higher and was achieved by using (25:37.5:37.5) (Experiment 8). Additionally, these total phenolic values were higher than those detected in milk thistle seeds reported by Stancheva et al. [317] and Lucini et al. [178]. According to the results presented in this work, the ternary mixture (25:37.5:37.5) was the most suitable solvent for the extraction of total phenolic compounds. This result was confirmed by M. Solana et al. who also obtained that the mixture water: ethanol in the same proportion gave the highest TPC [51]. The obtained TPC values did not show any correlation with the global yield.

4.2.3. GXL extraction vs other extraction methods

In order to compare the performance of the GXL extraction to recover silymarin the extraction rates obtained by other methods published during the previous 9 years are summarized in Table (2.4). In this sense, it is clear that none of them can obtain the same levels of flavonolignans of the silymarin complex than those obtained using GXL. For example, Wianowska et al. [181] developed a single step approach but based on PLE with organic solvent whose best extraction rate was 24.21 mg Silymarin per g of extract. Or Andrzejewska et al. [316] who developed a single step method based on ultrasounds and obtained up to 43.6 mg Silymarin per g dry weight of extract in their best conditions. Meanwhile, the method described in the present paper can achieve up to 704.5 mg of total silymarin per g of extract using condition #5 (50:25:25) on Algerian wild grown milk thistle. The values obtained by Giuliani et al. [173] were similar to those obtained in the present work using similar conventional extraction. Nevertheless, they were far from those obtained by Saleh et al. [318] using microwave assisted extraction. Drouet et al. [172] evaluated the SLY content using two extraction methods: ultrasound-assisted extraction (UAE) and maceration. They obtained the best results by means of UAE at 45 °C for 60 min, using 50 % of aqueous ethanol to obtain 8.61 (mg/g of DW) of SB_{a+b}. As can be seen in Figure (S.4 A), using GXL in the present study led to 545.73 (mg/g of extract) of SB_{a+b}, more than 63-fold the recovery of UAE reported in the literature. Furthermore, according to the reported values for the content of SC in *S. marianum* seeds (3 –6.2 mg/g of DW) [181] [318] [172] it seems that in the present work, the GXL-Extraction using ternary mixture of CO_2: EtOH:

H_2O provided a higher extraction rate (Figure (S.4 C)). It is worth mentioning that the content of SD in any of the GXL extracts was higher than that previously reported by Wianowska et al. using acetone for 10 min at 125 °C [181]. The IS content was higher in the present study compared to the studies reported by Andrzejewska et al. [316] and by Wianowska et al. [181]. All these results were far from the recoveries values that could be obtained using GXL, which could reach up to 702.3 mg silymarin in the conditions of Experiment 5. Besides, it's important to note the origin of *S. marianum* used in the present work, which was wild, meanwhile [181] and [316] used cultivated milk thistle.

4.2.4. Neuroprotective activities

Taking into account the multifactorial effects of neurological disorders like Alzheimer or Parkinson diseases [319], three in vitro activities were measured: antioxidant ($ABTS^{\cdot+}$), anti-inflammatory (LOX) and inhibition of acetylcholinesterase (AChE), besides the phenolic content (TPC) which was included due to the large implications of phenolic compounds in several health disorders. Results for *in-vitro* tests of *S. marianum* GXL extracts are presented in Table (4.13) (lower IC_{50} means higher inhibition capacity). In order to easily transfer the GXL technology to obtain bioactive extracts from *S. marianum* seeds, these measurements were not performed using the kinetic study previously presented. They were rather performed using the extraction time that led to obtain a yield with more than 85 % of extractable material. The main reason justifying this was to reduce the solvent and energy consumption of the optimal process.

The anti-inflammatory activity evaluated by the LOX assay ranged from 28.52 μg/mL (run 6 at 140 min) to 98.75 μg/mL (run 7 at 120 min). The strong inhibition of LOX enzyme confirmed the anti-inflammatory capacity, which was in agreement with previously results reported by Gupta et al. [194] , who demonstrated that *S. marianum* seeds and its active extract, silymarin complex (SLY), inhibited the formation of inflammatory mediators.

Table 4.13: IC50 values (µg/mL) for antioxidant (ABTS•+), anti-inflammatory (LOX) and inhibition of acetylcholinesterase (AChE) and Total Phenolic content (TPC, mg GAE/g extract) obtained for the S. marianum seeds using Gas Expanded Liquid extraction at different experimental conditions (see Table (3.2)) and reference-standards studied (Created by the author).

#	Extraction time	TPC*	AChE*	LOX*	ABTS*
1	120 min	325.15 ± 6.90	222.88 ± 8.01^{e,f}	94.15 ± 5.67^{e}	11.72 ± 0.22^{c}
2	120 min	317.41 ± 7.75	125.09 ± 5.74^{b}	56.96 ± 2.37^{d}	16.01 ± 0.06^{f}
3	140 min	418.36 ± 2.13	440.69 ± 35.44^{g}	64.46 ± 4.36^{d}	16.62 ± 0.20^{f}
4	80 min	222.00 ± 3.86	152.23 ± 4.51^{b,c}	53.48 ± 3.28^{c,d}	12.61 ± 0.37^{d}
5	40 min	138.11 ± 1.96	234.71 ± 7.24^{e,f}	53.38 ± 4.30^{c,d}	14.65 ± 0.01^{e}
6	140 min	318.87 ± 3.45	259.62 ± 15.66^{f}	28.52 ± 9.03^{a,b}	20.48 ± 0.30^{g}
7	120 min	170.38 ± 1.95	160.41 ± 6.43^{b,c}	98.75 ± 4.76^{e}	22.29 ± 0.09^{h}
8	60 min	223.62 ± 5.71	183.45 ± 15.26^{c,d}	56.96 ± 2.73^{d}	11.71 ± 0.06^{c}
9	120 min	595.43 ± 3.89	204.06 ± 12.67^{d,e}	37.65 ± 1.96^{b,c}	8.80 ± 0.09^{b}
10	20 min	76.67 ± 0.52	309.58 ± 20.76^{h}	54.73 ± 2.26^{c,d}	11.94 ± 0.15^{c}
11	20 min	151.70 ± 1.35	139.01 ± 8.55^{i}	53.24 ± 4.18^{c,d}	7.49 ± 0.01^{i}
12$^{\$}$	72 hours$^{\$}$	64 ± 1.18	427.28 ± 6.43^{g}	99.51 ± 1.98^{b,c}	29.84 ± 0.23^{c}
	Galantamine		0.45 ± 0.03^{a}	-	-
	Quercetin		-	19.71 ± 0.24^{a}	-
	Trolox		-	-	2.50 ± 0.02^{a}

* Lower IC_{50} values means higher activity. The results are expressed as the mean ± SD (n= 3). Different letters in the same column show significant statistical differences ($p< 0.05$) among them. $^{\$}$ two step classic solid-liquid extraction experiment.

Concerning the results of the AChE assay of *S. marianum* seeds extracts, they showed low AChE inhibitory capacity compared with Galantamine, which was used as reference. According to the general classification of potential natural extracts reported by Santos et al. [320] , which is in accordance with the AChE inhibition capacity (IC_{50} values), Experiments 2, 4, 7, 8 presented moderate potency ($20 < IC_{50} < 200$ µg/mL), while Experiments 1, 3, 5, 6, 9 presented low potency ($200 < IC_{50} < 1000$ µg/mL). The IC_{50} value of the strongest AChE inhibitor (experiment 2) was approximately three-and-a half-fold lower than the IC_{50} value of the weakest AChE inhibitor (Experiment 3). From Table (4.12) and the dendogram of correlations depicted in Figure (4.13) it can be concluded that the antioxidant and anti-inflammatory activities were related to the flovonolignans composition, while

acetylcholinesterase inhibitory activity was linked with phenolic compound and the flavonol taxifolin. These results were in line with Nausheen et al. [321] , who evaluated the inhibition of cholinesterase enzymes in-vitro of the methanolic extract obtained from milk thistle seeds, presenting an IC_{50} value of AChE is 110 µg/mL. However, the IC_{50} values obtained in the current study were higher than the ones found by Duan et al. [188]. The recent review reported by Haddadi et al. demonstrated the potential effects of SLY in vitro, vivo and animal models, as a neuroprotective agent in many neurodegenerative diseases [189].

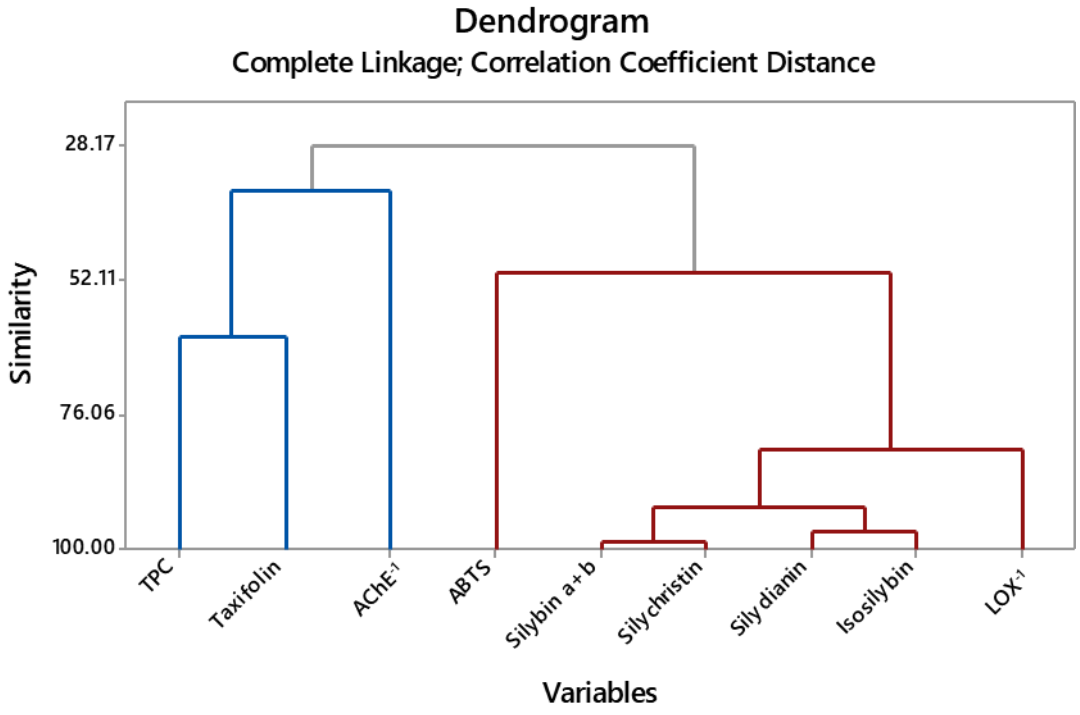

Figure 4.13: Dendogram of correlations between activities and composition of GXL extracts from S. marianum seeds (Created by the author).

Natural antioxidants have the capacity to scavenge free radicals, which have an important role in the progression of oxidative stress. It is very important to note that SLY demonstrated very high antioxidant capacity according to different antioxidant assays including CUPRAC, AGEs [172], DPPH, ORAC, FRAP [178], metal chelating [171] and ABTS [179], in comparison to synthetic antioxidants such as BHT and BHA [322]. Regarding the antioxidant activity, measured using the ABTS in vitro assay, the *S. marianum* extract showed promising radical scavenging activity against ABTS radical with IC_{50} values from 8.80 ± 0.09 µg/mL to 22.29 ± 0.09 µg/mL. Obviously, Trolox (positive control) showed the highest antioxidant activity (2.50 ± 0.02 µg/mL). This ABTS result was in agreement with

the value found by Koksal [68] who used a standard Silymarin purified by Sigma-Aldrich and found an EC_{50} value of 8.62 mg/mL and also with the reported value for *S. marianum* seed by Nowak [323]. It is remarkable that all the studied extracts in the present work were more active than the values obtained by Suárez-Montenegro et al. [324] with ascorbic acid (25.00 ± 0.30 μg/mL) (chemical standard) and rosemary (35.63 ± 1.14 μg/mL) (natural standard), hence an excellent antioxidant potential of the *S. marianum* extracts. Nausheen et al. [321] performed ABTS of methanolic extract of *S. marianum* seeds, obtaining the best IC_{50} of 31.25 μg/mL, which was higher (less antioxidant) than the values obtained in the present study using GXL for extraction, nevertheless the value reported by Nausheen et al. [321] was similar to the one obtained in the two-steps maceration method (Experiment 12). This indicates that despite dielectric constant of Experiment 12 was similar to that of Experiment 7, the extracted compounds were not the same, probably due to the long extraction times which might have affected the antioxidant capacity measured by both methods LOX and ABTS. Nausheen et al. attributed the biological activities of most plant extracts to the synergistic effect of many metabolites rather than to a single compound [321].

CHAPTER V: GENERAL CONCLUSION

CHAPTER V: GENERAL CONCLUSION

The main conclusions of the present thesis are presented in the following section. They are described according to the specific objectives proposed for each experimental part:

I. Pressurized liquid extraction from *Ammodaucus leucotrichus* seeds:

- The pressurized liquids extraction (PLE) using GRAS solvents (ethanol, and water) at different extraction temperatures (40, 110 and 180 °C) demonstrated to be an efficient and fast method for the recovery of high-added value compounds, from *Ammodaucus leucotrichus* biomass.
- The best yield was 44.44 %, and TPC and TCC were achieved using water at 180 °C. Moreover, under the same conditions, the extracts obtained presented a meaningful in vitro bioactivities AChE (IC_{50} = 55.598 µg/mL) and LOX (IC_{50} = 39.373 µg/mL) inhibition, as well as a good antioxidant capacity measured by DPPH (IC_{50} = 58.513 µg/mL).
- 94 compounds belonging to different groups, mainly free and glycosylated phenolic compounds, as well as lipids and organic acids were identified in the metabolite performed by ultra-high performance liquid chromatography coupled to quadrupole time-of-flight tandem mass spectrometry (UHPLC-ESI-q-TOF-MS/MS).
- The multivariate analysis allowed correlating the bioactivity with the compositions of phenolic compounds and carbohydrates, which are the main compounds, associated with high anti-inflammatory and neuroprotective activities.

II. Supercritical fluid extraction from *Ammodaucus leucotrichus* seeds:

- Multivariate response surface methodology using Box Behnken design was performed to optimize SFE as a function of pressure, temperature and percentage of co-solvent. Optimum extraction conditions were 15 % of ethanol at 300 bar and 70 °C, providing a yield of 19.814 (%), a TPC of 21.707 (mg GAE /g of extract), a TCC of 1007.557 (mg/ g of extract) and an AChE of 416.932 (µg/mL).
- A total of 12 compounds, mainly from the terpenoid class, were detected by gas chromatography mass spectrometry analysis. Remarkably, perillaldehyde emerged as the predominant compound in the SFE extracts, followed by β-caryophyllene.
- SFE extracts presented lower AChE inhibition than SWE extracts.
- A biorefinery approach using sequential PLE (180 °C and H_2O) and SFE (70 °C, 300bar and 15 % of EtOH) was able to provide a mean for the valorization of the different high added-value compounds with an antioxidant potential present in *A. leucotrichus.*

- Extracts resulting from successive SFE and PLE extractions showed higher total phenolic content and antioxidant activity than extracts obtained through separate operations. However, anti-inflammatory and anticholinesterase activities were reduced by fractionation, suggesting that bioactivity was related to synergies between polar and non-polar compounds.
- Using UHPLC-ESI-q-TOF-MS/MS, a total of 41 metabolites were detected in SFE extracts, 20 of which had already been detected in extracts obtained by PLE method. In addition, SFE extract under the optimal conditions was the most concentrated in bioactive compounds in comparison to the extracts obtained through the combined methods.
- A total of 32 compounds were detected, 24 of which were tentatively identified by GC-MS/MS analysis from sequential extraction fractions. Notably, compared to the SFE, the extracts obtained by sequential extraction have a very different chemical profile. Furthermore, it is worth mentioning that 14 compounds were reported for the first time in *A. leucotrichus* extracts.

III. Gas-Expanded liquid extraction of Silymarin complex from *Silybum marianum* seeds:

- Gas-expanded liquid extraction (GXLE) was shown to be an efficient and sustainable method for the release of silymarin complex from the Silybum marianum matrix without a defatting step. GXLE achieved higher extraction yields than other previously published research, namely, that using neat supercritical CO_2 at higher pressures and ethanol as a co-solvent.
- Solvent composition mixture (CO_2: EtOH: H_2O) influenced significantly in the global extraction yield, the total phenolic content and the presence of different target compounds at constant pressure and temperature. Maximum global yields were achieved in the more polar (higher dielectric constant) extraction conditions experiments that are the lower CO_2 ones. Nevertheless, among them the assay with solvent composition (25: 60: 15), provided the maximum extraction yield with a value of 55.97 % (g extract /100 g seeds).
- GXLE led to higher extraction yields than PLE performed at 75 °C, whose yield was 8.71 % ± 0.38. However, PLE performed at 175 °C provided a yield 22.71 % ± 0.39, which was close to less polar GXLE conditions.
- TPC exhibited a different behaviour depending on the extraction conditions and there was no correlation with the global yield. The lowest TPC value (236.93 mg GAE/g extract) was obtained with a (75: 20: 5) mixture, while the highest value (757.66 mg GAE/g extract), i.e. 3 times higher, was achieved by using (25: 37.5: 37.5).

- Extraction condition with a mixture of (50: 25: 25) provided the highest extraction rates for these compounds: silybin (a + b), silychristin, and silydianin, while the highest taxifolin content was obtained using a (75: 5: 20) mixture. Regarding isosilybin, the highest concentration was obtained with a (25: 37.5: 37.5) mixture.
- Silybin (A + B) (SB_{a+b}) was the predominant compound among the flavonolignans identified, its content ranged from 182.02 to 545.73 (mg/g of extract). In addition, the SB_{a+b} values obtained by GXLE were higher than those obtained by PLE and other previously published work.
- *Silybum marianum* extracts enriched in silymarin complex provide a moderate acetylcholinesterase inhibitory potential (IC_{50} = 125.09 µg/mL) and powerful anti-inflammatory activity measured by lipoxygenase inhibition (IC_{50} = 28.52 µg/mL). Additionally, the antioxidant activity of extracts was assessed by ABTS test, which confirmed stronger antioxidant properties (IC_{50} = 8.80 µg/mL).
- Antioxidant and anti-inflammatory capacities were related to the composition of silymarin complex, while acetylcholinesterase inhibition capacity was associated with phenolic compound and taxifolin.

Presepectives:

- Further investigations are essential to explore the full potential of used techniques and to determine its economic feasibility on an industrial scale for the utilization of these underutilized Algerian biomasses.
- In vivo studies should be carried out to confirm the potential described in the present thesis, also to understand the mechanism underlying the neuroprotective properties of these bioactive-rich extracts as promising sources of new functional foods and nutraceuticals with health-promoting properties against AD.

In summary, this PhD Thesis has significantly expanded the available knowledge about the development of sustainable processes using compressed fluids and green chemistry techniques such as supercritical fluid, pressurized liquid and gas expanded liquid extraction. This research can also be an opportunity to draw the attention regarding the potential of various neglected and unexploited Algerian biomasses, like *Ammodaucus leucotrichus* and *Silybuim marinum*, as promising natural sources for producing and recovering bioactive compounds associated with neuroprotection and other health benefits.

CHAPTER VI: BIBLIOGRAPHIC REFERENCES

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[1] Buenz EJ, Verpoorte R, Bauer BA. The Ethnopharmacologic Contribution to Bioprospecting Natural Products. Annu Rev Pharmacol Toxicol 2018;58:509–30. https://doi.org/10.1146/annurev-pharmtox-010617-052703.

[2] Rienth M, Vigneron N, Darriet P, Sweetman C, Burbidge C, Bonghi C, et al. Grape Berry Secondary Metabolites and Their Modulation by Abiotic Factors in a Climate Change Scenario – A Review 2021;12:1–26. https://doi.org/10.3389/fpls.2021.643258.

[3] Sebaa A, Marouf A, Kambouche N, Derdour A. Phytochemical composition, antioxidant and antimicrobial activities of Ammodaucus leucotrichus fruit from Algerian Sahara. Oriental Journal of Chemistry 2018;34:519–25. https://doi.org/10.13005/ojc/340158.

[4] Hamza N, Berke B, Umar A, Cheze C, Gin H, Moore N. A review of Algerian medicinal plants used in the treatment of diabetes. J Ethnopharmacol 2019;238:2–28. https://doi.org/10.1016/j.jep.2019.111841.

[5] Li P, Feng D, Yang D, Li X, Sun J, Wang G, et al. Protective effects of anthocyanins on neurodegenerative diseases. Trends Food Sci Technol 2021. https://doi.org/10.1016/j.tifs.2021.05.005.

[6] Auti ST, Kulkarni YA. A Systematic Review on the Role of Natural Products in Modulating the Pathways in Alzheimer's Disease. Vitamin and Nutrition Research 2017;87. https://doi.org/.org/10.1024/0300-9831/a000405.

[7] Hampel H, Mesulam MM, Cuello AC, Farlow MR, Giacobini E, Grossberg GT, et al. The cholinergic system in the pathophysiology and treatment of Alzheimer's disease. Brain 2018;141:1917–33. https://doi.org/10.1093/brain/awy132.

[8] Adams M, Gmünder F, Hamburger M. Plants traditionally used in age related brain disorders-A survey of ethnobotanical literature. J Ethnopharmacol 2007;113:363–81. https://doi.org/10.1016/j.jep.2007.07.016.

[9] Herrero M, Ibañez E. Green extraction processes, biorefineries and sustainability: Recovery of high added-value products from natural sources. Journal of Supercritical Fluids 2018;134:252–9. https://doi.org/10.1016/j.supflu.2017.12.002.

[10] Picot-Allain C, Mahomoodally MF, Ak G, Zengin G. Conventional versus green extraction techniques — a comparative perspective. Curr Opin Food Sci 2021;40:144–56. https://doi.org/10.1016/j.cofs.2021.02.009.

[11] Ahmad T, Masoodi FA, A. Rather S, Wani SM, Gull A. Supercritical Fluid Extraction: A Review. Journal of Biological and Chemical Chronicles 2019;5:114–22. https://doi.org/10.33980/jbcc.2019.v05i01.019.

[12] Khaw KY, Parat MO, Shaw PN, Falconer JR. Solvent supercritical fluid technologies to extract bioactive compounds from natural sources: A review. Molecules 2017;22. https://doi.org/10.3390/molecules22071186.

[13] Herrero M, Castro-Puyana M, Mendiola JA, Ibañez E. Compressed fluids for the extraction of bioactive compounds. TrAC - Trends in Analytical Chemistry 2013;43:67–83. https://doi.org/10.1016/j.trac.2012.12.008.

[14] Ballesteros-Vivas D, Ortega-Barbosa JP, Sánchez-Camargo A del P, Rodríguez-Varela LI, Parada-Alfonso F. Pressurized Liquid Extraction of Bioactives. Reference Module in Food Science, Elsevier; 2020. https://doi.org/10.1016/b978-0-08-100596-5.22817-5.

[15] Vinatoru M, Mason TJ, Calinescu I. Ultrasonically assisted extraction (UAE) and microwave assisted extraction (MAE) of functional compounds from plant materials. TrAC - Trends in Analytical Chemistry 2017;97:159–78. https://doi.org/10.1016/j.trac.2017.09.002.

[16] Coelho MC, Pereira RN, Rodrigues AS, Teixeira JA, Pintado ME. The use of emergent technologies to extract added value compounds from grape by-products. Trends Food Sci Technol 2020;106:182–97. https://doi.org/10.1016/j.tifs.2020.09.028.

[17] Belwal T, Ezzat SM, Rastrelli L, Bhatt ID, Daglia M, Baldi A, et al. A critical analysis of extraction techniques used for botanicals: Trends, priorities, industrial uses and optimization strategies. TrAC - Trends in Analytical Chemistry 2018;100:82–102. https://doi.org/10.1016/j.trac.2017.12.018.

[18] Niu D, Zeng XA, Ren EF, Xu FY, Li J, Wang MS, et al. Review of the application of pulsed electric fields (PEF) technology for food processing in China. Food Research International 2020;137:109715. https://doi.org/10.1016/j.foodres.2020.109715.

[19] Martínez JM, Delso C, Álvarez I, Raso J. Pulsed electric field-assisted extraction of valuable compounds from microorganisms. Compr Rev Food Sci Food Saf 2020;19:530–52. https://doi.org/10.1111/1541-4337.12512.

[20] Heydari M, Carbone K, Gervasi F, Parandi E, Rouhi M, Rostami O, et al. Cold Plasma-Assisted Extraction of Phytochemicals: A Review. vol. 12. 2023. https://doi.org/10.3390/foods12173181.

[21] Pankaj SK, Wan Z, Keener KM. Effects of cold plasma on food quality: A review. Foods 2018;7. https://doi.org/10.3390/foods7010004.

[22] Dalvi-Isfahan M, Hamdami N, Le-Bail A, Xanthakis E. The principles of high voltage electric field and its application in food processing: A review. Food Research International 2016;89:48–62. https://doi.org/10.1016/j.foodres.2016.09.002.

[23] Li Z, Fan Y, Xi J. Recent advances in high voltage electric discharge extraction of bioactive ingredients from plant materials. Food Chem 2019;277:246–60. https://doi.org/10.1016/j.foodchem.2018.10.119.

[24] Puri M, Sharma D, Barrow CJ. Enzyme-assisted extraction of bioactives from plants. Trends Biotechnol 2012. https://doi.org/10.1007/s10570-018-1982-1.

[25] Nadar SS, Rao P, Rathod VK. Enzyme assisted extraction of biomolecules as an approach to novel extraction technology: A review. Food Research International 2018. https://doi.org/10.1016/j.foodres.2018.03.006.

[26] Gallego R, Bueno M, Herrero M. Sub- and supercritical fluid extraction of bioactive compounds from plants, food-by-products, seaweeds and microalgae – An update. TrAC - Trends in Analytical Chemistry 2019;116:198–213. https://doi.org/10.1016/j.trac.2019.04.030.

[27] Dias ALB, de Aguiar AC, Rostagno MA. Extraction of natural products using supercritical fluids and pressurized liquids assisted by ultrasound: Current status and trends. Ultrason Sonochem 2021;74:105584. https://doi.org/10.1016/j.ultsonch.2021.105584.

[28] Sánchez-Camargo ADP, Montero L, Stiger-Pouvreau V, Tanniou A, Cifuentes A, Herrero M, et al. Considerations on the use of enzyme-assisted extraction in combination with pressurized liquids to recover bioactive compounds from algae. Food Chem 2016;192:67–74. https://doi.org/10.1016/j.foodchem.2015.06.098.

[29] Viganó J, Zabot GL, Martínez J. Supercritical fluid and pressurized liquid extractions of phytonutrients from passion fruit by-products: Economic evaluation of sequential multi-stage and single-stage processes. Journal of Supercritical Fluids 2017;122:88–98. https://doi.org/10.1016/j.supflu.2016.12.006.

[30] Gilbert-López B, Mendiola JA, Fontecha J, Van Den Broek LAM, Sijtsma L, Cifuentes A, et al. Downstream processing of Isochrysis galbana: a step towards microalgal biorefinery. Green Chemistry 2015;17:4599–609. https://doi.org/10.1039/c5gc01256b.

[31] Ameer K, Shahbaz HM, Kwon JH. Green Extraction Methods for Polyphenols from Plant Matrices and Their Byproducts: A Review. Compr Rev Food Sci Food Saf 2017;16:295–315. https://doi.org/10.1111/1541-4337.12253.

[32] Alvarez-Rivera G, Bueno M, Ballesteros-Vivas D, Mendiola JA, Ibañez E. Pressurized liquid extraction. Liquid-Phase Extraction, Elsevier; 2019, p. 375–98. https://doi.org/10.1016/B978-0-12-816911-7.00013-X.

[33] Mustafa A, Turner C. Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review. Anal Chim Acta 2011;703:8–18. https://doi.org/10.1016/j.aca.2011.07.018.

[34] Saha S, Singh AK, Keshari AK, Raj V, Rai A, Maity S. Modern Extraction Techniques for Drugs and Medicinal Agents. Ingredients Extraction by Physicochemical Methods in Food, Elsevier; 2018, p. 65–106. https://doi.org/10.1016/b978-0-12-811521-3.00002-8.

[35] Alarcon NÁ, Ortiz-Ramírez JS, Ortega-Barbosa JP, Ballesteros-Vivas D, Rodríguez-Varela LI, Alfonso PF. PRESSURIZED LIQUID EXTRACTION (PLE). In: Plaza M, Luisa Marina M, editors. Green ExtractionTechniques in Food analysis, Bentham Science Publishers Ltd.; 2023, p. 357–95. https://doi.org/10.2174/9789815049459123030010.

[36] Sun H, Ge X, Lv Y, Wang A. Application of accelerated solvent extraction in the analysis of organic contaminants, bioactive and nutritional compounds in food and feed. J Chromatogr A 2012;1237:1–23. https://doi.org/10.1016/j.chroma.2012.03.003.

[37] Leyva-Jiménez FJ, Lozano-Sánchez J, Borrás-Linares I, Arráez-Román D, Segura-Carretero A. Comparative study of conventional and pressurized liquid extraction for recovering bioactive compounds from Lippia citriodora leaves. Food Research International 2018;109:213–22. https://doi.org/10.1016/j.foodres.2018.04.035.

[38] Montero L, Socas-Rodríguez B, Mendiola JA, Ibáñez E. Extraction of marine phenolics using compressed fluids. 2023. https://doi.org/10.1016/B978-0-12-823589-8.00007-8.

[39] Avanza MV, Álvarez-Rivera G, Cifuentes A, Mendiola JA, Ibáñez E. Phytochemical and functional characterization of phenolic compounds from cowpea (Vigna unguiculata (l.) walp.) obtained by green extraction technologies. Agronomy 2021;11. https://doi.org/10.3390/agronomy11010162.

[40] Teo CC, Tan SN, Yong JWH, Hew CS, Ong ES. Pressurized hot water extraction (PHWE). J Chromatogr A 2010;1217:2484–94. https://doi.org/10.1016/j.chroma.2009.12.050.

[41] Dhouibi N, Manuguerra S, Arena R, Messina CM, Santulli A, Kacem S, et al. Impact of the Extraction Method on the Chemical Composition and Antioxidant Potency of Rosmarinus officinalis L. Extracts. Metabolites 2023;13. https://doi.org/10.3390/metabo13020290.

[42] Tabernero A, Martín del Valle EM, Galán MA. Supercritical fluids for pharmaceutical particle engineering: Methods, basic fundamentals and modelling. Chemical Engineering and Processing: Process Intensification 2012. https://doi.org/10.1016/j.cep.2012.06.004.

[43] Subramaniam B. Sustainable Processes With Supercritical Fluids. Encyclopedia of Sustainable Technologies, Elsevier; 2017, p. 653–62. https://doi.org/10.1016/B978-0-12-409548-9.10252-0.

[44] Yerena-Prieto BJ, Gonzalez-Gonzalez M, García-Alvarado MÁ, Casas L, Palma M, Rodríguez-Jimenes GDC, et al. Evaluation of the Effect of Different Co-Solvent Mixtures on the Supercritical CO2 Extraction of the Phenolic Compounds Present in Moringa oleifera Lam. Leaves. Agronomy 2022;12. https://doi.org/10.3390/agronomy12061450.

[45] Herrero M, Mendiola JA, Cifuentes A, Ibáñez E. Supercritical fluid extraction: Recent advances and applications. J Chromatogr A 2010;1217:2495–511. https://doi.org/10.1016/j.chroma.2009.12.019.

[46] Herrero M, Sánchez-Camargo A del P, Cifuentes A, Ibáñez E. Plants, seaweeds, microalgae and food by-products as natural sources of functional ingredients obtained using pressurized liquid extraction and supercritical fluid extraction. TrAC - Trends in Analytical Chemistry 2015. https://doi.org/10.1016/j.trac.2015.01.018.

[47] Carpentieri S, Soltanipour F, Ferrari G, Pataro G, Donsì F. Emerging green techniques for the extraction of antioxidants from agri-food by-products as promising ingredients for the food industry. Antioxidants 2021;10. https://doi.org/10.3390/antiox10091417.

[48] Gondo TF, Jönsson M, Karlsson EN, Sandahl M, Turner C. Extractability, selectivity, and comprehensiveness in supercritical fluid extraction of seaweed using ternary mixtures of carbon dioxide, ethanol, and water. J Chromatogr A 2023;1706. https://doi.org/10.1016/j.chroma.2023.464267.

[49] Guan M, Xu X, Tang X, Li Y. Optimization of supercritical CO2 extraction by response surface methodology, composition analysis and economic evaluation of bamboo green wax. J Clean Prod 2022;330:129906. https://doi.org/10.1016/j.jclepro.2021.129906.

[50] Herrero M, Sánchez-Camargo A del P, Cifuentes A, Ibáñez E. Plants, seaweeds, microalgae and food by-products as natural sources of functional ingredients obtained using pressurized liquid extraction and supercritical fluid extraction. TrAC - Trends in Analytical Chemistry 2015;71:26–38. https://doi.org/10.1016/j.trac.2015.01.018.

[51] Solana M, Boschiero I, Dall'Acqua S, Bertucco A. A comparison between supercritical fluid and pressurized liquid extraction methods for obtaining phenolic compounds from Asparagus officinalis L. Journal of Supercritical Fluids 2015;100:201–8. https://doi.org/10.1016/j.supflu.2015.02.014.

[52] Fraguela-Meissimilly H, Bastías-Monte JM, Vergara C, Ortiz-Viedma J, Lemus-Mondaca R, Flores M, et al. New Trends in Supercritical Fluid Technology and Pressurized Liquids for the Extraction and Recovery of Bioactive Compounds from Agro-Industrial and Marine Food Waste. Molecules 2023;28. https://doi.org/10.3390/molecules28114421.

[53] Týskiewicz K, Konkol M, Rój E. The application of supercritical fluid extraction in phenolic compounds isolation from natural plant materials. Molecules 2018;23. https://doi.org/10.3390/molecules23102625.

[54] Herrero M, Cifuentes A, Ibañez E. Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae - A review. Food Chem 2006;98:136–48. https://doi.org/10.1016/j.foodchem.2005.05.058.

[55] Machado BAS, Pereira CG, Nunes SB, Padilha FF, Umsza-Guez MA. Supercritical Fluid Extraction Using CO2: Main Applications and Future Perspectives. Separation Science and Technology (Philadelphia) 2013;48:2741–60. https://doi.org/10.1080/01496395.2013.811422.

[56] Getachew AT, Jacobsen C, Holdt SL. Emerging technologies for the extraction of marine phenolics: Opportunities and challenges. Mar Drugs 2020;18:1–22. https://doi.org/10.3390/MD18080389.

[57] Amador-Luna VM, Montero L, Herrero M. Compressed fluids for the extraction of bioactive compounds from plants, food by-products, seaweeds and microalgae – an update from 2019 to 2023. TrAC - Trends in Analytical Chemistry 2023;169:117410. https://doi.org/10.1016/j.trac.2023.117410.

[58] Sánchez-Camargo ADP, Mendiola JA, Ibáñez E. CHAPTER 17: Gas Expanded-liquids. RSC Green Chemistry, vol. 2018- Janua, Royal Society of Chemistry; 2018, p. 512–31. https://doi.org/10.1039/9781788013543-00512.

[59] Cunico LP, Turner C. Supercritical Fluids and Gas-Expanded Liquids. The Application of Green Solvents in Separation Processes, Elsevier Inc.; 2017, p. 155–214. https://doi.org/10.1016/B978-0-12-805297-6.00007-3.

[60] Jessop PG, Subramaniam B. Gas-Expanded Liquids. Chem Rev 2007;107:2666–94. https://doi.org/org/10.1021/cr040199o.

[61] Vásquez-Villanueva R, Plaza M, García MC, Turner C, Marina ML. A sustainable approach for the extraction of cholesterol-lowering compounds from an olive by-product based on CO2-expanded ethyl acetate. Anal Bioanal Chem 2019;411:5885–96. https://doi.org/10.1007/s00216-019-01970-4.

[62] Paudel A, Jessop MJ, Stubbins SH, Champagne P, Jessop PG. Extraction of lipids from microalgae using CO2-expanded methanol and liquid CO2. Bioresour Technol 2015;184:286–90. https://doi.org/10.1016/j.biortech.2014.11.111.

[63] Reyes FA, Mendiola JA, Ibañez E, Del Valle JM. Astaxanthin extraction from Haematococcus pluvialis using CO 2-expanded ethanol. Journal of Supercritical Fluids 2014;92:75–83. https://doi.org/10.1016/j.supflu.2014.05.013.

[64] Esquivel-Hernández DA, López VH, Rodríguez-Rodríguez J, Alemán-Nava GS, Cuéllar-Bermúdez SP, Rostro-Alanis M, et al. Supercritical carbon dioxide and microwave-assisted extraction of functional lipophilic compounds from Arthrospira platensis. Int J Mol Sci 2016;17. https://doi.org/10.3390/ijms17050658.

[65] del Pilar Sánchez-Camargo A, Pleite N, Herrero M, Cifuentes A, Ibáñez E, Gilbert-López B. New approaches for the selective extraction of bioactive compounds employing bio-based solvents and pressurized green processes. Journal of Supercritical Fluids 2017;128:112–20. https://doi.org/10.1016/j.supflu.2017.05.016.

[66] Fagundes MB, Alvarez-Rivera G, Mendiola JA, Bueno M, Sánchez-Martínez JD, Wagner R, et al. Phytosterol-rich compressed fluids extracts from Phormidium autumnale cyanobacteria with neuroprotective potential. Algal Res 2021;55. https://doi.org/10.1016/j.algal.2021.102264.

[67] Mendiola JA. Green Processes in Foodomics. Gas-Expanded Liquids Extraction of Bioactives. Comprehensive Foodomics, Elsevier; 2021, p. 744–53. https://doi.org/10.1016/b978-0-08-100596-5.22927-2.

[68] Gallego R, MENDIOLA JA, HERRERO M, CASTRO-PUYANA M, IBANEZ E. Supercritical Fluid Extraction. In: Prado J, Rostagno M, editors. Natural Product Extraction: Pricipales and Applications, 2022, p. 202–40. https://doi.org/org/10.1039/9781839165894-00202.

[69] Abbott AP, Hope EG, Mistry R, Stuart AM. Probing the structure of gas expanded liquids using relative permittivity, density and polarity measurements. Green Chemistry 2009;11:1530–5. https://doi.org/10.1039/b915570h.

[70] Aida T, Aizawa T, Kanakubo M, Nanjo H. Relation between volume expansion and hydrogen bond networks for CO 2-alcohol mixtures at 40 °C. Journal of Physical Chemistry B 2010;114:13628–36. https://doi.org/10.1021/jp1017339.

[71] Palafox-Hernandez JP, Mendis CH, Thompson WH, Laird BB. Pressure and Temperature Tuning of Gas-Expanded Liquid Structure and Dynamics. Journal of Physical Chemistry B 2019;123:2915–24. https://doi.org/10.1021/acs.jpcb.8b09826.

[72] Kamlet MJ, Taft RW. The Solvatochromic Comparison Method. I. The β-Scale Of Solvent Hydrogen-Bond Acceptor (HBA) Basicities. J Am Chem Soc 1976;98:377–83. https://doi.org/10.1021/ja00418a009.

[73] Reichardt C. Solvatochromic dyes as solvent polarity indicators. Chem Rev 1994;94:2319–58. https://doi.org/10.1021/cr00032a005.

[74] Mazzutti S, Rodrigues LGG, Mezzomo N, Venturi V, Ferreira SRS. Integrated green-based processes using supercritical CO2 and pressurized ethanol applied to recover antioxidant compouds from cocoa (Theobroma cacao) bean hulls. Journal of Supercritical Fluids 2018;135:52–9. https://doi.org/10.1016/j.supflu.2017.12.039.

[75] Gilbert-López B, Mendiola JA, van den Broek LAM, Houweling-Tan B, Sijtsma L, Cifuentes A, et al. Green compressed fluid technologies for downstream processing of Scenedesmus obliquus in a biorefinery approach. Algal Res 2017;24:111–21. https://doi.org/10.1016/j.algal.2017.03.011.

[76] Castejón N, Señoráns FJ. Simultaneous extraction and fractionation of omega-3 acylglycerols and glycolipids from wet microalgal biomass of Nannochloropsis gaditana using pressurized liquids. Algal Res 2019;37:74–82. https://doi.org/10.1016/j.algal.2018.11.003.

[77] Hirondart M, Rombaut N, Fabiano-Tixier AS, Bily A, Chemat F. Comparison between Pressurized Liquid Extraction and Conventional Soxhlet Extraction for Rosemary Antioxidants, Yield, Composition, and Environmental Footprint. Foods 2020;9:584. https://doi.org/10.3390/foods9050584.

[78] Muñoz-Almagro N, Gilbert-López B, Pozuelo-Rollón MC, García-Fernandez Y, Almeida C, Villamiel M, et al. Exploring the microalga euglena cantabrica by pressurized liquid extraction to obtain bioactive compounds. Mar Drugs 2020;18:1–17. https://doi.org/10.3390/md18060308.

[79] Liang X, Nielsen NJ, Christensen JH. Selective pressurized liquid extraction of plant secondary metabolites: Convallaria majalis L. as a case. Anal Chim Acta X 2020;4. https://doi.org/10.1016/j.acax.2020.100040.

[80] Xu JL, Kim TJ, Kim JK, Choi Y. Simultaneous roasting and extraction of green coffee beans by pressurized liquid extraction. Food Chem 2019;281:261–8. https://doi.org/10.1016/j.foodchem.2018.12.061.

[81] Hernández-Corroto E, Plaza M, Marina ML, García MC. Sustainable extraction of proteins and bioactive substances from pomegranate peel (Punica granatum L.) using pressurized liquids and deep eutectic solvents. Innovative Food Science and Emerging Technologies 2020;60. https://doi.org/10.1016/j.ifset.2020.102314.

[82] Otero P, Gutierrez-Docio A, Navarro del Hierro J, Reglero G, Martin D. Extracts from the edible insects Acheta domesticus and Tenebrio molitor with improved fatty acid profile due to ultrasound assisted or pressurized liquid extraction. Food Chem 2020;314. https://doi.org/10.1016/j.foodchem.2020.126200.

[83] Okiyama DCG, Soares ID, Cuevas MS, Crevelin EJ, Moraes LAB, Melo MP, et al. Pressurized liquid extraction of flavanols and alkaloids from cocoa bean shell using ethanol as solvent. Food Research International 2018;114:20–9. https://doi.org/10.1016/j.foodres.2018.07.055.

[84] Castejón N, Luna P, Señoráns FJ. Alternative oil extraction methods from Echium plantagineum L. seeds using advanced techniques and green solvents. Food Chem 2018;244:75–82. https://doi.org/10.1016/j.foodchem.2017.10.014.

[85] Guo C, Valdés A, Sánchez-Martínez JD, Ibáñez E, Bi J, Cifuentes A. Neuroprotective Potential of Thinned Peaches Extracts Obtained by Pressurized Liquid Extraction after Different Drying Processes. Foods 2022;11. https://doi.org/10.3390/foods11162464.

[86] Rudke AR, Mazzutti S, Andrade KS, Vitali L, Ferreira SRS. Optimization of green PLE method applied for the recovery of antioxidant compounds from buriti

(Mauritia flexuosa L.) shell. Food Chem 2019;298:125061. https://doi.org/10.1016/j.foodchem.2019.125061.

[87] Raspe DT, da Silva C, da Costa SC. Pressurized liquid extraction of compounds from Stevia leaf: Evaluation of process variables and extract characterization. Journal of Supercritical Fluids 2023;193. https://doi.org/10.1016/j.supflu.2022.105813.

[88] Bagatini L, Zandoná GP, Hoffmann JF, de Souza Cardoso J, Teixeira FC, Moroni LS, et al. Evaluation of Eugenia uniflora L. leaf extracts obtained by pressurized liquid extraction: Identification of chemical composition, antioxidant, antibacterial, and allelopathic activity. Sustain Chem Pharm 2023;35:101214. https://doi.org/10.1016/j.scp.2023.101214.

[89] Sanchez-Martinez JD, Bueno M, Alvarez-Rivera G, Tudela J, Ibanez E, Cifuentes A. In vitro neuroprotective potential of terpenes from industrial orange juice by-products. Food Funct 2021;12:302–14. https://doi.org/10.1039/d0fo02809f.

[90] Suárez-Montenegro ZJ, Ballesteros-Vivas D, Gallego R, Valdés A, Sánchez-Martínez JD, Parada-Alfonso F, et al. Neuroprotective Potential of Tamarillo (Cyphomandra betacea) Epicarp Extracts Obtained by Sustainable Extraction Process. Front Nutr 2021;8. https://doi.org/10.3389/fnut.2021.769617.

[91] Woloszyn N, Krabbe RD, Fischer B, Bernardi JL, Duarte PF, Puton BMS, et al. Use of pressurized liquid extraction technique to obtain extracts with biological and antioxidant activity from Mentha pulegium, Equisetum giganteum and Sida cordifolia. Chemical Papers 2022;76:5775–88. https://doi.org/10.1007/s11696-022-02289-8.

[92] Tejedor-Calvo E, García-Barreda S, Sánchez S, Morte A, Siles-Sánchez M de las N, Soler-Rivas C, et al. Application of Pressurized Liquid Extractions to Obtain Bioactive Compounds from Tuber aestivum and Terfezia claveryi. Foods 2022;11. https://doi.org/10.3390/foods11030298.

[93] Morcelli A, Cassel E, Vargas R, Rech R, Marcílio N. Supercritical fluid (CO2+ethanol) extraction of chlorophylls and carotenoids from Chlorella

sorokiniana: COSMO-SAC assisted prediction of properties and experimental approach. Journal of CO2 Utilization 2021;51. https://doi.org/10.1016/j.jcou.2021.101649.

[94] Ospina M, Castro-Vargas HI, Parada-Alfonso F. Antioxidant capacity of Colombian seaweeds: 1. Extracts obtained from Gracilaria mammillaris by means of supercritical fluid extraction. Journal of Supercritical Fluids 2017;128:314–22. https://doi.org/10.1016/j.supflu.2017.02.023.

[95] Jiao G, Kermanshahi pour A. Extraction of anthocyanins from haskap berry pulp using supercritical carbon dioxide: Influence of co-solvent composition and pretreatment. LWT 2018;98:237–44. https://doi.org/10.1016/j.lwt.2018.08.042.

[96] Rosas-Quina YE, Mejía-Nova FC. Supercritical fluid extraction with cosolvent of alkaloids from Lupinus mutabilis Sweet and comparison with conventional method. J Food Process Eng 2021;44:1–10. https://doi.org/10.1111/jfpe.13657.

[97] Zhou D, Zhou X, Shi Q, Pan J, Zhan H, Ge F. High-pressure supercritical carbon dioxide extraction of Idesia polycarpa oil: Evaluation the influence of process parameters on the extraction yield and oil quality. Ind Crops Prod 2022;188:115586. https://doi.org/10.1016/j.indcrop.2022.115586.

[98] Rodrigues VH, De Melo MMR, Portugal I, Silva CM. Extraction of added-value triterpenoids from acacia Dealbata leaves using supercritical fluid extraction. Processes 2021;9. https://doi.org/10.3390/pr9071159.

[99] Sharma A, Ray A, Singhal RS. Co-extraction of turmeric (Curcuma longa L.) and dried coconut shreds by supercritical fluid extraction (SFE): Chemical and bioactivity profile. J Clean Prod 2023;382:135313. https://doi.org/10.1016/j.jclepro.2022.135313.

[100] Moges A, Barik CR, Sahoo L, Goud V V. Optimization of polyphenol extraction from Hippophae salicifolia D. Don leaf using supercritical CO2 by response surface methodology. 3 Biotech 2022;12:1–13. https://doi.org/10.1007/s13205-022-03358-1.

[101] Coelho JP, Filipe RM, Paula Robalo M, Boyadzhieva S, Cholakov GS, Stateva RP. Supercritical CO2 extraction of spent coffee grounds. Influence of co-solvents and characterization of the extracts. Journal of Supercritical Fluids 2020;161. https://doi.org/10.1016/j.supflu.2020.104825.

[102] Šojić B, Tomović V, Kocić-Tanackov S, Kovačević DB, Putnik P, Mrkonjić Ž, et al. Supercritical extracts of wild thyme (Thymus serpyllum L.) by-product as natural antioxidants in ground pork patties. Lwt 2020;130. https://doi.org/10.1016/j.lwt.2020.109661.

[103] Coelho JP, Robalo MP, Fernandes IS, Stateva RP. Optimization of Oil Recovery from Japonica Luna Rice Bran by Supercritical Carbon Dioxide Applying Design of Experiments: Characterization of the Oil and Mass Transfer Modeling. ChemEngineering 2022;6. https://doi.org/10.3390/chemengineering6040063.

[104] Bueno M, Vitali C, Sánchez-Martínez JD, Mendiola JA, Cifuentes A, Ibáñez E, et al. Compressed CO2 Technologies for the Recovery of Carotenoid-Enriched Extracts from Dunaliella salina with Potential Neuroprotective Activity. vol. 8. 2020. https://doi.org/10.1021/acssuschemeng.0c03991.

[105] Luan G, Yang M, Nan X, Lv H, Liu Q, Wang Y, et al. Optimization and Comparative Study of Different Extraction Methods of Sixteen Fatty Acids of Potentilla anserina L. from Twelve Different Producing Areas of the Qinghai-Tibetan Plateau. Molecules 2022;27. https://doi.org/10.3390/molecules27175443.

[106] Muñoz AM, Casimiro-Gonzales S, Gómez-Coca RB, Moreda W, Best I, Cajo-Pinche MI, et al. Comparison of Four Oil Extraction Methods for Sinami Fruit (Oenocarpus mapora H. Karst): Evaluating Quality, Polyphenol Content and Antioxidant Activity. Foods 2022;11. https://doi.org/10.3390/foods11101518.

[107] Poletto P, Álvarez-Rivera G, López GD, Borges OMA, Mendiola JA, Ibáñez E, et al. Recovery of ascorbic acid, phenolic compounds and carotenoids from acerola by-products: An opportunity for their valorization. LWT 2021;146. https://doi.org/10.1016/j.lwt.2021.111654.

[108] Sakai H, Ono K, Tokunaga S, Sharmin T, Aida TM, Mishima K. Extraction of natural pigments from gardenia Jasminoides J.Ellis fruit pulp using CO2-expanded liquids and direct sonication. Separations 2021;8:1–14. https://doi.org/10.3390/separations8010001.

[109] Cunico LP, Acosta MC, Turner C. Experimental measurements and modeling of curcumin solubility in CO2-expanded ethanol. Journal of Supercritical Fluids 2017;130:381–8. https://doi.org/10.1016/j.supflu.2017.06.018.

[110] Cunico LP, Cobo AM, Al-Hamimi S, Turner C. Solubility and Thermal Degradation of Quercetin in CO2-Expanded Liquids. Molecules 2020;25. https://doi.org/10.3390/molecules25235582.

[111] Cunico LP, Sun M, Rui Y, Ghirmai S, Enekvist M, Lundegard S, et al. Enhanced distribution kinetics in liquid-liquid extraction by CO2-expanded solvents. Journal of Supercritical Fluids 2020;163. https://doi.org/10.1016/j.supflu.2020.104874.

[112] Li PL, Li HN, Jing KJ, David A, Lin JR, Deng G. Evaluation of lipid extraction from microalgae based on different phase regions of CO2-expanded ethanol. Chemical Engineering and Processing - Process Intensification 2019;138:1–6. https://doi.org/10.1016/j.cep.2019.02.011.

[113] Chhouk K, Uemori C, Wahyudiono, Kanda H, Goto M. Extraction of phenolic compounds and antioxidant activity from garlic husk using carbon dioxide expanded ethanol. Chemical Engineering and Processing: Process Intensification 2017;117:113–9. https://doi.org/10.1016/j.cep.2017.03.023.

[114] Hoang HN, Nagashima Y, Mori S, Kagechika H, Matsuda T. CO2-expanded bio-based liquids as novel solvents for enantioselective biocatalysis. Tetrahedron 2017;73:2984–9. https://doi.org/10.1016/j.tet.2017.04.024.

[115] Pilařová V, Kuda L, Vlčková HK, Nováková L, Gupta S, Kulkarni M, et al. Carbon dioxide expanded liquid: an effective solvent for the extraction of quercetin from South African medicinal plants. Plant Methods 2022;18. https://doi.org/10.1186/s13007-022-00919-6.

[116] Hollá M, Pilařová V, Švec F, Sklenářová H. Green Solvents in the Extraction of Bioactive Compounds from Dried Apple Cultivars. Foods 2023;12. https://doi.org/10.3390/foods12040893.

[117] Villanueva-Bermejo D, Calvo M V., Castro-Gómez P, Fornari T, Fontecha J. Production of omega 3-rich oils from underutilized chia seeds. Comparison between supercritical fluid and pressurized liquid extraction methods. Food Research International 2019;115:400–7. https://doi.org/10.1016/j.foodres.2018.10.085.

[118] Nastić N, Borrás-Linares I, Lozano-Sánchez J, Švarc-Gajić J, Segura-Carretero A. Comparative Assessment of Phytochemical Profiles of Comfrey (Symphytum officinale L.) Root Extracts Obtained by Different Extraction Techniques. Molecules 2020;25. https://doi.org/10.3390/molecules25040837.

[119] David A, Wang F, Sun X, Li H, Lin J, Li P, et al. Chemical Composition, Antioxidant, and Antimicrobial Activities of Vetiveria zizanioides (L.) Nash Essential Oil Extracted by Carbon Dioxide Expanded Ethanol. Molecules 2019;24. https://doi.org/10.3390/molecules24101897.

[120] Kraujalis P, Kraujalienė V, Kazernavičiūtė R, Venskutonis PR. Supercritical carbon dioxide and pressurized liquid extraction of valuable ingredients from Viburnum opulus pomace and berries and evaluation of product characteristics. Journal of Supercritical Fluids 2017;122:99–108. https://doi.org/10.1016/j.supflu.2016.12.008.

[121] Scopel E, Santos LC dos, Bofinger MR, Martínez J, Rezende CA. Green extractions to obtain value-added elephant grass co-products in an ethanol biorefinery. J Clean Prod 2020;274. https://doi.org/10.1016/j.jclepro.2020.122769.

[122] Melgosa R, Trigueros E, Sanz MT, Cardeira M, Rodrigues L, Fernández N, et al. Supercritical CO2 and subcritical water technologies for the production of bioactive extracts from sardine (Sardina pilchardus) waste. Journal of Supercritical Fluids 2020;164. https://doi.org/10.1016/j.supflu.2020.104943.

[123] Ballesteros-Vivas D, Alvarez-Rivera G, Ibánez E, Parada-Alfonso F, Cifuentes A. Integrated strategy for the extraction and profiling of bioactive

metabolites from Passiflora mollissima seeds combining pressurized-liquid extraction and gas/liquid chromatography–high resolution mass spectrometry. J Chromatogr A 2019;1595:144–57. https://doi.org/10.1016/j.chroma.2019.02.031.

[124] Rodríguez-Pérez C, Mendiola JA, Quirantes-Piné R, Ibáñez E, Segura-Carretero A. Green downstream processing using supercritical carbon dioxide, CO2-expanded ethanol and pressurized hot water extractions for recovering bioactive compounds from Moringa oleifera leaves. Journal of Supercritical Fluids 2016;116:90–100. https://doi.org/10.1016/j.supflu.2016.05.009.

[125] Gallego R, Martínez M, Cifuentes A, Ibáñez E, Herrero M. Development of a Green Downstream Process for the Valorization of Porphyridium cruentum Biomass. Molecules 2019;24. https://doi.org/10.3390/molecules24081564.

[126] Bobinaitė R, Kraujalis P, Tamkutė L, Urbonavičienė D, Viškelis P, Venskutonis PR. Recovery of bioactive substances from rowanberry pomace by consecutive extraction with supercritical carbon dioxide and pressurized solvents. Journal of Industrial and Engineering Chemistry 2020;85:152–60. https://doi.org/10.1016/j.jiec.2020.01.036.

[127] Viganó J, Aguiar AC, Moraes DR, Jara JLP, Eberlin MN, Cazarin CBB, et al. Sequential high pressure extractions applied to recover piceatannol and scirpusin B from passion fruit bagasse. Food Research International 2016;85:51–8. https://doi.org/10.1016/j.foodres.2016.04.015.

[128] Nastić N, Borrás-Linares I, Lozano-Sánchez J, Švarc-Gajić J, Segura-Carretero A. Optimization of the extraction of phytochemicals from black mulberry (Morus nigra L.) leaves. Journal of Industrial and Engineering Chemistry 2018;68:282–92. https://doi.org/10.1016/j.jiec.2018.07.055.

[129] Vardanega R, Fuentes FS, Palma J, Bugueño-Muñoz W, Cerezal-Mezquita P, Ruiz-Domínguéz MC. Extraction of valuable compounds from granadilla (Passiflora ligularis Juss) peel using pressurized fluids technologies. Sustain Chem Pharm 2023;34:101135. https://doi.org/10.1016/j.scp.2023.101135.

[130] Imbimbo P, Bueno M, D'Elia L, Pollio A, Ibañez E, Olivieri G, et al. Green Compressed Fluid Technologies To Extract Antioxidants and Lipids from Galdieria phlegrea in a Biorefinery Approach. ACS Sustain Chem Eng 2020;8:2939–47. https://doi.org/10.1021/acssuschemeng.9b07505.

[131] Ziani BEC, Calhelha RC, Barreira JCM, Barros L, Hazzit M, Ferreira ICFR. Bioactive properties of medicinal plants from the Algerian flora: Selecting the species with the highest potential in view of application purposes. Ind Crops Prod 2015;77:582–9. https://doi.org/10.1016/J.INDCROP.2015.09.034.

[132] Ziani BEC, Rached W, Bachari K, Alves MJ, Calhelha RC, Barros L, et al. Detailed chemical composition and functional properties of Ammodaucus leucotrichus Cross. & Dur. and Moringa oleifera Lamarck. J Funct Foods 2019;53:237–47. https://doi.org/10.1016/j.jff.2018.12.023.

[133] Hellal K, Maulidiani M, Ismail IS, Tan CP. Antioxidant, α-Glucosidase, and Nitric Oxide Inhibitory Activities of Six Algerian Traditional Medicinal Plant Extracts and 1H-NMR-Based Metabolomics Study of the Active Extract. Molecules 2020;25. https://doi.org/10.3390/molecules25051247.

[134] Benarba B. Medicinal plants used by traditional healers from South-West Algeria: An ethnobotanical study. J Intercult Ethnopharmacol 2016;5:320–30. https://doi.org/10.5455/jice.20160814115725.

[135] Hammiche V, Maiza K. Traditional medicine in Central Sahara: Pharmacopoeia of Tassili N'ajjer. J Ethnopharmacol 2006;105:358–67. https://doi.org/10.1016/j.jep.2005.11.028.

[136] Jamila F, Mostafa E. Ethnobotanical survey of medicinal plants used by people in Oriental Morocco to manage various ailments. J Ethnopharmacol 2014;154:76–87. https://doi.org/10.1016/j.jep.2014.03.016.

[137] Halla N, Boucherit K, Boucherit-Otmani Z, Touati FZ, Rahmani N, Aid I. Ammodaucus leucotrichus and Citrullus colocynthis from algerian Sahara: Ethnopharmacological application, phytochemical screening, polyphenols content

and antioxidant activity of hydromethanolic extracts. J King Saud Univ Sci 2018. https://doi.org/10.1016/j.jksus.2018.03.018.

[138] Chinsembu KC. Diabetes mellitus and nature's pharmacy of putative antidiabetic plants. J Herb Med 2019;15. https://doi.org/10.1016/j.hermed.2018.09.001.

[139] Velasco-Negueruela A, Pérez-Alonso MJ, Pérez De Paz PL, Palá-Paúl J, Sanz J. Analysis by gas chromatography-mass spectrometry of the volatiles from the fruits of Ammodaucus leucotrichus subsp. leucotrichus and subsp. nanocarpus grown in North Africa and the Canary Islands, respectively. J Chromatogr A 2006;1108:273–5. https://doi.org/10.1016/j.chroma.2006.01.031.

[140] Louail Z, Kameli A, Benabdelkader T, Bouti K, Hamza K, Krimat S. Antimicrobial and antioxidant activity of essential oil of Ammodaucus leucotrichus Coss. & Dur. seeds. vol. 7. 2016.

[141] Dahmane D, Dob T, Krimat S, Nouasri A, Metidji H, Ksouri A. Chemical composition, antioxidant and antibacterial activities of the essential oils of medicinal plant Ammodaucus leucotrichus from Algeria. Journal of Essential Oil Research 2017;29:48–55. https://doi.org/10.1080/10412905.2016.1201015.

[142] Halla N, Heleno SA, Costa P, Fernandes IP, Calhelha RC, Boucherit K, et al. Chemical profile and bioactive properties of the essential oil isolated from Ammodaucus leucotrichus fruits growing in Sahara and its evaluation as a cosmeceutical ingredient. Ind Crops Prod 2018;119:249–54. https://doi.org/10.1016/j.indcrop.2018.04.043.

[143] Naima B, Abdelkrim R, Ouarda B, Salah NN, Larbi BAM. Chemical composition, antimicrobial, antioxidant and anticancer activities of essential oil from Ammodaucus leucotrichus Cosson & Durieu (Apiaceae) growing in South Algeria. Bull Chem Soc Ethiop 2019;33:541–9. https://doi.org/10.4314/bcse.v33i3.14.

[144] Khaldi A, Meddah B, Moussaoui A, Sonnet P. Anti-mycotoxin Effect and Antifungal Properties of Essential Oil from Ammodaucus leucotrichus Coss. & Dur.

on Aspergillus flavus and Aspergillus ochraceus. Journal of Essential Oil-Bearing Plants 2017;20:36–44. https://doi.org/10.1080/0972060X.2017.1282840.

[145] Sadaoui N, Bec N, Barragan-Montero V, Kadri N, Cuisinier F, Larroque C, et al. The essential oil of Algerian Ammodaucus leucotrichus Coss. & Dur. and its effect on the cholinesterase and monoamine oxidase activities. Fitoterapia 2018;130:1–5. https://doi.org/10.1016/j.fitote.2018.07.015.

[146] Abu Zarga MH, Al-Jaber HI, Baba Amer ZY, Sakhrib L, Al-Qudah MA, Al-humaidi JYG, et al. Chemical Composition, Antimicrobial and Antitumor Activities of Essential Oil of Ammodaucus leucotrichus Growing in Algeria. Biologically Active Products from Nature 2013;3:224–31. https://doi.org/10.1080/22311866.2013.833469.

[147] Bekhechi C, Atik F, Abou B, Belkaid B, Mazari W, Belkaid AB. Antimicrobial Activity of Ammodaucus leucotrichus Fruit Oil from Algerian Sahara. Nat Prod Commun, 2014.

[148] Manssouri M, Znini M, El Harrak A, Majidi L. Antifungal activity of essential oil from the fruits of Ammodaucus leucotrichus Coss. & Dur., in liquid and vapour phase against postharvest phytopathogenic fungi in apples. J Appl Pharm Sci 2016;6:131–6. https://doi.org/10.7324/JAPS.2016.60520.

[149] Muckensturm B, Diyani F, Le Nolren D, Fkih-Tetouanit S, Reduron~ J-P. AMMOLACTONE, A GUAIANOLIDE FROM A MEDICINAL PLANT, AMMODAUCUS LEUCOTRICHUS. vol. 44. 1997.

[150] Sahara DW, Lamin M, Bellau A, Chiurato MA, Maietti A, Fantin G, et al. Nutrients and Main Secondary Metabolites Characterizing Extracts and Essential Oil from Fruits of Ammodaucus leucotrichus Coss. & Dur. (Western Sahara). Molecules 2022;27. https://doi.org/10.3390/molecules27155013.

[151] Miguel M, Bouchmaaa N, Smail A, Gaamoussi F, Lyoussi B. ANTIOXIDANT , ANTI-INFLAMMATORY AND ANTI-ACETYLCHOLINESTERASE ACTIVITIES OF ELEVEN EXTRACTS OF

MOROCCAN PLANTS. Fresenuis Environmental Bulletin 2014;23. https://doi.org/10.1007/s10068-014-0044-1.

[152] Mouderas F, Lahfa FB, Mezouar D, Benahmed NEH. Valorization and identification of bioactive compounds of a spice Ammodaucus leucotrichus. Orient Pharm Exp Med 2019. https://doi.org/10.1007/s13596-019-00390-0.

[153] Louail Z, Djemouai N, Ghardaia U De, Bouti K, Kameli A, Kouba EN De. BIOLOGICAL ACTIVITIES OF DIFFERENT EXTRACTS OF Ammodaucus leucotrichus subsp . leucotrichus COSSON & DURIEU FROM ALGERIAN. Analele Universităţii Din Oradea, Fascicula Biologie 2020;XXVII:215–23.

[154] Selama G, Ramdan B, Oulad Y, Majdoub E, Dugo P, Mondello L, et al. Determination of the polyphenolic content of Ammodaucus leucotrichus Cosson and Durieu by liquid chromatography coupled with mass spectrometry and evaluation of the antioxidant and antiglycation properties. Separation Science 2022;45. https://doi.org/10.1002/jssc.202200235.

[155] Lenzi M, Turrini E, Catanzaro E, Cocchi V, Guerrini A, Hrelia P, et al. In Vitro Investigation of the Anticancer Properties of Ammodaucus Leucotrichus Coss. & Dur. Pharmaceuticals 2022;15. https://doi.org/10.3390/ph15121491.

[156] Bouknana S, Daoudi NE, Bouhrim M, Ziyyat A, Legssyer A, Mekhfi H, et al. Ammodaucus leucotrichus Coss. & Durieu: Antihyperglycemic activity via the inhibition of α-amylase, α-glucosidase, and intestinal glucose absorption activities and its chemical composition. J Pharm Pharmacogn Res 2022;10:94–103. https://doi.org/10.56499/jppres21.1141_10.1.94.

[157] Hajib A, Danton O, Keller M, Potterat O, Bougrin K, Charrouf Z, et al. Polyacetylenic caffeoyl amides from Ammodaucus leucotrichus. Phytochemistry 2023;206:113555. https://doi.org/10.1016/j.phytochem.2022.113555.

[158] El Haci I A, Mazari W, Gherib M ABF. Evaluation of the Antioxidant Activities of Organic Extracts from Ammodaucus leucotrichus Coss & Dur Fruit Part Harvested from the Algerian Sahara. Advances in Complementary and Alternative Medicine 2018;1:1–5.

[159] Wallace SN, Carrier DJ, Clausen EC. Extraction of Nutraceuticals from Milk Thistle. Biotechnology for Fuels and Chemicals 2003;105:891–903. https://doi.org/10.1007/978-1-4612-0057-4_75.

[160] Cavaretta M. Therapeutic Review: Milk Thistle. J Exot Pet Med 2015. https://doi.org/10.1053/j.jepm.2015.09.001.

[161] Bhattacharya S. Milk Thistle Seeds in Health. Nuts and Seeds in Health and Disease Prevention 2020:429–38. https://doi.org/10.1016/b978-0-12-818553-7.00030-9.

[162] Karkanis A, Bilalis D, Efthimiadou A. Cultivation of milk thistle (Silybum marianum L. Gaertn.), a medicinal weed. Ind Crops Prod 2011;34:825–30. https://doi.org/10.1016/j.indcrop.2011.03.027.

[163] Khan MA, Abbasi BH, Ahmed N, Ali H. Effects of light regimes on in vitro seed germination and silymarin content in Silybum marianum. Ind Crops Prod 2013;46:105–10. https://doi.org/10.1016/j.indcrop.2012.12.035.

[164] Lynch KD, Montonye ML, Tian DD, Arman T, Oyanna VO, Bechtold BJ, et al. Hepatic organic anion transporting polypeptides mediate disposition of milk thistle flavonolignans and pharmacokinetic silymarin-drug interactions. Phytotherapy Research 2021. https://doi.org/10.1002/ptr.7049.

[165] Zhang ZS, Wang S, Liu H, Li BZ, Che L. Constituents and thermal properties of milk thistle seed oils extracted with three methods. LWT 2020;126. https://doi.org/10.1016/j.lwt.2020.109282.

[166] Wianowska D, Gil M. Silymarin Extraction From Silybum marianum L. Gaertner. Water Extraction of Bioactive Compounds: From Plants to Drug Development, Elsevier; 2017, p. 385–97. https://doi.org/10.1016/B978-0-12-809380-1.00015-2.

[167] Liu L, Zhang H. Milk Thistle Oil Extracted by Enzyme-Mediated Assisted Solvent Extraction Compared with n-Hexane and Cold-Pressed Extraction. Molecules 2023;28:2591. https://doi.org/10.3390/molecules28062591.

[168] Gilabadi S, Stanyon H, DeCeita D, Pendry BA, Galante E. Simple and effective method for the extraction of silymarin from Silybum marianum (L.) gaertner seeds. J Herb Med 2023;37:100619. https://doi.org/10.1016/j.hermed.2022.100619.

[169] Mukhtar S, Xiaoxiong Z, Khalid W, Moreno A, Lorenzo JM. In Vitro Antioxidant Capacity of Purified Bioactive Compounds in Milk Thistle Seed (Silybum marianum) Along with Phenolic Profile. Food Anal Methods 2023:651–63. https://doi.org/10.1007/s12161-023-02449-w.

[170] Mahgoub YA, Shawky E, Ghareeb DA, Darwish FA, El Sebakhy NA, El-Hawiet AM. UPLC-MS/MS multivariate data analysis reveals phenological growth stages affect silymarin bioactive components of the different organs of two Silybum marianum genotypes. Microchemical Journal 2023;187:108436. https://doi.org/10.1016/j.microc.2023.108436.

[171] Jabłonowska M, Ciganović P, Jablan J, Marguí E, Tomczyk M, Zovko Končić M. Silybum marianum glycerol extraction for the preparation of high-value anti-ageing extracts. Ind Crops Prod 2021;168. https://doi.org/10.1016/j.indcrop.2021.113613.

[172] Drouet S, Leclerc EA, Garros L, Tungmunnithum D, Kabra A, Abbasi BH, et al. A green ultrasound-assisted extraction optimization of the natural antioxidant and anti-aging flavonolignans from milk thistle silybum marianum (L.) gaertn. fruits for cosmetic applications. Antioxidants 2019;8. https://doi.org/10.3390/antiox8080304.

[173] Giuliani C, Tani C, Maleci Bini L, Fico G, Colombo R, Martinelli T. Localization of phenolic compounds in the fruits of Silybum marianum characterized by different silymarin chemotype and altered colour. Fitoterapia 2018;130:210–8. https://doi.org/10.1016/j.fitote.2018.09.002.

[174] Drouet S, Abbasi BH, Falguières A, Ahmad W, Sumaira, Ferroud C, et al. Single laboratory validation of a quantitative core shell-based LC separation for the evaluation of silymarin variability and associated antioxidant activity of pakistani ecotypes of milk thistle (silybum marianum L.). Molecules 2018;23. https://doi.org/10.3390/molecules23040904.

[175] Saleh IA, Vinatoru M, Mason TJ, Abdel-Azim NS, Shams KA, Aboutabl E, et al. Extraction of silymarin from milk thistle (Silybum marianum) seeds–a comparison of conventional and microwave-assisted extraction methods. Journal of Microwave Power and Electromagnetic Energy 2017;51:124–33. https://doi.org/10.1080/08327823.2017.1320265.

[176] Jahan N, Khalil-ur-Rahman, Basra SMA, Sajid S, Afzal I. Seed enhancement of Silybum marianum and optimization of silymarin extraction. Int J Agric Biol 2016;18:464–70. https://doi.org/10.17957/IJAB/15.0139.

[177] Abouzid SF, Chen SN, McAlpine JB, Friesen JB, Pauli GF. Silybum marianum pericarp yields enhanced silymarin products. Fitoterapia 2016;112:136–43. https://doi.org/10.1016/j.fitote.2016.05.012.

[178] Lucini L, Kane D, Pellizzoni M, Ferrari A, Trevisi E, Ruzickova G, et al. Phenolic profile and in vitro antioxidant power of different milk thistle [Silybum marianum (L.) Gaertn.] cultivars. Ind Crops Prod 2016;83:11–6. https://doi.org/10.1016/j.indcrop.2015.12.023.

[179] Ben Rahal N, Barba FJ, Barth D, Chevalot I. Supercritical CO2 extraction of oil, fatty acids and flavonolignans from milk thistle seeds: Evaluation of their antioxidant and cytotoxic activities in Caco-2 cells. Food and Chemical Toxicology 2015;83:275–82. https://doi.org/10.1016/j.fct.2015.07.006.

[180] Çelik HT, Gürü M. Extraction of oil and silybin compounds from milk thistle seeds using supercritical carbon dioxide. Journal of Supercritical Fluids 2015;100:105–9. https://doi.org/10.1016/j.supflu.2015.02.025.

[181] Wianowska D, Wiśniewski M. Simplified procedure of silymarin extraction from Silybum marianum L. Gaertner. J Chromatogr Sci 2015;53:366–72. https://doi.org/10.1093/chromsci/bmu049.

[182] Zhao F, Li XH. Ultrasonic-assisted enzymatic extraction of silymarin from the silybum marianum seed shell and evaluation of its antioxidant activity in vitro. EXCLI J 2015;14:861–74. https://doi.org/10.17179/excli2015-257.

[183] Polyak SJ, Morishima C, Lohmann V, Pal S, Lee DYW, Liu Y, et al. Identification of hepatoprotective flavonolignans from silymarin. Proc Natl Acad Sci U S A 2010;107:5995–9. https://doi.org/10.1073/pnas.0914009107.

[184] Federico A, Dallio M, Loguercio C. Silymarin/Silybin and chronic liver disease: A marriage of many years. Molecules 2017;22. https://doi.org/10.3390/molecules22020191.

[185] Wang X, Zhang Z, Wu SC. Health Benefits of Silybum marianum: Phytochemistry, Pharmacology, and Applications. J Agric Food Chem 2020;68:11644–64. https://doi.org/10.1021/acs.jafc.0c04791.

[186] Vilahur G, Casaní L, Peña E, Crespo J, Juan-Babot O, Ben-Aicha S, et al. Silybum marianum provides cardioprotection and limits adverse remodeling post-myocardial infarction by mitigating oxidative stress and reactive fibrosis. Int J Cardiol 2018;270:28–35. https://doi.org/10.1016/j.ijcard.2018.06.030.

[187] Pérez-H J, Carrillo-S C, García E, Ruiz-Mar G, Pérez-Tamayo R, Chavarría A. Neuroprotective effect of silymarin in a MPTP mouse model of Parkinson's disease. Toxicology 2014;319:38–43. https://doi.org/10.1016/j.tox.2014.02.009.

[188] Duan S, Guan X, Lin R, Liu X, Yan Y, Lin R, et al. Silibinin inhibits acetylcholinesterase activity and amyloid β peptide aggregation: A dual-target drug for the treatment of Alzheimer's disease. Neurobiol Aging 2015;36:1792–807. https://doi.org/10.1016/j.neurobiolaging.2015.02.002.

[189] Haddadi R, Shahidi Z, Eyvari-Brooshghalan S. Silymarin and neurodegenerative diseases: Therapeutic potential and basic molecular mechanisms. Phytomedicine 2020;79. https://doi.org/10.1016/j.phymed.2020.153320.

[190] Kazazis CE, Evangelopoulos AA, Kollas A, Vallianou NG. The therapeutic potential of milk thistle in diabetes. Review of Diabetic Studies 2014;11:167–74. https://doi.org/10.1900/RDS.2014.11.167.

[191] Qin N bo, Jia C cui, Xu J, Li D hong, Xu F xing, Bai J, et al. New amides from seeds of Silybum marianum with potential antioxidant and antidiabetic activities. Fitoterapia 2017;119:83–9. https://doi.org/10.1016/j.fitote.2017.04.008.

[192] Wang Y, Yuan AJ, Wu YJ, Wu LM, Zhang L. Silymarin in cancer therapy: Mechanisms of action, protective roles in chemotherapy-induced toxicity, and nanoformulations. J Funct Foods 2023;100:105384. https://doi.org/10.1016/j.jff.2022.105384.

[193] Hosseinabadi T, Lorigooini Z, Tabarzad M, Salehi B, Rodrigues CF, Martins N, et al. Silymarin antiproliferative and apoptotic effects: Insights into its clinical impact in various types of cancer. Phytotherapy Research 2019;33:2849–61. https://doi.org/10.1002/ptr.6470.

[194] Gupta OP, Sing S, Bani S, Sharma N, Malhotra ; S, Gupta BD, et al. Anti-inflammatory and anti-arthritic activities of silymarin acting through inhibition of 5-lipoxygenase. Phytomedicine 1999;7:21–4.

[195] Köksal E, Gülçin I, Beyza S, Sarikaya Ö, Bursal E. In vitro antioxidant activity of silymarin. J Enzyme Inhib Med Chem 2009;24:395–405. https://doi.org/10.1080/14756360802188081.

[196] Dos Santos TC, Gomes TM, Pinto BAS, Camara AL, De Andrade Paes AM. Naturally occurring acetylcholinesterase inhibitors and their potential use for Alzheimer's disease therapy. Front Pharmacol 2018;9:1–14. https://doi.org/10.3389/fphar.2018.01192.

[197] Li P, Feng D, Yang D, Li X, Sun J, Wang G, et al. Protective effects of anthocyanins on neurodegenerative diseases. Trends Food Sci Technol 2021. https://doi.org/10.1016/j.tifs.2021.05.005.

[198] 2022 Alzheimer's disease facts and figures. Alzheimer's and Dementia 2022;18:700–89. https://doi.org/10.1002/alz.12638.

[199] Ardura-Fabregat A, Boddeke EWGM, Boza-Serrano A, Brioschi S, Castro-Gomez S, Ceyzériat K, et al. Targeting Neuroinflammation to Treat Alzheimer's Disease. CNS Drugs 2017;31:1057–82. https://doi.org/10.1007/s40263-017-0483-3.

[200] Auti ST, Kulkarni YA. A Systematic Review on the Role of Natural Products in Modulating the Pathways in Alzheimer's Disease. Vitamin and Nutrition Research 2017;87. https://doi.org/.org/10.1024/0300-9831/a000405.

[201] Atlante A, Amadoro G, Bobba A, Latina V. Functional foods: An approach to modulate molecular mechanisms of alzheimer's disease. Cells 2020;9:1–62. https://doi.org/10.3390/cells9112347.

[202] Gordon BA, Blazey TM, Su Y, Hari-Raj A, Dincer A, Flores S, et al. Spatial patterns of neuroimaging biomarker change in individuals from families with autosomal dominant Alzheimer's disease: a longitudinal study. Lancet Neurol 2018;17:241–50. https://doi.org/10.1016/S1474-4422(18)30028-0.

[203] Anand R, Gill KD, Mahdi AA. Therapeutics of Alzheimer's disease: Past, present and future. Neuropharmacology 2014;76:27–50. https://doi.org/10.1016/j.neuropharm.2013.07.004.

[204] Lee WS, Lee HJ, Yang JY, Shin HL, Choi SW, Kim JK, et al. The Potential Neuroprotective Effects of Extracts from Oat Seedlings against Alzheimer's Disease. Nutrients 2022;14. https://doi.org/10.3390/nu14194103.

[205] Zhu J, Yi X, Zhang J, Chen S, Wu Y. Rapid screening of brain-penetrable antioxidants from natural products by blood-brain barrier specific permeability assay combined with DPPH recognition. J Pharm Biomed Anal 2018;151:42–8. https://doi.org/10.1016/j.jpba.2017.12.055.

[206] Elmaleh DR, Farlow MR, Conti PS, Tompkins RG. Developing Effective Alzheimer ' s Disease Therapies : Clinical Experience and Future Directions. Alzheimer's Disease 2019;71:715–32. https://doi.org/10.3233/JAD-190507.

[207] Sharma K. Cholinesterase inhibitors as Alzheimer ' s therapeutics (Review). MOLECULAR MEDCINE REPORTS 2019;20:1479–87. https://doi.org/10.3892/mmr.2019.10374.

[208] Atlante A, Amadoro G, Bobba A, Latina V. Functional foods: An approach to modulate molecular mechanisms of alzheimer's disease. Cells 2020;9:1–62. https://doi.org/10.3390/cells9112347.

[209] Pérez-Hernández J, Zaldívar-Machorro VJ, Villanueva-Porras D, Vega-Ávila E, Chavarría A. A Potential Alternative against Neurodegenerative Diseases: Phytodrugs. Oxid Med Cell Longev 2016;2016. https://doi.org/10.1155/2016/8378613.

[210] Gallego R, Valdés A, Suárez-Montenegro ZJ, Sánchez-Martínez JD, Cifuentes A, Ibáñez E, et al. Anti-inflammatory and neuroprotective evaluation of diverse microalgae extracts enriched in carotenoids. Algal Res 2022;67. https://doi.org/10.1016/j.algal.2022.102830.

[211] Xu X, Li S, Zhang R, Le W. Neuroprotective effects of naturally sourced bioactive polysaccharides : an update 2022;17:1907–12.

[212] Kerch G. The potential of chitosan and its derivatives in prevention and treatment of Age-related diseases. Mar Drugs 2015;13:2158–82. https://doi.org/10.3390/md13042158.

[213] Gali L, Bedjou F. Antioxidant and anticholinesterase effects of the ethanol extract, ethanol extract fractions and total alkaloids from the cultivated Ruta chalepensis. South African Journal of Botany 2019;120:163–9. https://doi.org/10.1016/j.sajb.2018.04.011.

[214] Rafiquzzaman SM, Kim EY, Lee JM, Mohibbullah M, Alam MB, Soo Moon I, et al. Anti-Alzheimers and anti-inflammatory activities of a glycoprotein purified from the edible brown alga Undaria pinnatifida. Food Research International 2015;77:118–24. https://doi.org/10.1016/j.foodres.2015.08.021.

[215] Sangha JS, Wally O, Banskota AH, Stefanova R, Hafting JT, Critchley AT, et al. A cultivated form of a red seaweed (Chondrus crispus), suppresses β-amyloid-induced paralysis in caenorhabditis elegans. Mar Drugs 2015;13:6407–24. https://doi.org/10.3390/md13106407.

[216] Rahbardar MG, Hosseinzadeh H. Therapeutic effects of rosemary (Rosmarinus officinalis L.) and its active constituents on nervous system disorders. Iran J Basic Med Sci 2020;23:1100–12. https://doi.org/10.22038/ijbms.2020.45269.10541.

[217] Habtemariam S. The Therapeutic Potential of Rosemary (Rosmarinus officinalis) Diterpenes for Alzheimer's Disease. Evidence-Based Complementary and Alternative Medicine 2016;2016. https://doi.org/10.1155/2016/2680409.

[218] Cutillas AB, Carrasco A, Martinez-Gutierrez R, Tomas V, Tudela J. Rosmarinus officinalis L. essential oils from Spain: composition, antioxidant capacity, lipoxygenase and acetylcholinesterase inhibitory capacities, and antimicrobial activities. Plant Biosyst 2018;152:1282–92. https://doi.org/10.1080/11263504.2018.1445129.

[219] Fagundes MB, Alvarez-Rivera G, Mendiola JA, Bueno M, Sánchez-Martínez JD, Wagner R, et al. Phytosterol-rich compressed fluids extracts from Phormidium autumnale cyanobacteria with neuroprotective potential. Algal Res 2021;55. https://doi.org/10.1016/j.algal.2021.102264.

[220] Ahmed SI, Hayat MQ, Zahid S, Tahir M, Mansoor Q, Ismail M, et al. Isolation and identification of flavonoids from anticancer and neuroprotective extracts of trigonella foenum graecum. Tropical Journal of Pharmaceutical Research 2017;16:1391–8. https://doi.org/10.4314/tjpr.v16i6.25.

[221] Guo C, Valdés A, Sánchez-Martínez JD, Ibáñez E, Bi J, Cifuentes A. Neuroprotective Potential of Thinned Peaches Extracts Obtained by Pressurized Liquid Extraction after Different Drying Processes. Foods 2022;11. https://doi.org/10.3390/foods11162464.

[222] Kolodziejczyk-Czepas J, Nowak P, Wachowicz B, Piechocka J, Głowacki R, Moniuszko-Szajwaj B, et al. Antioxidant efficacy of Kalanchoe daigremontiana bufadienolide-rich fraction in blood plasma in vitro. Pharm Biol 2016;54:3182–8. https://doi.org/10.1080/13880209.2016.1214740.

[223] Bogucka-Kocka A, Zidorn C, Kasprzycka M, Szymczak G, Szewczyk K. Phenolic acid content, antioxidant and cytotoxic activities of four Kalanchoë species. Saudi J Biol Sci 2016;25:622–30. https://doi.org/10.1016/j.sjbs.2016.01.037.

[224] de Armas-Ricard M, Quinán-Cárdenas F, Sanhueza H, Pérez-Vidal R, Mayorga-Lobos C, Ramírez-Rodríguez O. Phytochemical screening and antioxidant activity of seven native species growing in the forests of southern chilean patagonia. Molecules 2021;26. https://doi.org/10.3390/molecules26216722.

[225] Orhan I, Şener B, Choudhary MI, Khalid A. Acetylcholinesterase and butyrylcholinesterase inhibitory activity of some Turkish medicinal plants. J Ethnopharmacol 2004;91:57–60. https://doi.org/10.1016/j.jep.2003.11.016.

[226] Marinas IC, Oprea E, Geana EI, Chifiriuc C, Lazar V. Antimicrobial and antioxidant activity of the vegetative and reproductive organs of Robinia pseudoacacia. Journal of the Serbian Chemical Society 2014;79:1363–78. https://doi.org/10.2298/JSC140304049M.

[227] Kim HS, Jang JM, Yun SY, Zhou D, Piao Y, Ha HC, et al. Effect of Robinia pseudoacacia Leaf Extract on Interleukin-1β-mediated Tumor Angiogenesis. In Vivo (Brooklyn) 2019;33:1901–10. https://doi.org/10.21873/invivo.11684.

[228] Ha SK, Moon E, Lee P, Ryu JH, Oh MS, Kim SY. Acacetin attenuates neuroinflammation via regulation the response to LPS stimuli in Vitro and in Vivo. Neurochem Res 2012;37:1560–7. https://doi.org/10.1007/s11064-012-0751-z.

[229] Costa ASG, Alves RC, Vinha AF, Barreira SVP, Nunes MA, Cunha LM, et al. Optimization of antioxidants extraction from coffee silverskin, a roasting by-product, having in view a sustainable process. Ind Crops Prod 2014;53:350–7. https://doi.org/10.1016/j.indcrop.2014.01.006.

[230] Soccio M, Laus MN, Flagella Z, Pastore D. Assessment of antioxidant capacity and putative healthy effects of natural plant products using soybean lipoxygenase-based methods. An overview. Molecules 2018;23. https://doi.org/10.3390/molecules23123244.

[231] Zengin G, Sinan KI, Mahomoodally MF, Angeloni S, Mustafa AM, Vittori S, et al. Chemical composition, antioxidant and enzyme inhibitory properties of different extracts obtained from spent coffee ground and coffee silverskin. Foods 2020;9. https://doi.org/10.3390/FOODS9060713.

[232] Rebollo-Hernanz M, Zhang Q, Aguilera Y, Martín-Cabrejas MA, de Mejia EG. Relationship of the phytochemicals from coffee and cocoa by-products with their potential to modulate biomarkers of metabolic syndrome in vitro. Antioxidants 2019;8. https://doi.org/10.3390/antiox8080279.

[233] Eddin LB, Jha NK, Meeran MFN, Kesari KK, Beiram R, Ojha S. Neuroprotective potential of limonene and limonene containing natural products. Molecules 2021;26. https://doi.org/10.3390/molecules26154535.

[234] 'Chistiane MF, 'Rivelilson M de F, 'Lidiane da SA, 'Valeria LS, 'Cassio HS de M, 'Felipe PDSS. Citrus A Prespective of Developing Phytomedicines for neuroprotective Diseases. In: Harsimran Gill, Harsh Garg, editors. Citrus Pathology, London: IntechOpen Limited; 2017.

[235] Barreca D, Gattuso G, Laganà G, Leuzzi U, Bellocco E. C- and O-glycosyl flavonoids in Sanguinello and Tarocco blood orange (Citrus sinensis (L.) Osbeck) juice: Identification and influence on antioxidant properties and acetylcholinesterase activity. Food Chem 2016;196:619–27. https://doi.org/10.1016/j.foodchem.2015.09.098.

[236] Rafiq S, Kaul R, Sofi SA, Bashir N, Nazir F, Ahmad Nayik G. Citrus peel as a source of functional ingredient: A review. Journal of the Saudi Society of Agricultural Sciences 2018;17:351–8. https://doi.org/10.1016/j.jssas.2016.07.006.

[237] Ademosun AO, Oboh G. Anticholinesterase and antioxidative properties of water-extractable phytochemicals from some citrus peels. J Basic Clin Physiol Pharmacol 2014;25:199–204. https://doi.org/10.1515/jbcpp-2013-0027.

[238] Rebollo-Hernanz M, Zhang Q, Aguilera Y, Martín-Cabrejas MA, de Mejia EG. Relationship of the phytochemicals from coffee and cocoa by-products with their potential to modulate biomarkers of metabolic syndrome in vitro. Antioxidants 2019;8. https://doi.org/10.3390/antiox8080279.

[239] Park JH, Jung JH, Yang JY, Kim HS. Olive leaf down-regulates the oxidative stress and immune dysregulation in streptozotocin-induced diabetic mice. Nutrition Research 2013;33:942–51. https://doi.org/10.1016/j.nutres.2013.07.011.

[240] Lins PG, Marina S, Pugine P, Arcio Scatolini AM, Pires De Melo M. In vitro antioxidant activity of olive leaf extract (Olea europaea L.) and its protective effect on oxidative damage in human erythrocytes. Heliyon 2018;4:e00805. https://doi.org/10.1016/j.heliyon.2018.

[241] Omar SH, Scott CJ, Hamlin AS, Obied HK. Olive biophenols reduces alzheimer's pathology in SH-SY5Y cells and APPswe mice. Int J Mol Sci 2019;20. https://doi.org/10.3390/ijms20010125.

[242] De La Cruz JP, Ruiz-Moreno MI, Guerrero A, Reyes JJ, Benitez-Guerrero A, Espartero JL, et al. Differences in the Neuroprotective Effect of Orally Administered Virgin Olive Oil (Olea europaea) Polyphenols Tyrosol and Hydroxytyrosol in Rats. J Agric Food Chem 2015;63:5957–63. https://doi.org/10.1021/acs.jafc.5b00627.

[243] Difonzo G, Russo A, Trani A, Paradiso VM, Ranieri M, Pasqualone A, et al. Green extracts from Coratina olive cultivar leaves: Antioxidant characterization and biological activity. J Funct Foods 2017;31:63–70. https://doi.org/10.1016/j.jff.2017.01.039.

[244] Bulotta S, Celano M, Lepore SM, Montalcini T, Pujia A, Russo D. Beneficial effects of the olive oil phenolic components oleuropein and hydroxytyrosol: Focus on protection against cardiovascular and metabolic diseases. J Transl Med 2014;12. https://doi.org/10.1186/s12967-014-0219-9.

[245] Gallego R, Suárez-Montenegro ZJ, Ibáñez E, Herrero M, Valdés A, Cifuentes A. In vitro Neuroprotective Potential and Lipidomics Study of Olive Leaves Extracts Enriched in Triterpenoids. Front Nutr 2021;8. https://doi.org/10.3389/fnut.2021.769218.

[246] Suárez-Montenegro ZJ, Álvarez-Rivera G, Sánchez-Martínez JD, Gallego R, Valdés A, Bueno M, et al. Neuroprotective effect of terpenoids recovered from olive oil by-products. Foods 2021;10. https://doi.org/10.3390/foods10071507.

[247] El-Baz FK, Salama A, Salama RAA, Gabr M. Dunaliella salina Attenuates Diabetic Neuropathy Induced by STZ in Rats: Involvement of Thioredoxin. Biomed Res Int 2020;2020. https://doi.org/10.1155/2020/1295492.

[248] Mohamad Agus Salim, Muhammad Subandi, Yeni Yuniarti. Neuroprotective Efficacy of Dunaliella salina Against ParaquatInduced Neurotoxicity in Drosophila melanogaster. Jordan Journal of Biological Science 2021;14:239–43. https://doi.org/10.54319/jjbs/140206.

[249] Bueno M, Vitali C, Sánchez-Martínez JD, Mendiola JA, Cifuentes A, Ibáñez E, et al. Compressed CO2 Technologies for the Recovery of Carotenoid-Enriched Extracts from Dunaliella salina with Potential Neuroprotective Activity. vol. 8. 2020. https://doi.org/10.1021/acssuschemeng.0c03991.

[250] El-Baz FK, Abdel Jaleel GA, Hussein RA, Saleh DO. Dunalialla salina microalgea and its isolated zeaxanthin mitigate age-related dementia in rats: Modulation of neurotransmission and amyloid-β protein. Toxicol Rep 2021;8:1899–908. https://doi.org/10.1016/j.toxrep.2021.11.021.

[251] Hassan SHA, Bakar MFA. Antioxidative and anticholinesterase activity of cyphomandra betacea fruit. The Scientific World Journal 2013;2013. https://doi.org/10.1155/2013/278071.

[252] Li N, Li W. Cytotoxicity and Anti-Inflammatory Activity of Tamarillo (Solanum betaceum Cav.) Peel Extract in Lipopolysaccharide Stimulated RAW 264.7 Cells. E-GiGi 2021;9. https://doi.org/10.35790/eg.9.1.2021.32847.

[253] Wang S, Zhu F. Tamarillo (Solanum betaceum): Chemical composition, biological properties, and product innovation. Trends Food Sci Technol 2020;95:45–58. https://doi.org/10.1016/j.tifs.2019.11.004.

[254] Suárez-Montenegro ZJ, Ballesteros-Vivas D, Gallego R, Valdés A, Sánchez-Martínez JD, Parada-Alfonso F, et al. Neuroprotective Potential of Tamarillo (Cyphomandra betacea) Epicarp Extracts Obtained by Sustainable Extraction Process. Front Nutr 2021;8. https://doi.org/10.3389/fnut.2021.769617.

[255] Salazar MAR, Costa JV, Urbina GRO, Cunha VMB, Silva MP, Bezerra P do N, et al. Chemical composition, antioxidant activity, neuroprotective and anti-inflammatory effects of cipó-pucá (Cissus sicyoides L.) extracts obtained from supercritical extraction. Journal of Supercritical Fluids 2018;138:36–45. https://doi.org/10.1016/j.supflu.2018.03.022.

[256] Santos DT, Vardanega R, Albarelli JQ, Ensinas A V., Maréchal F, Meireles MAA. Energy consumption versus antioxidant activity of pressurized fluid extracts from pfaffia glomerata roots. Chemical Engineering Transactions, vol. 35, Italian Association of Chemical Engineering - AIDIC; 2013, p. 1099–104. https://doi.org/10.3303/CET1335183.

[257] Costa ARM, Freitas LAP, Mendiola J, Ibáñez E. Copaifera langsdorffii supercritical fluid extraction: Chemical and functional characterization by LC/MS and in vitro assays. Journal of Supercritical Fluids 2015;100:86–96. https://doi.org/10.1016/j.supflu.2015.02.028.

[258] Zhang Y, Yang J, Yu YX. Dielectric constant and density dependence of the structure of supercritical carbon dioxide using a new modified empirical potential model: A Monte Carlo simulation study. Journal of Physical Chemistry B 2005;109:13375–82. https://doi.org/10.1021/jp045741r.

[259] Sengwa RJ, Chaudhary R, Mehrotra SC. Static Dielectric Constant and Relaxation Time for the Binary Mixture of Water, Ethanol, N,N-Dimethylformamide, Dimethylsulphoxide, and N,N-Dimethylacetamide with 2-Methoxyethanol. Korean Chemical Scociety 2001:357–61. https://doi.org/org/10.5012/bkcs.2001.22.4.357.

[260] Chien YW. Solubilization of Metronidazole by Water-Miscible Multi-Cosolvents and Water-Soluble Vitamins. Journal of Pharmaceutical Science and Technology 1984;38:32–6.

[261] Martinelli T, Whittaker A, Benedettelli S, Carboni A, Andrzejewska J. The study of flavonolignan association patterns in fruits of diverging Silybum marianum (L.) Gaertn. chemotypes provides new insights into the silymarin biosynthetic pathway. Phytochemistry 2017;144:9–18. https://doi.org/10.1016/j.phytochem.2017.08.013.

[262] Machmudah S, Wahyudiono, Kanda H, Goto M. Hydrolysis of Biopolymers in Near-Critical and Subcritical Water. Elsevier Inc.; 2017. https://doi.org/10.1016/B978-0-12-809380-1.00003-6.

[263] Koşar M, Dorman HJD, Hiltunen R. Effect of an acid treatment on the phytochemical and antioxidant characteristics of extracts from selected Lamiaceae species. Food Chemistry 2005;91:525–33. https://doi.org/10.1016/j.foodchem.2004.06.029.

[264] Woisky RG, Salatino A. Analysis of propolis: Some parameters and procedures for chemical quality control. Journal of Apicultural Research 1998;37:99–105. https://doi.org/10.1080/00218839.1998.11100961.

[265] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric Method for Determination of Sugars and Related Substances. Analytical Chemistry, 1956, p. 350–6. https://doi.org/doi.org/10.1021/ac60111a017.

[266] Whent M, Ping T, Kenworthy W, Yu L. High-throughput assay for detection of soybean lipoxygenase-1. Journal of Agricultural and Food Chemistry 2010;58:12602–7. https://doi.org/10.1021/jf1028784.

[267] Brand-Williams W, Cuvelier ME, Berset C. Use of a Free Radical Method to Evaluate Antioxidant Activity. Lebensmittel-Wissenschaft Und -Technologie- Food Science and Technology 1995;28:25–30. https://doi.org/10.1016/S0023-6438(95)80008-5.

[268] Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine 1999;26:1231–7.

[269] Ballesteros-Vivas D, Álvarez-Rivera G, Ibáñez E, Parada-Alfonso F, Cifuentes A. A multi-analytical platform based on pressurized-liquid extraction, in vitro assays and liquid chromatography/gas chromatography coupled to high resolution mass spectrometry for food by-products valorisation. Part 2: Characterization of bioactive compound. Journal of Chromatography A 2019;1584:144–54. https://doi.org/10.1016/j.chroma.2018.11.054.

[270] Aron AT, Gentry EC, McPhail KL, Nothias LF, Nothias-Esposito M, Bouslimani A, et al. Reproducible molecular networking of untargeted mass spectrometry data using GNPS. Nature Protocols 2020;15:1954–91. https://doi.org/10.1038/s41596-020-0317-5.

[271] Kim HW, Wang M, Leber CA, Nothias LF, Reher R, Kang K Bin, et al. NPClassifier: A Deep Neural Network-Based Structural Classification Tool for Natural Products. Journal of Natural Products 2021;84:2795–807. https://doi.org/10.1021/acs.jnatprod.1c00399.

[272] Setyaningsih W, Saputro IE, Palma M, Barroso CG. Pressurized liquid extraction of phenolic compounds from rice (Oryza sativa) grains. Food Chemistry 2016;192:452–9. https://doi.org/10.1016/j.foodchem.2015.06.102.

[273] de Santana ÁL, Osorio-Tobón JF, Cárdenas-Toro FP, Steel CJ, Meireles MA de A. Partial-hydrothermal hydrolysis is an effective way to recover bioactives from turmeric wastes. Food Science and Technology 2018;38:280–92. https://doi.org/10.1590/1678-457X.22016.

[274] Plaza M, Amigo-Benavent M, del Castillo MD, Ibáñez E, Herrero M. Neoformation of antioxidants in glycation model systems treated under subcritical water extraction conditions. Food Research International 2010;43:1123–9. https://doi.org/10.1016/j.foodres.2010.02.005.

[275] Plaza M, Abrahamsson V, Turner C. Extraction and neoformation of antioxidant compounds by pressurized hot water extraction from apple byproducts. Journal of Agricultural and Food Chemistry 2013;61:5500–10. https://doi.org/10.1021/jf400584f.

[276] Mašković PZ, Veličković V, Đurović S, Zeković Z, Radojković M, Cvetanović A, et al. Biological activity and chemical profile of Lavatera thuringiaca L. extracts obtained by different extraction approaches. Phytomedicine 2018;38:118–24. https://doi.org/10.1016/j.phymed.2017.11.010.

[277] Soria AC, Sanz ML, Villamiel M. Determination of minor carbohydrates in carrot (Daucus carota L.) by GC-MS. Food Chemistry 2009;114:758–62. https://doi.org/10.1016/j.foodchem.2008.10.060.

[278] Song J, Yan Y, Wang X, Li X, Chen Y, Li L, et al. Characterization of fatty acids, amino acids and organic acids in three colored quinoas based on untargeted and targeted metabolomics. LWT 2021;140. https://doi.org/10.1016/j.lwt.2020.110690.

[279] Mišan AČ, Mimica-Dukić NM, Mandić AI, Sakač MB, Milovanović IL, Sedej IJ. Development of a Rapid Resolution HPLC method for the separation and determination of 17 phenolic compounds in crude plant extracts. Central European Journal of Chemistry 2011;9:133–42. https://doi.org/10.2478/s11532-010-0126-8.

[280] Farag MA, Sharaf El-Din MG, Aboul-Fotouh Selim M, Owis AI, Abouzid SF. Mass spectrometry-based metabolites profiling of nutrients and anti-nutrients in major legume sprouts. Food Bioscience 2021;39. https://doi.org/10.1016/j.fbio.2020.100800.

[281] Pi JJ, Wu X, Rui W, Feng YF, Guo J. Identification and fragmentation mechanisms of two kinds of chemical compositions in Eucommia ulmoides by UPLC-ESI-Q-TOF-MS/MS. Chemistry of Natural Compounds 2016;52:144–8. https://doi.org/10.1007/s10600-016-1574-y.

[282] Barnaba C, Nardin T, Pierotti A, Malacarne M, Larcher R. Targeted and untargeted characterisation of free and glycosylated simple phenols in cocoa beans

using high resolution-tandem mass spectrometry (Q-Orbitrap). Journal of Chromatography A 2017;1480:41–9. https://doi.org/10.1016/j.chroma.2016.12.022.

[283] Bettaieb Rebey I, Bourgou S, Ben Slimen Debez I, Jabri Karoui I, Hamrouni Sellami I, Msaada K, et al. Effects of Extraction Solvents and Provenances on Phenolic Contents and Antioxidant Activities of Cumin (Cuminum cyminum L.) Seeds. Food and Bioprocess Technology 2012;5:2827–36. https://doi.org/10.1007/s11947-011-0625-4.

[284] Fan JJ, Li CH, Hu YJ, Chen H, Yang FQ. Comparative assessment of in vitro thrombolytic and fibrinolysis activity of four aloe species and analysis of their phenolic compounds by LC–MS. South African Journal of Botany 2018;119:325–34. https://doi.org/10.1016/j.sajb.2018.10.001.

[285] Lim HS, Kim OS, Kim BY, Jeong SJ. Apigetrin from scutellaria baicalensis georgi inhibits neuroinflammation in BV-2 microglia and exerts neuroprotective effect in HT22 hippocampal cells. Journal of Medicinal Food 2016;19:1032–40. https://doi.org/10.1089/jmf.2016.0074.

[286] Silva AS, Reboredo-Rodríguez P, Süntar I, Sureda A, Belwal T, Loizzo MR, et al. Evaluation of the status quo of polyphenols analysis: Part I—phytochemistry, bioactivity, interactions, and industrial uses. Comprehensive Reviews in Food Science and Food Safety 2020;19:3191–218. https://doi.org/10.1111/1541-4337.12629.

[287] Antunes-Ricardo M, García-Cayuela T, Mendiola JA, Ibañez E, Gutiérrez-Uribe JA, Cano MP, et al. Supercritical CO2 enzyme hydrolysis as a pretreatment for the release of isorhamnetin conjugates from Opuntia ficus-indica (L.) Mill. Journal of Supercritical Fluids 2018;141:21–8. https://doi.org/10.1016/j.supflu.2017.11.030.

[288] Machado A, Bento AFML, Rubem M, Scheffel TB, Rockenbach L, Mendonça F, et al. Use of supercritical CO2 to obtain Baccharis uncinella extracts with antioxidant and antitumor activity. CO2 Utilization 2021;49. https://doi.org/10.1016/j.jcou.2021.101563.

[289] Putra NR, Idham ZB, Machmudah S, Hazwan S, Azizi M, Yunus C. Extraction of peanut skin oil by modified supercritical carbon dioxide : Empirical modelling and optimization. Separation Science and Technology 2018;53:2695–703. https://doi.org/10.1080/01496395.2018.1459705.

[290] Zou X, Liu Y, Tao C, Liu Y, Liu M, Wu J, et al. CO2 supercritical fluid extraction and characterization of polysaccharide from bamboo (Phyllostachys heterocycla) leaves. Journal of Food Measurement and Characterization 2018;12:35–44. https://doi.org/10.1007/s11694-017-9614-2.

[291] Ratiu IA, Al-Suod H, Ligor M, Ligor T, Railean-Plugaru V, Buszewski B. Complex investigation of extraction techniques applied for cyclitols and sugars isolation from different species of Solidago genus. Electrophoresis 2018;39:1966–74. https://doi.org/10.1002/elps.201700419.

[292] Gong T, Liu S, Wang H, Zhang M. Supercritical CO2 fluid extraction, physicochemical properties, antioxidant activities and hypoglycemic activity of polysaccharides derived from fallen Ginkgo leaves. Food Bioscience 2021;42:101153. https://doi.org/10.1016/j.fbio.2021.101153.

[293] Dos Santos TC, Gomes TM, Pinto BAS, Camara AL, De Andrade Paes AM. Naturally occurring acetylcholinesterase inhibitors and their potential use for Alzheimer's disease therapy. Frontiers in Pharmacology 2018;9. https://doi.org/10.3389/fphar.2018.01192.

[294] N. Rajha H, Darra N El, Vorobiev E, Louka N, Maroun RG. An Environment Friendly, Low-Cost Extraction Process of Phenolic Compounds from Grape Byproducts. Optimization by Multi-Response Surface Methodology. Food and Nutrition Sciences 2013;04:650–9. https://doi.org/10.4236/fns.2013.46084.

[295] Vera Candioti L, De Zan MM, Cámara MS, Goicoechea HC. Experimental design and multiple response optimization. Using the desirability function in analytical methods development. Talanta 2014;124:123–38. https://doi.org/10.1016/j.talanta.2014.01.034.

[296] Soulaimani B, Laghzaoui EM, Babram MA, Spooner-Hart R, Hassani L, Abbad A. Optimization of the insecticidal response of combined essential oils from Satureja alpina, Rosmarinus officinalis and Ammodaucus leucotrichus using a designed mixtures approach. International Journal of Tropical Insect Science 2022;42:2501–12. https://doi.org/10.1007/s42690-022-00778-7.

[297] Jung K-H, Yoo SK, Moon S-K, Lee U-S. Furfural from Pine Needle Extract Inhibits the Growth of a Plant Pathogenic Fungus, Alternaria mali. Mycobiology 2007;35:39. https://doi.org/10.4489/myco.2007.35.1.039.

[298] Bazalar Pereda MS, Nazareno MA, Viturro CI. Volatile compound profile and sensory features of cape gooseberry (Physalis peruviana Linnaeus): comparative study between cultivated and wild fruits. European Food Research and Technology 2022;249:1007–21. https://doi.org/10.1007/s00217-022-04191-9.

[299] Li W, Dou D, Koike K. Revised absolute stereochemistry of rhodiolosides A-D, rhodiolol A and sachalinol A from Rhodiola rosea. Chemical and Pharmaceutical Bulletin 2008;56:1047–8. https://doi.org/10.1248/cpb.56.1047.

[300] Hazrati S, Salimi-Sabour E, Mojab F, Talebi SM, Matsyura A. Phytochemical investigation of Carex praecox (C.A. Mey.) Rech. f.(Asteraceae). Acta Biologica Sibirica 2022;184:175–84. https://doi.org/10.5281/ zenodo.7686835.

[301] Idm'hand E, Msanda F, Cherifi K. Medicinal uses, phytochemistry and pharmacology of Ammodaucus leucotrichus. Clinical Phytoscience 2020;6. https://doi.org/10.1186/s40816-020-0154-7.

[302] Karabagias IK, Koutsoumpou M, Liakou V, Kontakos S, Kontominas MG. Characterization and geographical discrimination of saffron from Greece, Spain, Iran, and Morocco based on volatile and bioactivity markers, using chemometrics. European Food Research and Technology 2017;243:1577–91. https://doi.org/10.1007/s00217-017-2866-6.

[303] Suresh S, Pradheesh G, Ramani VA. Phytochemical Screening and GCMS Studies of the Medicinal Plant Pavetta indica Linn. American Journal of Ethnomedicine 2015;2:347–55.

[304] Zidorn C. Bioprospecting of plant natural products in Schleswig-Holstein (Germany) I: chemodiversity of the Cichorieae tribe (Asteraceae) in Schleswig-Holstein. vol. 18. Springer Netherlands; 2019. https://doi.org/10.1007/s11101-019-09609-z.

[305] Wang M, Carver JJ, Phelan V V., Sanchez LM, Garg N, Peng Y, et al. Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. Nature Biotechnology 2016;34:828–37. https://doi.org/10.1038/nbt.3597.

[306] Wang L, Li D, Bao C, You J, Wang Z, Shi Y, et al. Ultrasonic extraction and separation of anthraquinones from Rheum palmatum L. Ultrasonics Sonochemistry 2008;15:738–46. https://doi.org/10.1016/j.ultsonch.2007.12.008.

[307] Abderrezag N, David J, Louaer O, Meniai A, Mendiola JA. Optimization of Pressurized Liquid Extraction and In Vitro Neuroprotective Evaluation of Ammodaucus leucotrichus. Untargeted Metabolomics Analysis by UHPLC-MS/MS. Molecules 2021;26. https://doi.org/10.3390/molecules26226951.

[308] Lang-Illievich K, Klivinyi C, Lasser C, Brenna CTA, Szilagyi IS, Bornemann-Cimenti H. Palmitoylethanolamide in the Treatment of Chronic Pain: A Systematic Review and Meta-Analysis of Double-Blind Randomized Controlled Trials. Nutrients 2023;15:1350. https://doi.org/10.3390/nu15061350.

[309] Okai Y, Higashi-Okai K. Potent anti-inflammatory activity of pheophytin a derived from edible green alga, Enteromorpha prolifera (Sujiao-nori). International Journal of Immunopharmacology 1997;19:355–8. https://doi.org/10.1016/S0192-0561(97)00070-2.

[310] Golmakani MT, Mendiola JA, Rezaei K, Ibáñez E. Expanded ethanol with CO 2 and pressurized ethyl lactate to obtain fractions enriched in γ-Linolenic Acid from Arthrospira platensis (Spirulina). Journal of Supercritical Fluids 2012;62:109–15. https://doi.org/10.1016/j.supflu.2011.11.026.

[311] Paula JT, Paviani LC, Foglio MA, Sousa IMO, Cabral FA. Extraction of anthocyanins from Arrabidaea chica in fixed bed using CO2 and CO2/ethanol/water

mixtures as solvents. Journal of Supercritical Fluids 2013;81:33–41. https://doi.org/10.1016/j.supflu.2013.04.009.

[312] Zulkafli ZD, Wang H, Miyashita F, Utsumi N, Tamura K. Cosolvent-modified supercritical carbon dioxide extraction of phenolic compounds from bamboo leaves (Sasa palmata). Journal of Supercritical Fluids 2014;94:123–9. https://doi.org/10.1016/j.supflu.2014.07.008.

[313] Dalmolin I, Skovroinski E, Biasi A, Corazza ML, Dariva C, Oliveira JV. Solubility of carbon dioxide in binary and ternary mixtures with ethanol and water. Fluid Phase Equilibria 2006;245:193–200. https://doi.org/10.1016/j.fluid.2006.04.017.

[314] Fornari T, Hernández EJ, Ruiz-Rodriguez A, Javier Señorans F, Reglero G. Phase equilibria for the removal of ethanol from alcoholic beverages using supercritical carbon dioxide. Journal of Supercritical Fluids 2009;50:91–6. https://doi.org/10.1016/j.supflu.2009.05.012.

[315] Kritzer P, Dinjus E. An assessment of supercritical water oxidation (SCWO) Existing problems, possible solutions and new reactor concepts. vol. 83. 2001.

[316] Andrzejewska J, Sadowska K, Mielcarek S. Effect of sowing date and rate on the yield and flavonolignan content of the fruits of milk thistle (Silybum marianum L. Gaertn.) grown on light soil in a moderate climate. Industrial Crops and Products 2011;33:462–8. https://doi.org/10.1016/j.indcrop.2010.10.027.

[317] Stancheva I, Georgiev G, Geneva M, Ivanova A, Dolezal M, Tumova L. Influence of foliar fertilization and growth regulator on milk thistle seed yield and quality. Journal of Plant Nutrition 2010;33:818–30. https://doi.org/10.1080/01904161003654063.

[318] Saleh IA, Vinatoru M, Mason TJ, Abdel-Azim NS, Shams KA, Aboutabl E, et al. Extraction of silymarin from milk thistle (Silybum marianum) seeds–a comparison of conventional and microwave-assisted extraction methods. Journal of Microwave Power and Electromagnetic Energy 2017;51:124–33. https://doi.org/10.1080/08327823.2017.1320265.

[319] Wahid M, Ali A, Saqib F, Aleem A, Bibi S, Afzal K, et al. Pharmacological exploration of traditional plants for the treatment of neurodegenerative disorders. Phytotherapy Research 2020;34:3089–112. https://doi.org/10.1002/ptr.6742.

[320] Dos Santos TC, Gomes TM, Pinto BAS, Camara AL, De Andrade Paes AM. Naturally occurring acetylcholinesterase inhibitors and their potential use for Alzheimer's disease therapy. Frontiers in Pharmacology 2018;9. https://doi.org/10.3389/fphar.2018.01192.

[321] Nazir N, Karim N, Abdel-Halim H, Khan I, Wadood SF, Nisar M. Phytochemical analysis, molecular docking and antiamnesic effects of methanolic extract of Silybum marianum (L.) Gaertn seeds in scopolamine induced memory impairment in mice. Journal of Ethnopharmacology 2018;210:198–208. https://doi.org/10.1016/j.jep.2017.08.026.

[322] Donald Armstrong, Robert D. Stratton. Oxidative Stress and Antioxidant Protection The Science of Free Radical Biology and Disease. John Wiley Sons, Inc.; 2016. https://doi.org/doi/book/10.1002/9781118832431.

[323] Nowak A, Florkowska K, Zielonka-Brzezicka J, Duchnik W, Muzykiewicz A, Klimowicz A. the Effects of Extraction Techniques on the Antioxidant Potential of Extracts of Different Parts of Milk Thistle (Silybum Marianum L.). Acta Scientiarum Polonorum, Technologia Alimentaria 2021;20:37–46. https://doi.org/10.17306/J.AFS.2021.0817.

[324] Suárez-Montenegro ZJ, Álvarez-Rivera G, Sánchez-Martínez JD, Gallego R, Valdés A, Bueno M, et al. Neuroprotective effect of terpenoids recovered from olive oil by-products. Foods 2021;10. https://doi.org/10.3390/foods10071507.

APPENDIX

APPENDIX

Appendix A.

-Reagents, chemical products and enzymes-

Table S.1: Reagents used in this work.

Chemical product	Company	Purity	Masse (g/mol)
2,2-diphenyl-1-picrylhydrazyl (DPPH)	Sigma Aldrich, made in Spain	99,99%	394.32
2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt	Sigma Aldrich, made in USA	98%	548.68
(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox)	Sigma Aldrich	97%	250.29
7-Fluorobenzofurazan-4-sulfonamide ABD-F	Alfa Aesar, Thermo Fisher scientific, Japan.	98 %	217.17
Ethanol absolute	VWR CHEMICAL, France	99.5 %	46.07
Methanol	VWR CHEMICALS, Netherlands.	99 %	
Hexane mixture of isomers	VWR CHEMICALS, made in Poland	99 %	86.18
Dimethyl sulphoxide (DMSO)	VWR CHEMICAL, France.	99 %	78.14
Formic acid	VWR CHEMICAL,EC	99 %	46.03

BUTYLATED HYDROXYTOLUENE (BHT) C15H24O	Sigma Aldrich, USA	99 %	
Acetylthiocholine iodide ACth	Sigma Aldrich, Germany	>98 %	289.18
Trizma base (2-amino-2-(hydroxymethyl)-1,3-propanediol) C4H11NO3	Sigma aldrich, USA	>99.9 %	121.14
Linoleic acid (LA)	Sigma aldrich, USA	>99.9 %	280.45
Galantamine hydrobromide	TCI, Japan.	>98.0 %	368.27
Fluorescein sodium salt	Sigma Aldrich, USA	99 %	376.27
Sand 0.1-0.6mm (SIO2)	LabKem, Bercelona Spain.		60.1
Folin-Ciocalteu's phenol reagent	Merck KGaA , Germany	-	-
Galic acid	Sigma-Aldrich, made in Steinheim, Germany	-	170.12
Aluminum chloride anhydrous AlCl3	Merck KGaA , made in Germany	-	241.43
phenol	Sigma Aldrich, USA		94.11
Sulfric acid	VWR CHEMICAL, EC	95 %	
D-(+)-Glucose	Sigma Aldrich, Germany		180.16

Acetonitrile	VWR chemicals (Radnor, Pensilvania, USA)	99 %	41.05
Soduim carbonate	AppliChem Panreac ITW Companies, made in Barcelona, Spain	-	105.99
Quercetin anhydrous	Sigma-Aldrich (Madrid, Spain)		302.24
Ascorbic acid	Sigma-Aldrich (Madrid, Spain)		
Monopotassium phosphate (KH2PO4)		≥99.0 %,	
Soduim phosphate dibasic Na2HPO4		≥99.0 %,	
Standards			
ISOSLYBIN	Chengdu Biopurify Phytochemicals Ltd, made in China.	98 %	482.441
SILYCHRISTIN	Chengdu Biopurify Phytochemicals Ltd, made in China.	98 %	482.441
SIYDIANIN	Chengdu Biopurify Phytochemicals Ltd, made in China.	98 %	
SILYBIN A+B	Chengdu Biopurify Phytochemicals Ltd, made in China.	98 %	
TAXIFOLIN	TargetMol	98.90 %	304.25
Enzyme			

Acetylcholinesterase (AChE) from Electrophorus electricus (electric eel) type VI-S	Sigma Aldrich, USA		
Lipoxidase from glycine max(soybean) type 1-B	Sigma Aldrich, UK		

Appendix B.

-Supplemetary results-

Table S.2: Extraction yield, total Phenolics (mg GAE/g extract) and total carbohydrates (mg/g extract) determined in the pressurized liquid extracts of Ammodaucus leucotrichus fruits obtained using 10 min of extraction time at the indicated conditions.

Solvent	Temperature (°C)	Total yield (%, w/w)	Total Phenolics (mg GAE/g Extract)	Total Carbohydrates (mg/g Extract)
Ethanol	40	15.55 ± 0,89^{a}	22.3 ± 0.3^{a}	133.63 ± 13.42^{a}
	110	21.61 ± 1.70^{b}	25.8 ± 0.4^{c}	177.52 ± 1.24^{a}
	180	28.71 ± 0.36^{d}	23.6 ± 0.4^{b}	165.02 ± 1.60^{a}
Water	40	24.38 ± 0.70^{c}	23.6 ± 0.5^{b}	160.34 ± 2.18^{a}
	110	27.25 ± 0.25^{d}	26.0 ± 0.5^{c}	342.12 ± 6.39^{b}
	180	44.44 ± 0.82^{e}	43.5 ± 0.8^{d}	489.36 ± 6.64^{c}

Note: Each data point represents the mean ± SD of replicates. Different superscript letters indicate statistically significant differences ($p < 0.05$) per column.

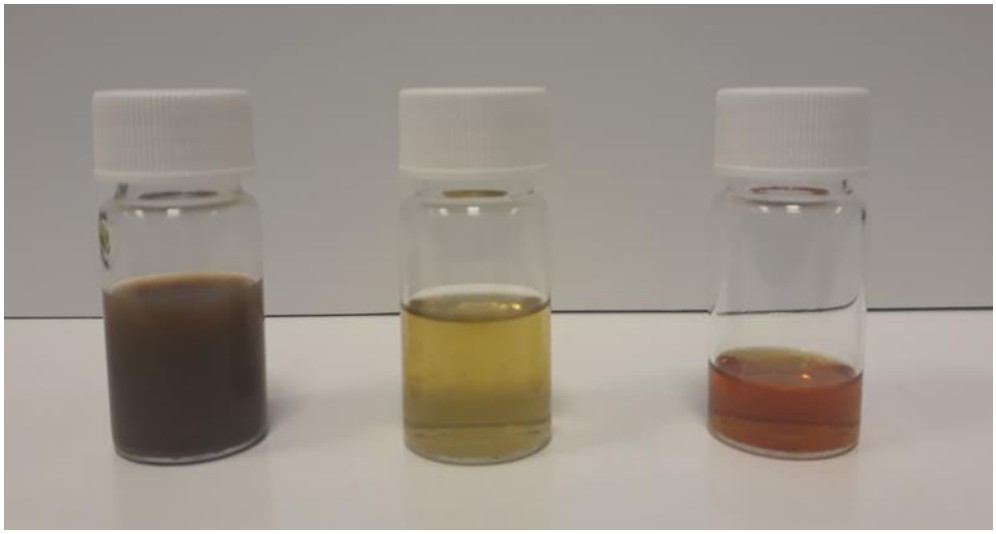

Figure S.1: Ammodaucus leucotrichus extracts obtained using biorefinery approach. From left to right, Extract 1, Extract 2.1 and Extract 1.1.

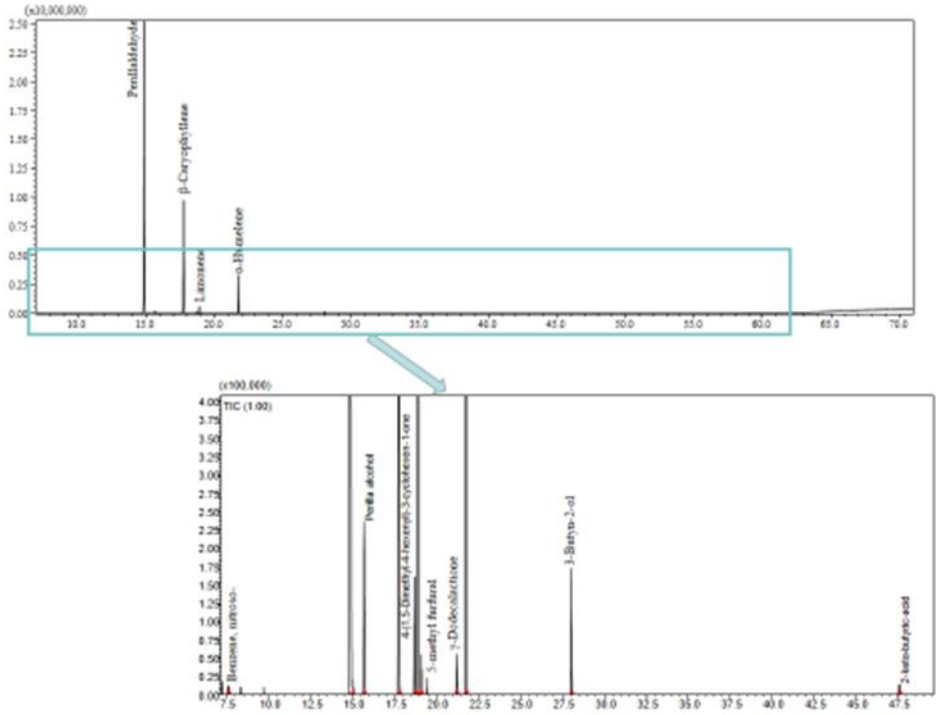

Figure S.2: GC-MS spectra of the optimal point of SFE.

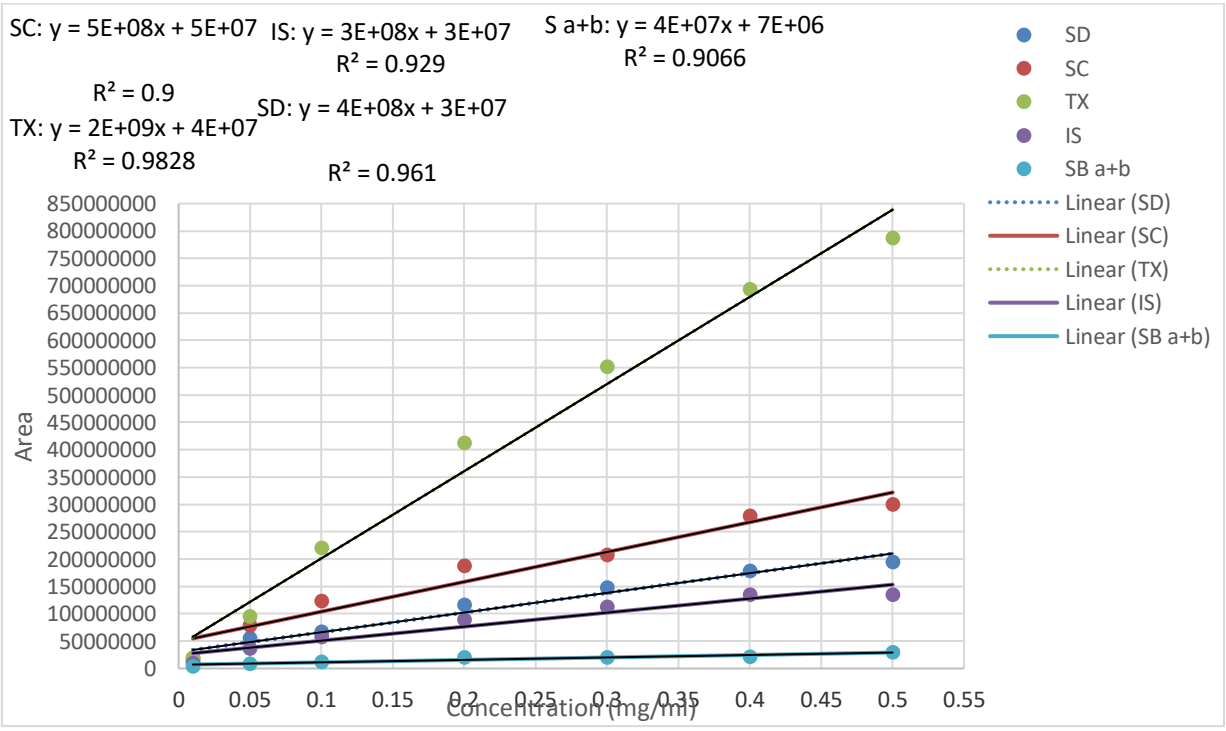

Figure S.3: Calibration curves of Silymarin compounds.

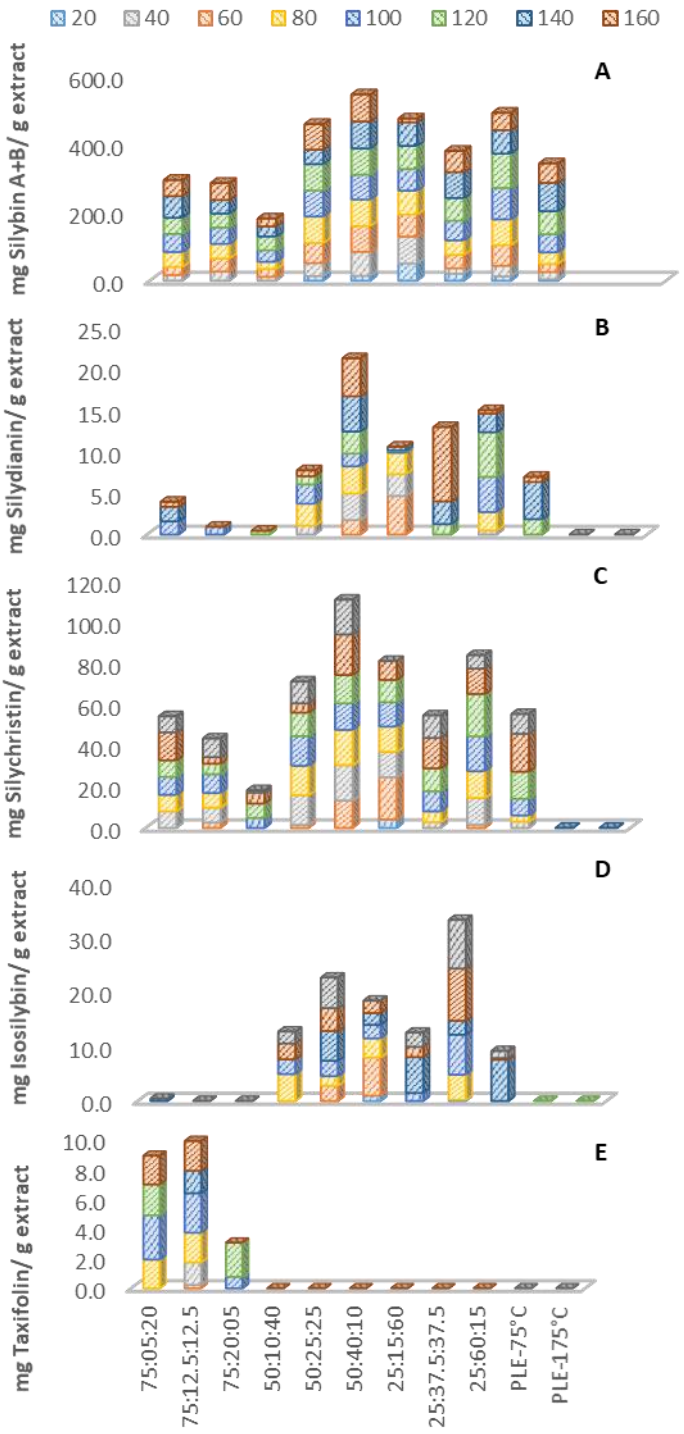

Figure S.4: Silymarin and taxifolin in *SM* extracts quantified by liquid chromatography mass spectrometry (UHPLC-ESI-MS/MS). (A): Silybin $_{(A+B)}$, (B): Silydianin, (C): silychristin, (D): isosilybin and (E): taxifolin.

Appendix

For Yield

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
A:Pressure	43,6837	1	43,6837	8,58	0,0327
B:Temperature	12,0234	1	12,0234	2,36	0,1850
C:% ETOH	38,0803	1	38,0803	7,48	0,0411
AA	0,113492	1	0,113492	0,02	0,8872
AB	25,4848	1	25,4848	5,00	0,0755
AC	1,75271	1	1,75271	0,34	0,5830
BB	5,13787	1	5,13787	1,01	0,3613
BC	0,638241	1	0,638241	0,13	0,7378
CC	9,35513	1	9,35513	1,84	0,2334
Total error	25,4687	5	5,09375		
Total (corr.)	162,941	14			

For TPC

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
A:Pressure	10,5699	1	10,5699	1,82	0,2351
B:Temperature	181,27	1	181,27	31,22	0,0025
C:% ETOH	186,757	1	186,757	32,17	0,0024
AA	0,411683	1	0,411683	0,07	0,8006
AB	0,131298	1	0,131298	0,02	0,8863
AC	16,3592	1	16,3592	2,82	0,1541
BB	2,60196	1	2,60196	0,45	0,5329
BC	6,43865	1	6,43865	1,11	0,3405
CC	57,3081	1	57,3081	9,87	0,0256
Total error	29,0287	5	5,80573		
Total (corr.)	488,932	14			

For Total Carbohydrate

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
A:Pressure	5941,32	1	5941,32	0,18	0,6868
B:Temperature	115539,	1	115539,	3,55	0,1181
C:% ETOH	945377,	1	945377,	29,08	0,0030
AA	374759,	1	374759,	11,53	0,0193
AB	51,0796	1	51,0796	0,00	0,9699
AC	52562,7	1	52562,7	1,62	0,2594
BB	2102,5	1	2102,5	0,06	0,8094
BC	2665,71	1	2665,71	0,08	0,7861
CC	80889,0	1	80889,0	2,49	0,1755
Total error	162521,	5	32504,2		
Total (corr.)	1,72828E6	14			

For AChE

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
A:Pressure	12,1796	1	12,1796	0,30	0,6370
B:Temperature	56601,0	1	56601,0	1410,78	0,0007
C:% ETOH	52241,6	1	52241,6	1302,12	0,0008
AA	19860,0	1	19860,0	495,01	0,0020
AB	2964,53	1	2964,53	73,89	0,0133
AC	4877,21	1	4877,21	121,56	0,0081
BB	6299,1	1	6299,1	157,00	0,0063
BC	40134,7	1	40134,7	1000,36	0,0010
CC	14267,8	1	14267,8	355,62	0,0028
Lack-of-fit	44732,8	3	14910,9	371,65	0,0027
Pure error	80,2408	2	40,1204		
Total (corr.)	245198,	14			

Figure S.5: Analysis of variance for diffrent single response.

	Estimate cofficients values			
Coefficient	*For yield*	*For TPC*	*For TCC*	*For AChE*
constant	37,7668	-51,1569	1280,69	2482,86
A:Pressure	-0,0894355	0,0719406	-14,8946	1,22468
B:Temperature	-0,884784	0,921066	15,7595	-42,7862
C:% ETOH	1,15204	5,85738	-114,441	-153,316
AA	0,0000175321	-0,0000333912	0,0318586	-0,00733399
AB	0,00168275	-0,000120783	0,00238233	0,0181492
AC	0,0013239	-0,00404465	0,229265	0,069837
BB	0,00524276	-0,00373094	-0,106056	0,183573
BC	0,005326	-0,0169163	0,344203	1,33558
CC	-0,0636702	-0,157587	5,92046	2,4865

Figure S.6: Regression coeffisions of single diffrent response.

Factor	*Low*	*High*	*Optimum for maximize the yield*	*Optimum for maximize the TPC*	*Optimum for maximize the TCC*	*Optimum for minimize the IC50 of AChE*
Pressure	100,0	300,0	300,0	236,086	299,688	156,955
Temperature	40,0	70,0	69,699	70,0	70,0	40,0
% ETOH	5,0	15,0	14,8346	11,7991	15,0	5,0
Optimum value of single response			25,9775	30,0972	1319,25	808,12

Figure S.7: Optimums of single diffrent response.

Appendix C.

-Scientific production-

Publications

- **N. Abderrezag**, J. D. Sánchez-Martínez, O. Louaer, A. H. Meniai, J. A. Mendiola. **Optimization of Pressurized Liquid Extraction and In Vitro Neuroprotective Evaluation of Ammodaucus leucotrichus. Untargeted Metabolomics Analysis by UHPLC-MS/MS.** Molecules 2021, 26, 6951.
- **N. Abderrezag**, Z. J. Suárez Montenegro, O. Louaer, A. H. Meniai, A. Cifuentes, E. Ibáñez, J. A. Mendiola. **One-step sustainable extraction of Silymarin compounds of wild Algerian milk thistle (Silybum marianum) seeds using Gas Expanded Liquids.** Journal of Chromatography A 1675 (2022) 463147.
- **N. Abderrezag**, F. Sanchez Bragagnolo, O. Louaer, A. H. Meniai, A. Cifuentes, E. Ibáñez, J. A. Mendiola. **Optimization of supercritical fluid extraction of bioactive compounds from Ammodaucus leucotrichus fruits by using multivariate response surface methodology.** Journal of Supercritical fluids. (**Accepted 05/02/2024).**

Oral communications

- 6th PATh Spring workshop, September 30th 2021, Aveiro, Portugal.

Poster communications

- **2nd European Sample Preparation Conference EuSP2022** (online event), March, 14th -16th 2022.
- 2nd Iberian meeting on supercritical fluids (online event), from 28 February to 2 March 2022, Coimbra, Portugal.
- Green Chemistry Postgraduate Summer School (online event), from 4-10 July 2021 Venezia, Italy. (grant).
- The XXIII International Symposium on Advances in Extraction Technologies (online event), from 29 June to 2 July 2021 in Alicante, Spain. (grant)
- The 1st European Sample Preparation Conference (online event), March, 11th -12th 2021.

Appendix

- Challenge day, Faculty of Culture and Art, University of Salah Boubnider Constantine 3, June 19th-20th 2019 in Constantine, Algeria.
- National seminar of Process Engineering, Faculty of Technology, University of Saad Dahlab Blida1, November 7th-8th2018 in Blida, Algeria.

molecules

MDPI

Article

Optimization of Pressurized Liquid Extraction and In Vitro Neuroprotective Evaluation of *Ammodaucus leucotrichus*. Untargeted Metabolomics Analysis by UHPLC-MS/MS

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Abstract: *Ammodaucus leucotrichus* is a spontaneous plant endemic of the North African region. An efficient selective pressurized liquid extraction (PLE) method was optimized to concentrate neuroprotective extracts from *A. leucotrichus* fruits. Green solvents were tested, namely ethanol and water, within a range of temperatures between 40 to 180 °C. Total carbohydrates and total phenolics were measured in extracts, as well as in vitro antioxidant capacity (DPPH radical scavenging), anti-cholinesterase (AChE) and anti-inflammatory (LOX) activities. Metabolite profiling was carried out by ultra-high-performance liquid chromatography coupled to quadrupole time-of-flight tandem mass spectrometry (UHPLC-ESI-q-TOF-MS/MS), identifying 94 compounds. Multivariate analysis was performed to correlate composition with bioactivity. A remarkable effect of the temperature using water was observed: the higher temperature, the higher extraction yield, the higher total phenolic content, as well as the higher total carbohydrates content. The water extract obtained at 180 °C, 10.34 MPa and 10 min showed meaningful anti-inflammatory ($IC50_{LOX}$ = 39.4 µg/mL) and neuroprotective activities ($IC50_{AChE}$ = 55.6 µg/mL). The Principal Components Analysis (PCA) and the cluster analysis correlated these activities with the presence of carbohydrates and phenolic compounds.

Keywords: *Ammodaucus leucotrichus*; bioprospecting; pressurized liquid extraction; UHPLC-q-TOF-MS/MS; neuroprotective potential; anti-inflammatory activity

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Citation: Abderrezag, N.; Sánchez-Martínez, J.D.; Louaer, O.; Meniai, A.-H.; Mendiola, J.A. Optimization of Pressurized Liquid Extraction and In Vitro Neuroprotective Evaluation of *Ammodaucus leucotrichus*. Untargeted Metabolomics Analysis by UHPLC-MS/MS. *Molecules* **2021**, *26*, 6951. https://doi.org/10.3390/molecules26226951

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1. Introduction

The bioprospecting of the North African plants, especially from desert areas, is almost nonexistent compared with those on other continents, although the desert has a rich heritage of medicinal plants of huge diversity. Furthermore, the ability of plant adaptation to the extreme conditions of desert climate led to the synthesis of new molecules, which possess a wide range of interesting biological activities [1].

Ammodaucus leucotrichus (AL) is a spontaneous endemic plant belonging to Apiaceae family that is native of the Saharan and sub-Saharan countries of north and tropical Africa [2]. Morphologically, it is a small yearly wild and cultivated plant from 10 to 12 cm tall with fine and little fleshy leaves and white flowers grouped in umbels of 2, until 4 branches with 5 free petals. The fruit is diachene 6 to 10 mm long and has a dense, soft and white hair [3,4]; in fact, it is known in some areas as *hairy cumin*. Traditionally, it has been used in infusions or decoctions to treat cardiac disease [5], digestive problems [1,2,6] diabetes [7,8], aphrodisiac and tonic [6], in addition to being used as condiment and flavoring agent in tea and food seasonings [4]. AL fruit extract is reported to exhibit antioxidant [1,9], antibacterial [1,10], anti-inflammatory [10] and neuroprotective activities [11].

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One-step sustainable extraction of Silymarin compounds of wild Algerian milk thistle (*Silybum marianum*) seeds using Gas Expanded Liquids

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Green solvents
Neuroprotective effect

ABSTRACT

This work reports the application of Gas Expanded Liquid (GXL) extraction to concentrate the flavonolignan fraction (silymarin) and taxifolin from Silybum marianum seeds, which have proven to be highly valuable health-promoting compounds. GXL using green solvents was used to isolate silymarin with the objective of replacing conventional methods. In one hand, the effect of different compositions of solvents, aqueous ethanol (20%, 50% or 80% (v/v)) at different CO_2/liquid (25, 50 and 75%) ratios, on the GXL extraction was investigated. The obtained extracts have been chemically and functionally characterized by means of UHPLC-ESI-MS/MS (triple quadrupole) and in-vitro assays such as anti-inflammatory, anti-cholinergic and antioxidant. Results revealed that the operating conditions influenced the extraction yield, the total phenolic content and the presence of the target compounds. The best obtained yield was 55.97% using a ternary mixture of solvents composed of CO_2:EtOH:H_2O (25:60:15) at 40 °C and 9 MPa in 160 min. Furthermore, the results showed that obtained extracts had significant antioxidant and anti-inflammatory activities (with best IC_{50} value of 8.80 μg/mL and 28.52 μg/mL, respectively) but a moderate anti-cholinesterase activity (with best IC_{50} value of 125.09 μg/mL). Otherwise, the concentration of silymarin compounds in extract can go up to 59.8% using the present one-step extraction method without further purification, being $silybin_{A+B}$ the predominant identified compound, achieving value of 545.73 (mg silymarin/g of extract). The obtained results demonstrate the exceptional potential of GXL to extract high-added values molecules under sustainable conditions from different matrices.

1. Introduction

Silybum marianum, formerly known as *Carduus marianus*, is now commonly referred in English as milk thistle or Mary thistle. It belongs to the Asteraceae family and is widespread around the world in wild or cultivated forms, but mainly in the Mediterranean area [1], particularly in the north African region where it grows spontaneously. This has mainly guided the selection of this plant into the framework of its valorization.

Previous scientific research showed that the milk thistle seeds may be an excellent source of fatty acids (20-30%), mainly linoleic acid (42-54%) followed by oleic acid (23-36%) and palmitic acid (7-8%). The seeds also contain silymarin (SLY), which is considered the main fraction responsible for bioactivity [2]. In fact, approximately, the seeds extract obtained with ethanol consists of 65% to 80% SLY [3]. SLY is essentially composed of a mixture of flavonolignans comprising silybinin (an equimolar mixture of diastereoisomers A and B), silychristin, silydianin, isosilybinin (an equimolar mixture of diastereoisomers A and B) and one flavonoid taxifolin (dihydroquercetin). The amount of each individual compound in SLY depend on several factors such as environmental conditions, geographic, genetic and time of sowing and harvesting of the

Abbreviations: SLY, silymarin; GXL, Gas Expanded Liquid; IC_{50}, half-maximal inhibitory concentration; LOX, Lipoxygenase; AChE, acetylcholinesterase; SFE, Supercritical Fluid Extraction; PLE, Pressurized Liquid Extraction; SB_{A+B}, $Silybin_{A+B}$; IS, Isosilybin; SD, Silydianin; SC, Silychristin; TX, Taxifolin; ABTS, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) cation radical; EtOH, Ethanol.

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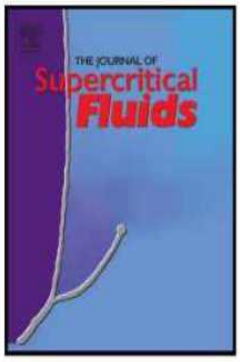

Optimization of supercritical fluid extraction of bioactive compounds from *Ammodaucus leucotrichus* fruits by using multivariate response surface methodology

Norelhouda Abderrezag, Felipe Sanchez Bragagnolo, Ouahida Louaer, Abdeslam-Hassen Meniai, Alejandro Cifuentes, Elena Ibáñez, Jose A. Mendiola

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Permission:

Phytosterol-rich compressed fluids extracts from Phormidium autumnale cyanobacteria with neuroprotective potential

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Title: IMPACT OF HIGH-PRESSURE EXTRACTION TECHNIQUES ON THE QUALITY OF EXTRACTS OBTAINED FROM LOCAL PLANTS.

To apply for the Degree of: **Doctor in Process Engineering.**

Abstract

In the present work, a multi-analytical approach was proposed to valorise two underused local Algerian plants from arid and non-arid zones, namely *Ammodaucus Leucotrichus* seeds and *Silybum marianum* seeds, and study their therapeutic performances. The first part was dedicated to the extraction of oils from the two plant using green processes based on the application of compressed fluids, such as pressurised liquid extraction (PLE), gas-expanded liquid extraction (GXLE) and supercritical fluid extraction (SFE), followed by the identification of the different constituents of the extracts by gas chromatography and liquid chromatography coupled with mass spectrometry. The second part of this thesis was reserved for the study of the neuroprotective activity of the different extracts, through acetylcholinesterase (AChE) and lipoxygenase (LOX) inhibition tests and the antioxidant activity, through free radical scavenging tests (DPPH and ABTS).

PLE using overheated liquid water at 180 °C proved to be a very effective extraction method for obtaining *Ammodaucus leucotrichus* extract with remarkable neuroprotective activity (IC50 (AChE) = 55, 6 µg/mL, IC50 (LOX)= 39.4 µg/mL and IC50 (DPPH)= 58.51 µg/mL). UHPLC-Q-TOF-MS/MS analysis allowed the preliminary identification of 94 compounds, mainly free and glycosylated phenols, lipids and organic acids. Furthermore, the extracts obtained by the ESF technique, optimized by the Box-Behnken design, showed a high carbohydrate content with low AChE inhibition. In addition, the extracts obtained by sequential PLE-SFE extractions possess relevant antioxidant activity compared to that of the extracts obtained by separate extraction processes. The results of the GC-MS analysis revealed the presence of 32 metabolites, 14 of which were reported for the first time in *Ammodaucus leucotrichus.* Concerning the seeds of *Silybum marianum*, the GXLE technique allowed the simultaneous extraction of the five favonolignans composing silymarin with predominance of silybin A + B (545.73 mg of silymarin/g of extract). Furthermore, the extract showed relevant antioxidant and anti-inflammatory potential with IC50 values equal to 8.80 µg/mL and 28.52 µg/mL, respectively, but a moderate AChE inhibition capacity (IC50 = 125.09 µg/mL).

Through this work, we have highlighted the promising potential of *Ammodaucus leucotrichus* and *Silybum marianum* extracts in the treatment of Alzheimer's and inflammatory diseases.

Key words: High pressure extraction, Silybum marianum, Ammodaucus leucotrichus, neuroprotective, antioxidant, anti-inflammatory.

Academic supervisor: LOUAER Ouahida-University of Constantine 3 Salah BOUBNIDER

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